PROCEEDINGS OF THE FIFTY-FOURTH WESTERN POULTRY DISEASE CONFERENCE

April 25-27, 2005 Vancouver, B.C., Canada

THE 54th WESTERN POULTRY DISEASE CONFERENCE DEDICATION

Willis D. “Woody” Woodward

Woody was born January 9, 1922 and died July 26, 2004 after a long battle with cancer. He graduated from Washington State University veterinary school in 1943, and subsequently served in the U.S. Army from 1943-44. After entering the Air Force in 1951, Captain Woodward was put in charge of the public health of two military bases during the Korean War.

Dr. Woodward is the father of one son and two daughters. He has five grandchildren and two great grandchildren. His career in private practice began in 1944. Although primarily a dairy veterinarian, Woody became a self-educated poultry pathologist, working in this field from 1960 until his death.

Woody is best known as the founder of Veterinary Service, Inc. (VSI) in 1960. As an incentive, he allowed his employees to participate in an Employee Stock Ownership Program. At the time of his passing, VSI employed 180 people in six locations within two states (California and Oregon). Woody practiced veterinary medicine for over 56 years and was active in VSI affairs until a short time ago. He also developed numerous products including autogenous bacterins, disinfectants, and a topical salve for wounds.

In 2003, the industry and Modesto community recognized Woody for his community service by bestowing upon him the "Good Egg Award." Dr. Woodward, through VSI, was a longtime member of the Pacific Egg & Poultry Association, and a great supporter of the poultry industry.

Woody was a subtle man who did much behind the scenes with little fanfare. He provided many free veterinary services for those who couldn’t pay. Many 4-H and FFA participants are deeply indebted for his veterinary assistance over many years.

It is with well-deserved appreciation that we dedicate the 54th Western Poultry Disease Conference to Dr. Willis D. “Woody” Woodward.

WPDC SPECIAL RECOGNITION AWARD

R. KEITH McMILLAN

The Western Poultry Disease Conference is proud to present the 2005 WPDC Special Recognition Award to Dr. R. Keith McMillan.

R. Keith McMillan is a proud Canadian who has spent almost his entire career servicing the poultry industry in Western Canada. Keith was born and raised in Saskatchewan, and is married to Julie and is proud to be a father of four children and a grandfather to three children, with a fourth grandchild expected. Keith and his wife Julie also work hard in coordinating and presenting information for couples in Marriage Encounter and Marriage Preparation courses.

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.

Keith and his wife Julie have been an invaluable reference to the western Canadian poultry industry.

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 especially thankful to the many Canadian organizations that contributed to our meeting this year.

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

Dr. Stewart J. Ritchie wishes to thank his support staff. As usual, behind the front person are the people that do the real work. Mrs. Catherine McAllan and Mrs. Shirley Fast, of Canadian Poultry Consultants Ltd., were instrumental in putting this program together and Dr. Ritchie is sincerely grateful for their hard work. The WPDC has a fantastic history and tradition that has been established through the hard work of Dr. Arnold Rosenwald, Mrs. Jo Rosenwald and their California team. "Rosy" and Jo are extremely generous and Dr. Ritchie is proud to be involved with the WPDC tradition. Dr. Richard Chin provided unlimited guidance and his skills and generosity were instrumental in all operational areas involved with the WPDC. Dr. Ritchie offers a sincere thanks to Rich.

We would also like to recognize the Title Review Committee; Dr. Jim Andreasen, Dr. Carol Cardona, Dr. Richard Chin, Dr. David Frame, Dr. Stewart J. Ritchie, Dr. Craig Riddell, Dr. Joan Schrader, Dr. Ken Takeshita, Dr. Dave Willoughby and Dr. Peter Woolcock. We appreciate the work done by this committee.

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 and Ms. Jennifer Thayer for their work with our conference.

We thank Dr. David Frame for editing and producing another outstanding Proceedings of this meeting. Dr. Frame is indebted to Ms. Sherry Nielson, Staff Assistant III of The Utah State University Turkey Research Center, for her dedicated service proofreading and formatting the Proceedings for publication and CD replication. We express our gratitude to all authors who submitted manuscripts – especially those who followed the instructions and submitted their papers on time! We again acknowledge and thank Ominpress (Madison, WI) for the handling and printing of this year's Proceedings, and to CDMan (Vancouver, BC, Canada) for reproduction of the CD-ROM for this year's meeting. Once again, we acknowledge Bruce Patrick (Graphic Communications, Brigham Young University) for the cover design, and Dr. Rocio Crespo (CAHFS-Fresno) for designing the CD label.

54TH WPDC CONTRIBUTORS LIST

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The **Proceedings** of the 54th Western Poultry Disease Conference are <u>not</u> refereed, but are presented as a service and a source of information to those attending the conference and to others who wish to gain some insight as to the information presented. Copies of the Proceedings are available in either hardcopy or electronic (CD) formats.

Copies of these Proceedings are available from: Dr. R. P. Chin
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Price per copy (includes shipping & handling): <u>Book and CD (sold together)</u>
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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		
3rd WPDC – 1954	C. M. Hamilton	Kermit Schaaf		
4th WPDC – 1955	E. M. Dickinson	W. H. Armstrong		
5th WPDC – 1956	D. E. Stover	E. E. Jones		
6th WPDC – 1957	D. V. Zander	H. E. Adler		
7th WPDC – 1958	H. E. Adler	E. E. Jones		
8th WPDC – 1959	R. D. Conrad	L. G. Raggi		
9th WPDC – 1960	L. G. Raggi	A. S. Rosenwald		
10th WPDC – 1961	A. S. Rosenwald	D. V. Zander		
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. Hammerlund	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		
15th PHS				
31st WPDC – 1982	M. Kumar	Robert Schock		
16th PHS				
32nd WPDC – 1983	R. Schock	George B. E. West		
33rd WPDC – 1984	G. B. E. West	Gregg J. Cutler		
34th WPDC – 1985	G. J. Cutler	Don W. Waldrip		Bryan Mayeda
35th WPDC – 1986	D. W. Waldrip (WPDC)	Duncan A. McMartin(WPDC)	J. A. Allen	

	PRESIDENT	PROGRAM CHAIR	DEDICATION	RECOGNITION
11th ANECA	Jorge Basurto (ANECA)	Mario Padron (ANECA)	A. Tellez – G. Rode	
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	
16th ANECA	Martha Silva M.(ANECA)	David Sarfati M.(ANECA)	A. S. Rosenwald	
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
44th WPDC – 1995	H. A. Medina	David D. Frame	W. M. Dungan (posthumous)	A. J. DaMassa Gabriel Galvan Walter F. Hughes W. D. Woodward R. Yamamoto
45th WPDC – 1996	D. D. Frame (WPDC)	Mark Bland (WPDC)	Don Zander (WPDC)	Pedro Villegas
21st ANECA	R. Salado C. (ANECA)	G. Tellez I. (ANECA)	M. A. Marquez (ANECA)	Ben Lucio M. Mariano Salem Victor Mireles Craig Riddell
46th WPDC – 1997	Mark Bland	James Andreasen, Jr.	Bryan Mayeda	Roscoe Balch Paul DeLay J. W. Dunsing Don Helfer D. E. Stover
47th WPDC – 1998	J. Andreasen, Jr.	H. L. Shivaprasad	W. J. Mathey	Marcus Jensen Duncan Martin
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	
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		

MINUTES OF THE 53rd WPDC ANNUAL BUSINESS MEETING

President Willoughby called the meeting to order on Monday, 8th March 2004, at 4:45 PM, at the Holiday Inn Capitol Plaza hotel. There were 27 people in attendance.

APPROVAL OF 52nd WPDC BUSINESS MEETING MINUTES

The minutes from the 52nd WPDC business meeting were reviewed and a motion was carried to approve them as printed in the Proceedings of the 53rd WPDC.

ANNOUNCEMENTS

President Willoughby acknowledged all the contributors; in particular, those contributing at the Benefactor level, which included the American Association of Avian Pathologists and Merial Select, Inc. He also thanked all the contributors for their generous donations. President Willoughby acknowledged the efforts of the current WPDC officers for their work and participation in the organization of this year's meeting. President Willoughby honored Dr. Ben Pomeroy, who passed away this year, for his tremendous contribution to the poultry industry.

REPORT OF THE SECRETARY-TREASURER

Dr. R.P. Chin presented the Secretary-Treasurer report. There were 196 registrants for the 52nd WPDC held at the Capitol Plaza Holiday Inn, Sacramento, CA, March 9-11, 2003. Contributions for the 52nd WPDC were $25,825, with a total income of $54,589. There were expenses of $55,622 for WPDC for the meeting, resulting in a net loss of $1,033. The current balance in the WPDC account is $47,437. The good news is that the loss was less than anticipated. The loss was again due to the low number of registrants for WPDC, decrease in contributions and an increase in costs. Last year, we had 195 registrants. In previous meetings in Sacramento, registration has been around 225.

This year's preliminary budget again looks even graver with a projected loss of $16,000. One week prior to the meeting, there were only 165 registered (though at the time of the meeting we had 205 registered) and our contributions were again lower, at only $23,100, noting that this is the first year that Rosy is not the Contributions Chair. Additionally, hotel costs have increased. Dr. Chin stated that WPDC is going to need to look at ways to increase our registration numbers and contributions to stop the loss (2 years in a row). There will be an increase in registration fees next year. The Secretary-Treasurer's report was approved.

REPORT OF THE PROCEEDINGS EDITOR

Dr. D. Frame presented the Proceedings Editor report. There were 62 papers and a total of 116 pages in this year's proceedings. For this conference, 400 hard copies and 400 electronic copies of the Proceedings were produced. The CD's cost approximately $7.75 each (much higher than last year's $3.00 each) and the books cost $4.78 each. As was done last year, the books were produced by Omnipress and the CD's were produced by Microsearch Corporation. The total cost came to $6143 for editing and publishing of the proceedings. Dr. Frame suggested we look into a web-based proceedings that would allow people to download an electronic file rather than continue with the production of a CD.

OLD BUSINESS

There was a brief discussion on whether to produce a CD of the proceedings every year. Following up to Dr. Frame's suggestion that we look into web-based proceedings, Dr. Schrader made a motion to form a sub-committee that would look into web-based proceedings. It was suggested that WPDC approach the AAAP and different universities (e.g., Utah State University or University of California, Davis) about this, and to look into other technologies that could be used. The motion was seconded and passed unanimously with Dr. Frame as the committee chair.

NEW BUSINESS

President Willoughby reported that the WPDC Executive Committee nominated Dr. Peter Woolcock for Program Chair-elect of the 55th WPDC in 2006. A motion was made by Dr. A. Bickford and seconded by Dr. Y. Ghazikhanian to close nominations. The motion was passed and Dr. Woolcock was elected unanimously as program chair-elect. Dr. Carol Cardona announced that she is resigning as Local Arrangements Coordinator. Since next year's meeting will be in Vancouver, BC, Canada, Dr. Stew Ritchie volunteered to be Local Arrangements Coordinator. President Willoughby nominated the following officers for 2004-2005:

Program Chair: Dr. Stewart Ritchie
President: Dr. Joan Schrader
Local Arrangement Coordinator: Dr. Stewart Ritchie
Contributions Chair: Dr. Ken Takeshita
Proceedings Editor: Dr. David Frame
Secretary-Treasurer: Dr. Rich Chin
Program Chair-elect: Dr. Peter Woolcock

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

There was then a discussion on how to do local arrangements. First, Dr. Frame thanked Dr. Cardona for her years of service as Local Arrangements Coordinator. Dr. Schrader suggested we investigate the cost and feasibility of outside companies to manage the entire conference and to do all arrangements. It was stated that Rosy was able to do everything himself with the help of a student. However, Rosy gave his whole career to working on WPDC, and those in academics cannot justify this type of work for merit or promotion in the academic ranks. Dr. Ritchie looked into some companies in Vancouver and found that it would double the cost of organizing the event. Dr. Cardona suggested that possibly industry organizations can donate their time to helping organizing the meeting. Dr. Schrader moved that a subcommittee be formed to look into hiring a company to provide conference organization for WPDC. The motion was seconded by Dr. Takeshita and approved. Dr. Chin will serve as the committee chair.

There was a discussion on ways to improve attendance to WPDC. Dr. Chin stated that in 2002, there were only 195 registrants, and in 2003 there were 196 registrants. This year, we currently had 200 registrants, though 2 weeks prior to the meeting, there were about 180 registered. In previous years, WPDC had been averaging about 225 registrants. He anticipates another outstanding attendance next year in Vancouver and noted that there were almost 300 registrants at the previous meeting in Vancouver in 1998. Dr. Takeshita suggested better advertisement of the meeting in various publications. Dr. Chin said that he has not done that recently since the meeting information is mailed to about 2400 addresses worldwide.

It was suggested that people announce the next WPDC whenever they attend other meetings or travel. Dr. Takeshita said that he would work on the advertisement and help designate people who are attending different meetings as WPDC spokes-persons.

It was asked if simultaneous translation would increase the attendance from Latin America. Dr. Chin stated that translation was costing WPDC about $5000 and the last time it was used, there were only about 10 people using the translation services. Dr. Cardona suggested that we might translate the proceedings.

Dr. Takeshita moved and it was seconded that we obtain a list of people who use to attend WPDC and find out why they are no longer attending. Dr. Eckroade commented that people choose to attend meetings because they know in advance when the meeting will occur and the quality of the program. He said that next year's meeting in Vancouver will have unique opportunities.

Dr. Edson asked if we have to have the meeting in the Davis/Sacramento area? There were comments that this was an excellent location because it is close to many tourist activities, e.g., Napa valley, Tahoe, San Francisco and Yosemite. However, other locations were also mentioned, e.g., Las Vegas, San Diego and Palm Springs.

Dr. Takeshita made a motion that we elect a temporary local arrangements coordinator until a company or site has been identified for the 2006 meeting. The motion was seconded and approved. Dr. Jim Andreasen volunteered as the temporary local arrangements coordinator and Dr. Bickford offered to help him.

It was also suggested that we improve our fund raising by contacting more production companies, especially within California. Those of us who know people within such companies should contact them to encourage them to contribute to WPDC. It was also suggested we do silent and/or verbal auctions at the meeting.

Dr. Cardona felt that the ACPV workshop prior to WPDC was not attracting people to WPDC, but rather just the opposite. She suggested WPDC approach ACPV to ask them to enhance their workshops to make them more attractive as a draw for WPDC. It was stated that a specialty college should be a leader in their continuing education programs with high academic standards and not be dependent upon a regional meeting.

Dr. Takeshita motioned that we ask ACPV to commit to improving their scientific program and covering the expenses of all invited speakers whenever their workshop is held in conjunction with WPDC. This motion was seconded and unanimously approved.

President Willoughby announced that next year's WPDC will be held in Vancouver, BC, Canada, April 25-27, 2005, at the Fairmont Hotel Vancouver. These dates are Monday through Wednesday, rather than our usual Sunday through Tuesday. Dr. Chin stated that this was due to the fact that another conference was already scheduled for Sunday and the hotel offered us more meeting rooms if we moved our meeting.

President Willoughby announced that the 55th WPDC is tentatively scheduled for March 4-7, 2006 at the Holiday Inn in Sacramento. However, this is dependent upon the information the Dr. Andreasen provides on other possible sites.

Dr. Chin discussed Feed Info News Service and Zootecnica International. These are internet news agency that have asked to publish WPDC papers on their web site. WPDC does not copyright their proceedings. Last year, WPDC agreed that papers could be published on the internet if the company received an agreement from the authors. It was agreed to continue with the same policy.

In addition, Dr. Chin mentioned that booksellers have always purchased WPDC proceedings at the regular price. He did not understand what booksellers do with the WPDC proceedings or why they would only buy one copy. Since booksellers obviously must make a profit, it was thought that they may be copying the proceedings and selling numerous copies. It was suggested that WPDC have an institutional price of $1000/proceedings. A motion was made and seconded that we charge an institutional rate of $1000/proceedings to booksellers. The motion passed. (NOTE: Dr. Chin found that most university libraries use booksellers to purchase books for their collections. It was decided that it would be better to continue selling to booksellers at the regular rate.) In addition, it was suggested that Dr. Frame look into copyrighting the proceedings.

Dr. Cutler noted that the University of California, Davis, School of Veterinary Medicine, is fund raising for new buildings on campus. Rooms can be named after an individual if enough funds are raised, e.g., contributions totaling $15,000 can name a small classroom. He suggested that members of WPDC contribute to the building fund in honor of Dr. Rosenwald so we can name a small classroom after Rosy. Many people thought this was an excellent idea. Dr. Cutler will be contacting folks who are interested in contributing.

President Willoughby passed the presidency to Dr. Joan Schrader who thanked those involved in the organization of the meeting. Dr. Yan made a motion that we adjourn the meeting. Dr. Frame seconded it and the meeting was adjourned at 6:00 PM.

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Note: Both the oral and poster presentations of the 54th 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-FOURTH WESTERN POULTRY DISEASE CONFERENCE

SELECTION OF THERAPEUTICALLY EFFICACIOUS LACTIC ACID BACTERIA CULTURES FOR PROBIOTIC USE IN COMMERCIAL POULTRY

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ABSTRACT

During the last four years, our laboratory has worked toward the identification of probiotic candidates for poultry which can actually displace *Salmonella* and other enteric pathogens which have colonized the gastrointestinal tract of chicks and poults. Published studies (Bielke *et al*., 2001 *Poultry Science*) indicated that by screening more than eight million enteric organisms for competition in vitro, 36 organisms were identified that had the ability to exclude *Salmonella* in neonatal poultry. Further screening allowed the identification of 11 lactic acid bacteria (of the genus *Lactobacillus* or related) that were even more efficacious in the treatment of *Salmonella* infected chicks and poults (Vicente *et al*., in submission, *JAPR*). In laboratory challenge studies, 80-90% reductions in *Salmonella* recovery rates from challenged chicks treated with the candidate probiotic culture were typical. By selecting *Salmonella* infected flocks pre-slaughter, we have demonstrated that treating such flocks, approximately two weeks prior to slaughter, can markedly reduce environmental *Salmonella* recovery in commercial turkeys and broilers (Vicente *et al*. submitted). Treatment of idiopathic enteritis in commercial poults also compared favorably to selected antibiotic therapy in recent studies (Higgins *et al*., In Press, *JAPR*). Large scale commercial trials have indicated that appropriate administration of this probiotic mixture to turkeys increased body weight gain at processing by approximately 230 grams with over 120 flocks evaluated (Torres-Rodriguez *et al*., submitted), with similar performance gains observed in more limited commercial trials with broilers. Administration of dietary lactose at a very small concentration (0.1%) greatly enhanced the growth rates of probiotic turkeys under commercial conditions and furthered reduced total production costs (Torres-Rodriguez *et al.* in preparation). These data indicate that selection of therapeutically efficacious probiotic cultures with marked performance benefits in poultry is possible, and that defined cultures can sometimes provide an attractive alternative to conventional antimicrobial therapy. The specific results of these studies will be presented.

FROM BURGER KING TO THE EUROPEAN UNION – NEW DEVELOPMENTS IN ANIMAL WELFARE AND EMERGING STANDARDS

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THE RECENT EMERGENCE OF FARM ANIMAL WELFARE STANDARDS

In the past several years, North America and Europe have entered a phase of rapid change in farm animal welfare standards. In 1999, the European Union (EU) passed a directive to phase out the standard battery cage for laying hens within 12 years. In 2000, McDonald's Restaurants in the United States (US) announced animal welfare standards that their suppliers would be required to meet, mainly in the slaughter and egg industries, with Burger King and Wendy's adopting similar programs soon after. In 2001 the EU passed a directive to ban the gestation stall for sows effective in 2013, and the national associations representing the chain restaurant and grocery industries in the US began developing harmonized animal welfare standards on behalf of their member companies. In 2002 the United Egg Producers in the US announced a program to certify producers as conforming to the industry's new animal welfare standards, and the 162

member countries of the World Organization for Animal Health (OIE) unanimously voted to begin developing internationally harmonized animal welfare standards.

In these examples we see a remarkably rapid movement toward explicit animal welfare standards, combined with harmonization of standards among different companies and different nations. In this paper I will examine some of the cultural changes in the West that led to the development of animal welfare standards, some of the science underlying the standards, and how different approaches to assessing animal welfare have led to the development of quite different standards, all claiming to promote animal welfare.

CULTURAL CHANGES IN THE WESTERN VIEW OF ANIMALS

In a sense, the recent move toward farm animal welfare standards represents one step in a gradual, historical change in Western culture (10). One of the longest-running debates in Western thought — dating back to ancient Greece, and re-emerging repeatedly over the centuries — is whether humans are distinctly different from animals, or whether humans are simply one species among many, closely related to the animal world. On one side of the debate were (for example) the Stoics in ancient Greece, Christian theologians such as St. Augustine, and philosophers such as Descartes and Kant, who saw humans as unique and considered that we have only minimal responsibilities toward other species. Opposing this view were (for example) the Pythagoreans in ancient Greece, Christian theologians such as St. Francis of Assisi, and philosophers such as Voltaire and Goethe, who emphasized our relatedness to the rest of nature, and were strongly opposed to treating animals as if they were purely for human use.

In medieval times, the pendulum seemed to have swung in the direction of seeing humans as different. Humans were seen as unique in appearance, being the only creature fashioned to resemble God; humans had been created separately from animals to serve as stewards of, not merely a part of, the natural world; and humans were not simply mortal bodies but conscious, reflective, immortal souls. Thus humans were seen as unique in appearance, in origin, and in mental/spiritual life.

Over recent centuries, however, the pendulum has been moving slowly in the opposite direction, driven partly by science. Beginning in the 1200s, comparative anatomy became one of the frontiers of scientific study in Europe, and through centuries of anatomical research the homologies of the vertebrate body gradually came to be recognized. According to historian Dix Harwood, by the year 1700 it had become relatively common knowledge that humans share a common anatomical template with the other vertebrates (16).

A second change arose from the evolutionary thinking of the 1800s. Through Charles Darwin and his contemporaries, people began to see the human species sharing not only a common anatomical template with other species, but a common origin as well.

During the late 1900s, the study of animal behavior led to a third change in the popular view of animals. Since about 1960, primatologists such as Jane Goodall have been studying animals partly in the manner of cultural anthropology, viewing animals not simply as sources of data to estimate normal or average trends, but more as "persons" possessing individuality, unique life histories, and complex social and emotional lives. Other work, in the tradition of psychology, has explored the cognitive abilities of animals, even using the classic approach of child psychologist Piaget to study stages of cognitive development in other species. The result is a tendency to see at least some species as having complex mental and emotional lives, thus further reducing the perceived difference between humans and non-human animals.

Through these three scientific developments — in anatomy, evolutionary biology, and animal behavior — popular understanding of animals has gradually shifted toward seeing humans as bearing strong commonalities with other animal species. This cultural shift has stimulated a major rethinking of what constitutes ethical conduct toward animals. By the early 1800s, Western countries had begun passing laws for the protection of animals. By 1900 there was vigorous opposition to the use of animals in science in many parts of Europe. Concern over animals went into decline during the two World Wars and the Great Depression of the 1930s. However, it re-emerged soon after 1950 and has exerted an important influence on all aspects of animal use, including animal-based research, management of zoos and wildlife, and the use of animals for entertainment and companionship. The shift toward farm animal welfare standards can be seen partly as an extension to agriculture of the growing trend to pay increased attention to animals and their welfare.

CHANGES IN ANIMAL-BASED AGRICULTURE

A second cultural change, this one specific to animal agriculture, has also helped set the stage for farm animal welfare standards (12). Until about 1950 poultry and swine in the industrialized countries were raised using fairly traditional methods which relied on labor to accomplish routine tasks such as feeding and manure removal, and which generally involved keeping animals in outdoor or semi-outdoor environments.

After the Second World War, there emerged a new generation of "confinement" animal production which used hardware and automation instead of human labor for many routine tasks, and the animals were generally kept in specialized indoor environments.

At the same time as the technology was changing, farm size was increasing. Larger farms had certain economies of scale and were able to sell animal products at lower prices. To remain competitive, other producers had to expand their operations, and the newer, more automated production systems helped to make this feasible. Eventually, for certain commodities in certain regions (such as egg production in the US), family-sized units ceased to be economically viable at all.

These changes in technology and farm size contributed to a change in how animal production is perceived by the public. Traditionally, farmers in the West have enjoyed a strongly positive public image. As described by philosopher Paul Thompson (20), the traditional "agrarian" life-style — whereby a family works together to farm the land where it lives — has been a cherished ideal, evoking images of virtuous living, good citizenship, and harmony with nature. Similarly, the cultural values of pastoralism, kept alive in the West by the Bible, involved strongly positive images of people who provide diligent care for domestic animals, to the point that a conscientious shepherd was a common metaphor for God. As long as animal production was viewed as conforming to agrarian living and pastoralist animal care, it was almost guaranteed a positive public image.

However, the move toward confinement rearing of animals and large production units has led the public to see animal production more as an industrial process. Industrialists, unlike farmers, have traditionally been seen in a more negative light — as fully capable of pursuing personal wealth at the expense of workers, the environment and the public good, and therefore needing to be restrained. Thus, as animal production came to be viewed less as a form of agrarianism and pastoralism, and more as an industrial activity, the public became more willing to see standards and regulations imposed on animal agriculture for the protection of animals.

DIFFERENT VIEWS OF ANIMAL WELFARE

As social concern for animal welfare grew, debate arose over what constitutes acceptable welfare for farm animals. Three different views emerged (7). One view emphasizes the basic health and functioning of animals in the sense of survival, growth, and freedom from injury and disease. This interpretation of animal welfare is commonly heard among farmers and veterinarians involved in animal production, and it fits with a value system that sees highly efficient production as the guiding principle of animal agriculture. According to this view, confinement production methods, however unnatural and restrictive they may seem, should be considered to provide satisfactory animal welfare as long as the animals are healthy, growing, and reproducing well.

A second view emphasizes the "affective states" of animals — especially pain, suffering, and other unpleasant feelings and emotions. This view is commonly heard among humanitarians concerned about animal welfare. According to this view, production methods should be judged on the basis of how happy the animals are, or conversely how much the animals are caused to suffer.

A third view focuses on "natural living". It holds that animals should be allowed to live in as natural circumstances as possible and to express their normal behavior. This view is commonly heard among consumers and critics of confinement systems (18).

These three views often lead to similar conclusions. For example, keeping animals in temperatures to which they are well adapted should be good for their welfare by all three criteria: because the animals should not develop stress and disease problems due to cold (a basic health and functioning criterion), nor suffer from feeling cold (an affective state criterion), and because the temperatures are natural for the species (a natural living criterion). However, the different views of animal welfare sometimes lead to different conclusions, and they have led to some quite different approaches in the scientific study of animal welfare.

ANIMAL WELFARE RESEARCH

Basic health and functioning. In many cases, scientists seeking to understand and improve animal welfare have focused on basic measures of injury and disease. For example, Ragnar Tauson (19) made detailed comparisons of the health and performance of laying hens in different types of cages. The research found that in cages with steeply sloped floors and poor quality galvanizing, the majority of birds had significant foot lesions, whereas foot health was good with plastic-coated floors of more moderate slope. Similarly, most birds developed severe lesions of the neck when feeding from deep troughs with sharp lips installed too high for comfortable access, whereas the problem was largely eliminated by a shallower trough located more conveniently for the birds. The use of solid side partitions reduced feather damage due to wear and pecking; installation of abrasive strips helped prevent overgrown claws; and design improvements to the cage fronts led to fewer birds becoming trapped and killed. These results were very influential with cage

manufacturers and formed the basis of regulations on cage design in Sweden and elsewhere.

Where we lack that sort of experimental control, it may still be possible to use epidemiology to see what environmental features promote good health and functioning. Many animal welfare standards used for broiler chickens put particular emphasis on space allowance. Marian Dawkins and co-workers (6) in the UK recently did a study involving 10 chicken production companies which agreed to house birds at a wide range of stocking densities, while Dawkins and co-workers measured a large number of environmental parameters, as well as basic health and functioning variables such as survival, lameness, growth and aggression. They found that space allowance did influence the health and functioning of the birds as expected, but the really striking differences were not between different stocking rates but between different companies for reasons other than stocking rate. Taking growth rate as an example, they found that growth rate was most strongly correlated with the number of visits per day by the stockmen, and with the humidity in the barn especially in the first week, whereas the stocking density, along with other variables such as the age of the building and the moisture of the litter, had correlations of a lower level. The authors concluded that stocking density is important, but that in trying to improve bird welfare in the sense of basic health and functioning, the major focus should be on other variables.

Natural living. Although a high level of health is an important element of animal welfare, many people feel that acceptable welfare requires other factors to be taken into account. The use of natural living criteria is an attempt to move beyond simple health measures, but the approach must be used with caution. Simply providing a "natural" environment is not a solution, partly because so-called natural environments include hardships such as cold weather and predators. Performance of a full range of natural behavior is not a solution because natural behavior includes such elements as shivering and fleeing. Instead, animal welfare scientists have tried to identify those types of natural behavior that the animals themselves are highly motivated to perform, and those environmental features that the animals themselves prefer.

A common approach has been to train animals to perform "instrumental" tasks, such as pecking on a key, for access to various environmental features, and then determine which features the birds will work to obtain. Research of this type has shown that hens will expend considerable effort to obtain a perch for resting at night (14), a nest box where they can retreat to lay eggs (8) and, perhaps to a lesser extent, litter for dust bathing (21). One study also found that hens would work to enlarge the floor space up to about 750 cm^2 per bird (13).

Affective states. A third approach used in the scientific study of animal welfare has been to identify unpleasant affective states such as fear, pain, hunger, and distress. One example arose from research on catching broiler chickens. When mechanical chicken harvesters first appeared, there was concern that they would cause fear and distress among the birds. However Ian Duncan and colleagues (9) monitored birds when they were captured by hand or by machine. They found that heart rate, monitored as an index of emotional reaction, actually returned to normal faster for birds caught by machine rather than by hand. The researchers also tested the birds for tonic immobility as a more specific test of fear. Again the chickens that had been caught by hand before the test remained in tonic immobility longer than those that had been caught by machine. Both lines of evidence suggested that the birds were actually less upset when moved by the machine.

CONTRASTING STANDARDS

Thus we see three rather different views of animal welfare, each with its scientific proponents and each leading to research that makes a contribution to animal welfare. We are also beginning to see quite different types of animal welfare standards, each influenced by a different mixture of basic health and functioning, affective states, and natural living criteria (11).

Standards for laying hens provide a particularly clear example (Table 1). Recent decades have seen dozens of scientific studies examining the effects of different space allowances for laying hens (1, 17), mostly using basic functioning variables such as survival, rate of lay, and feed conversion efficiency. The studies generally show that when space allowance drops below about 450 cm^2 per bird, survival is reduced, rate of lay declines, and feed efficiency drops. On that basis, approximately 450 cm^2 per bird was adopted as a standard by several chain restaurants in the United States and by the European Union until 2003. What we might call "basic" standards require about this amount of floor space, combined with sufficient access to food and water to ensure a high level of basic health and functioning.

The standards for "enriched cages", approved by the European Union in 1999, are based on a broader conception of animal welfare. Such cages support a high level of basic health and functioning (2). They also accommodate specific elements of natural behavior that birds are highly motivated to perform, namely perching, dust-bathing and laying eggs in a nest box. Also, by providing an amount of space that the

birds will work to obtain, distress or frustration associated with crowding should be reduced.

The standards for "alternative" production systems, such as the Freedom Foods program in the UK and various organic production standards, take a third approach. These standards generally prohibit all use of cages, requiring instead that birds be free to move in a large area with a generous space allowance and access to the outdoors, natural light, and other amenities that provide a seemingly more natural environment. These standards appear to give special emphasis to natural living criteria.

Thus we see, in a sense, three different kinds of standards summarized briefly in Table 1. All are claimed to protect the welfare of the birds, but they set very different requirements at least partly because they are based on different degrees of emphasis on basic health and functioning, affective states and natural living as criteria for animal welfare.

CONCLUSIONS

With a wide variety of programs, all claiming to ensure a high standard of animal welfare and all claiming to be based on science, there is a risk that the public will become confused and disillusioned by the conflicting claims. How can we reduce the chance of this happening?

First, we need to be clear about the mixture of science and values that go into animal welfare standards. Science has a key role to play, for example by demonstrating how different housing and handling practices affect the growth and health of animals, by helping us understand their affective states, and by identifying environmental features and types of natural behavior that are important for the animals themselves. Science does not, however, answer or trump value-based questions about the relative importance of the different criteria of animal welfare. Hence, we need to recognize (and communicate) that animal welfare standards have a basis in both science and values. The science does not replace the values; rather, the values underlie and help to shape the science.

Second, we need to strike reasonable balances among the different elements of animal welfare. Standards that emphasize natural living (e.g., organic, free-range) need to provide adequate protection against disease and harsh environmental conditions. Standards that emphasize basic health and functioning (e.g., basic standards endorsed by producer organizations) must not ignore concerns over affective states. Perhaps no welfare standards will maintain public trust unless they take the different conceptions of animal welfare into account to some degree.

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Table 1. Examples illustrating three types of animal welfare standards for laying hens.

Type of standard*	Floor space (cm^2/bird)	Feed trough (cm/bird)	Water sources	Perch, litter, nestbox	Outdoors, natural light
Basic	450	10	2 nipples	no	no
Enhanced	750	12	2 nipples	yes	no
Alternative	2300	8	1 round	yes	6 h/day

*Sources: "Basic" standards include those of various food companies and producer organizations; the example is the standard approved by Council of the European Communities (1988). The "enhanced" standard shown is the standard for enriched cages approved by Council of the European Union (1999). "Alternative" standards include many free-range, organic and specialty standards; the example is the organic standard of the Certified Organic Associations of British Columbia (2003). The table shows only certain elements of the standards, selected for purposes of comparison.

EXPERIENCES USING H7 AVIAN INFLUENZA VACCINATION IN CONNECTICUT

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SUMMARY

Early 2003, a low pathogenic H7N2 avian influenza (LPAI) was isolated at four out of our seven farms in Connecticut. It has the same genetic sequence as the low pathogenic H7N2 avian influenza that has been circulating in the live-bird markets in the Northeast United States for several years now. Clinical signs observed were rapid drop in feed consumption followed by severe drop in egg production (10 to 20% per week for two weeks). Mortality is slightly elevated and respiratory distress was also observed. Kofkoff Egg Farms have seven farms in Southeastern Connecticut with a capacity of 3.5 million layers and 1.1 million replacement pullets. All the farms are located within 20 miles from the feed mill. When NVSL confirmed the H7N2 LPAI infection last February 27, 2003, three farms were already infected.

With the recent successes on the use of vaccination as part of the eradication plan of H7N1 low pathogenic avian influenza (LPAI) in Italy in 2000 and H7N3 LPAI in Utah in 1995, the option to vaccinate in addition to enhanced biosecurity and active surveillance was immediately recommended. The option to vaccinate to eradicate the infection has been preferred over depopulation mainly because we believe it is a very viable option, and that the State of Connecticut does not want to adversely affect the local economy where the farms are located nor does it have the resources to compensate for the depopulation of more than four million birds. After almost a month of meetings and negotiations, the United States Department of Agriculture (USDA) gave us their authorization to vaccinate with modification of our proposal, most notably, by requiring us to also vaccinate all previously infected layers and under very strict conditions. The authorization for use of the vaccine will be withdrawn if one of the following conditions emerges:

1. There is genetic evidence that H7 isolates have mutated toward a highly pathogenic form;
2. There is failure to meet protocol requirements;
3. There is an indication after six months that vaccine will not eradicate the infection;
4. There is spread of virus to a new premises; and/or
5. There are significant trade bans imposed on the United States.

With close coordination with USDA, Connecticut Department of Agriculture and University of Connecticut, stricter biosecurity procedures were put in place. This is very critical in breaking the cycle of infection so that the replacement pullets are not exposed to the virus before they are vaccinated. We were also very fortunate that the State of Pennsylvania agreed to sell us their H7N2 vaccine in storage at the Lohmann Animal Health Laboratories in Maine for our immediate use. Although the vaccine was six years old, it was tested during the outbreak in Virginia in 2002 and was found to still be potent. At the same time, Lohmann Animal Health International has been approved to produce a heterologous H7N3 Utah strain vaccine for the completion of the program.

Farm biosecurity. Aside from the usual farm biosecurity procedures, the farm biosecurity was enhanced by doing the following:

1. Traffic on and off the farms was controlled. Unnecessary visits were denied and most business that can be conducted over the phone was used. Necessary visits were coordinated with company management. All vehicles coming in and out of the farms and feed mill have their tires and wheel wells thoroughly disinfected with either DC&R or Tektrol. Disposable coveralls and boots are being used before entering the chicken houses. Disposable coveralls and boots are discarded in a receptacle at the gate entrance before leaving the farm.
2. Foot baths and hand disinfectants were provided in all the chicken houses and doorways. All pullet houses have their own portable toilets to prevent co-mingling of farm personnel. Farm equipment and tools are washed and disinfected if they are to be used in another chicken house or farm.
3. Farm personnel must use their work clothes and shoes while working and change to their street clothes and shoes when leaving the farm.
4. Spent hens are humanely euthanized with CO_2. Birds are placed in leak proof vehicle lined with tough disposable polyethylene plastic sheeting large enough to cover the carcasses and be sealed at top. The spent hens are sent to the incineration plant using a pre-approved route. Vehicle is disinfected immediately prior to leaving the farm and before entering public roadways. Upon leaving the incineration plant, the vehicle is again cleaned and disinfected. If possible, the driver should remain in the vehicle with the windows closed. If the driver exits the vehicle he must used coveralls and boots. The daily mortalities are also put in a leak proof container and are sent to the rendering plant.
5. Poultry manure is first stacked in the farm and covered with a tarp and is tested virus-negative before it can be spread in the fields.
6. All houses are cleaned and disinfected and are tested virus negative before any placement of day-old chicks or replacement pullets.
7. We have separate feed trucks for infected and non-infected farms. The trucks are disinfected before leaving the feed mill and again before they enter the farm.

Vaccination and Surveillance. The pullets are vaccinated twice (six and 13 weeks old), and the previously infected layers once by either subcutaneous or intramuscular injection. Vaccination started last April 16, 2003. Pullets older than six weeks during the start of vaccination were vaccinated once. Sentinel birds are bled every other week for hemagglutination inhibition test (HI), and dead birds are swabbed every week for RRT-PCR test. Manure and house environmental swabs were also performed as needed. All samples are sent to NVSL for testing. Any dead

sentinel birds are sent to the University of Connecticut for postmortem testing.

Following is the farm-by-farm update of the low pathogenic avian influenza infection at Kofkoff Egg Farms.

Lebanon Layer Farm

- Low pathogenic H7N2 avian influenza was diagnosed by Cornell diagnostic laboratory and confirmed by NVSL on Feb. 27, 2003. All the flocks in the farm were positive.
- The farm was immediately placed under enhanced biosecurity under close supervision of Connecticut Department of Agriculture and USDA.
- Vaccination of previously infected flocks and replacement pullets with inactivated H7N2 avian influenza vaccine started on April 16, 2003.
- There were only 2 flocks that were PCR positive during the start of vaccination. Also, the virus could no longer be isolated in any of the flocks during the start of vaccination.
- There was no re-circulation of the virus observed and no new cases diagnosed since the start of vaccination. Vaccination of all the layer flocks was completed on August 28, 2003.
- Sentinel birds and dead bird surveillance were routinely done by Connecticut Department of Agriculture and USDA. The sentinel birds have remained negative since the start of vaccination and no virus was ever isolated from the tracheal swabs.
- Since April 2003, the virus could no longer be isolated from the farm.

Bozrah Layer Farm

- Low pathogenic H7N2 avian influenza was diagnosed by NVSL on March 3, 2003. All the flocks in the farm were positive.
- The farm was immediately placed under enhanced biosecurity under close supervision of Connecticut Department of Agriculture and USDA.
- Vaccination of previously infected flocks and replacement pullets with inactivated H7N2 avian influenza vaccine started on April 28, 2003.
- All the flocks were already PCR and virus isolation negative during the start of vaccination.
- There was no re-circulation of the virus observed and no new cases diagnosed since the start of vaccination. Vaccination of all layer flocks was completed last August 23, 2003.
- Sentinel birds and dead bird surveillance were routinely done by Connecticut Department of Agriculture and USDA. The sentinel birds have remained negative since the start of vaccination and no virus was ever isolated from the dead birds.
- Since April 2003, the virus could no longer be isolated from the farm.

Bozrah Pullet Farm

- Low pathogenic H7N2 avian influenza was diagnosed by NVSL on March 5, 2003.
- The farm was immediately placed under enhanced biosecurity under close supervision of Connecticut Department of Agriculture and USDA.
- Vaccination of pullets with inactivated H7N2 avian influenza vaccine started on April 16, 2003.
- The oldest three pullet flocks were avian influenza positive during the start of vaccination.
- There was no re-circulation of the virus observed and no new cases diagnosed since the start of vaccination.
- Sentinel birds and dead bird surveillance were routinely done by Connecticut Department of Agriculture and USDA. The sentinel birds have remained negative since the start of vaccination and no virus was ever isolated from the dead birds.
- Since the vaccination started in April 2003, the virus could no longer be isolated from the farm.

Country Acres Layer Farm - Franklin, CT

- Low pathogenic H7N2 avian influenza was diagnosed by NVSL on April 30, 2003. All the flocks (four houses) were PCR positive and one house was virus isolation positive.
- The farm was immediately placed under quarantine, under close supervision of Connecticut Department of Agriculture and USDA.
- June 12, 2003 routine surveillance showed two houses are PCR positive and one house virus isolation positive.
- June 26, 2003 routine surveillance showed only house both PCR and virus isolation positive.

- July 15, 2003 routine surveillance showed all houses negative to avian influenza by both PCR and virus isolation.
- Vaccination started on September 2, 2003 and finished in October. There are no sentinel birds yet in the farm as the 4 houses have been previously infected, but dead bird surveillance and tracheal swabs are routinely done.
- The virus could no longer be isolated from the farm since July.

Lebanon Pullet Farm - Goshen Hill, Lebanon, CT. The farm has never been infected and has remained negative to avian influenza up to the present. Routine serology and dead bird surveillance is being conducted by Connecticut Department of Agriculture and USDA every other week and every week respectively.

Hebron Layer Farm - East Street, Hebron, CT. The farm has never been infected and has remained negative to avian influenza up to the present. Connecticut Department of Agriculture and USDA are conducting routine dead bird surveillance every week.

Colchester Layer Farm - Shailor Hill, Colchester, CT. The farm has never been infected and has remained negative to avian influenza up to the present. Routine serology and dead bird surveillance is being conducted by Connecticut Department of Agriculture and USDA every other week and every week respectively.

In summary, vaccination in conjunction with solid biosecurity and routine surveillance could eradicate AI. Sentinel birds have remained negative for virus infection since the start of vaccination. Not a single sentinel bird got infected. No virus was re-isolated from the infected farms since the start of vaccination indicating no virus re-circulation. It took us two months from the start of vaccination to completely eradicate AI in all the infected farms. Three farms have never been infected and have remained negative to present. It is also very critical and important that a vaccine bank for AI is readily available to prevent the infection from getting out of hand. Also, the impact of the live bird market in the Northeast is still very important in the control AI in the area.

COMPARISON OF SEROLOGICAL METHODS FOR THE DETECTION OF ANTIBODIES TO AVIAN INFLUENZA VIRUS

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ABSTRACT

Influenza type A viruses can infect avian, porcine, equine, and other species including humans. Avian influenza virus (AIV), also known as Fowl Plague, is a viral disease of domestic and wild birds that is characterized by a full range of responses from almost no signs of the disease to very high mortality. Fifteen serologically distinct hemagglutinin and nine neuraminidase subtypes of Influenza type A virus have been isolated from avian species. Subtypes H5 and H7 can be associated with significant to catastrophic losses. Disease signs in poultry range from only a slight decrease in egg production to a highly fatal fulminating infection. Signs of infection may include respiratory problems, edema of the head and face, or diarrhea.

The objective of this study is to improve the sensitivity and specificity of the current commercial AIV ELISA as compared with standard serological methods for the detection of antibodies to AIV.

A novel ELISA for the detection of antibody to AIV using a histidine-tagged recombinant H9N2 nucleoprotein was developed. The ability of the new ELISA kit to detect antibodies to AIV is compared with standard serological methods and the current commercial ELISA. Our studies demonstrate that performance is enhanced with the use of the recombinant nucleoprotein. The improved kit allows for the detection of lower levels of antibodies to AIV as well as greater specificity as compared to the current commercial ELISA.

AN OVERVIEW OF THE 2004 OUTBREAK OF HIGHLY PATHOGENIC AVIAN INFLUENZA IN BRITISH COLUMBIA

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SUMMARY

In February 2004, low pathogenicity avian influenza (LPAI), subtype H7N3, was diagnosed on a commercial broiler breeder farm in Abbotsford, British Columbia. Within 10 days of the onset of relatively mild clinical signs in one barn, the virus mutated into a highly pathogenic form (HPAI) when it infected the birds in an adjacent barn causing massive acute mortality. HPAI is a List A notifiable disease which has never before been diagnosed as a multi-farm outbreak in Canada. The Canadian Food Inspection Agency (CFIA) is mandated with the responsibility for timely eradication of all List A diseases to ensure minimal disruption to international trade of agricultural livestock products exported from Canada. Through the combined efforts of multi-level government agencies and especially the poultry industry itself, this HPAI outbreak was contained within the Fraser Valley and the objective of eradication was achieved within three months. It is fair to say that this outbreak turned out to be one of the most significant veterinary challenges that Canadian animal health officials have ever had to face. A detailed step-by-step review of the chronology of this HPAI outbreak illustrates the unpredictable evolving challenges facing disease control strategies and eradication efforts. HPAI is a formidable foe.

INTRODUCTION

The Fraser Valley of British Columbia (BC), located in the far southwest corner of the province just outside of Vancouver, is a concentrated multi-commodity agricultural area encompassing approximately 400 square miles. Eighty percent of the provincial domestic poultry market is raised here, an anytime total of 17 million commercial birds. In addition, there is a well established "specialty poultry" sector that produces organic free-range chicken & eggs, squab, pheasant, tinamou, gamebirds, ducks and geese for both domestic and export markets. The Fraser Valley is also home to hundreds of small backyard mixed species flocks.

Despite sharing territory with the migratory waterfowl of the Pacific Flyway, passive surveillance for avian influenza (AI) through routine diagnostic submissions to the provincial veterinary diagnostic laboratory (Animal Health Centre) has detected non H5 and H7 strains in commercial poultry approximately once every three years. There has never been any active targeted surveillance for AI in either poultry or wild birds.

INDEX FARM

The farm identified as the index case in this outbreak is a very well managed broiler breeder operation with two lay barns of approximately 9,000 birds each and a pullet-rearing barn that was empty at the time. The first barn to be affected contained 52-week-old birds that experienced a sudden onset of reduced feed consumption rate and a slight increase in mortality. An H7N3 avian influenza virus of low pathogenicity was isolated from this flock. Within 10 days the adjacent barn containing 24-week-old birds experienced an alarming increase in overnight mortality and a HPAI virus containing a 21-base insertion sequence at the HA-cleavage site was eventually recovered indicating that the LPAI had mutated at some point between the first and second barns (1). It was initially believed that HPAI had been contained on the index farm due to the strict "self-quarantine" immediately implemented by the producer and poultry veterinarian but 12 days following the depopulation of the index farm, and three weeks after onset of the first clinical signs of HPAI, a second broiler breeder farm 1.5km away broke with HPAI. The infection has now become an outbreak.

OUTBREAK CHRONOLOGY[1]

February

6 (Day -12): Producer notices that his older flock of birds (Flock A) is taking much longer to eat the allotted feed (feed consumption ultimately remains steady). Mortality is 8 (4 is expected). Owner suspects a problem with a recently delivered load of feed and notifies his poultry veterinarian, Dr. Stewart Ritchie.

7 (Day-11): Veterinarian visits the flock. Feed is replaced but without resolution of feed consumption rate. On farm necropsy reveals severe deep pectoral myopathy but no obvious reason for feed refusal. Weekend mortality is 9 and 11.

9 (Day-9): Birds from Flock A are submitted to the provincial veterinary diagnostic lab (Animal Health Centre) for diagnostic work-up. Gross necropsy findings are non-specific (slightly meaty & congested lungs and slightly reddened tracheas), further tests pending. Mortality is 16. Over the next few days the 0.5% mortality rate and feed refusal resolves and the production drop of 20% is improved by 10%. *Flock B shows no clinical signs*.

13 (Day-5): Lung samples test suspect for Influenza A virus by PCR. This information is phoned to the veterinarian, who in turn phones his client and suggests a "self-quarantine" until diagnostics are completed. The owner complies.

15 (Day-3): Vet visits farm to investigate an overnight increase in mortality in the younger Flock B thought to be related to a barn "disturbance" (novel weekend egg collectors). Flock A has returned to normal. *Flock B mortality is 100.*

16 (Day-2): An AI virus is isolated and identified from the older Flock A birds. The CFIA district veterinarian is notified.

17 (Day-1): Overnight mortality in Flock B is reported as another 100. The referring and CFIA veterinarians visit the index farm and examine the flock. *No clinical signs noted at that time*. Flock B birds are submitted to the Animal Health Centre (AHC) and the CFIA vet attends the necropsy. The lung and tracheal lesions are similar but more pronounced than those seen in the previous submission of Flock A birds. The CFIA veterinarian is provided with virus culture isolated from Flock A and fresh affected lung tissue from Flock B. It is not realized at that time that those samples contain two very different viruses. *Overnight mortality in Flock B is 200.*

18 (Day 1): Diagnostic samples arrive at the National Centre for Foreign Animal Disease Laboratory (NCFAD) in Winnipeg, Manitoba. Testing is expedited and NCFAD confirms AI in Flock A, pathogenicity designation pending but assumed at this point to be LPAI. Regardless, the index farm is placed under official quarantine. National teleconference held at 4 pm. Public Health officials voice concern this may be an introduction of the Asian H5 strain. *Overnight mortality in Flock B is 800.*

19 (Day 2): National teleconference called by Public Health officials at 4AM in anticipation of the subtyping test results. Later that morning CFIA confirms the isolate from Flock A to be subtype H7 with an HA gene sequence compatible with LPAI. Test results pending for N subtype, IVPI pathogenicity testing and isolation of the Flock B virus. Based on the finding of an H7 subtype, despite low pathogenicity, CFIA moves to depopulate the index farm. This is a precedent-setting decision since HPAI has not yet been confirmed. This was a direct result of the current OIE discussions making all H5 and H7 AI subtypes subject to eradication regardless of pathogenicity since recent global occurrences of HPAI have indicated the ease with which H5 and H7 LPAI can mutate into HPAI. A 5km zone of active surveillance is established around the farm. Good natural boundaries. *Overnight mortality in Flock B is 1000.*

20 (Day 3): Major news conference involving Public Health, CFIA and BCMAFF. That night media broadcasts from outside the index farm (biosecurity??). The virus from Flock A is confirmed to be LPAI with N3 subtype.

20/21/22/23 (Day 3/4/5/6): The index farm is depopulated by CO_2 gas euthanasia. On-farm composting site reaches capacity and the remaining 40% of carcasses are placed into sealed totes in reefer trucks. Huge operational challenges due to lack of protocols and experience in mass euthanasia of poultry barns and biocontainment during the disposal process.

24 (Day 7): Reefer trucks take carcasses to a curtain burn site in Princeton, BC 200 km (135 miles) away since the local municipal incinerator operator refused to cooperate without order to do so (public health messaging has been alarming and has caused them to back away).

March

1 (Day 13): H7N3 now confirmed in Flock B. Pathotype has not been designated although highly suspicious for HPAI clinically.

9 (Day 21): Birds submitted to the AHC for a routine lameness work-up on March 4 test positive for AI by PCR. This farm is 1.5 km from the index farm. It has four barns of four ages of broiler breeder chickens (3w, 13w, 33w, 45w), totaling 24,000. NCFAD confirms HPAI from index Flock B, (IVPI=2.87). **CFIA officially declares an outbreak of HPAI to the OIE.** Thirty-six countries immediately sanction Canada.

11 (Day 23): A 5 km High Risk Region (HRR) is established. Also a surveillance zone and outer control zone with *severe movement restrictions of poultry products outside of the entire Fraser Valley.* CFIA Emergency Operations Centres (EOC) set up in Abbotsford and Ottawa.

15 (Day 27): The City of Abbotsford assists with road closures and sanitation stations at the border of HRR but there are reports of waiving vehicles through without tire disinfection. Public Information notices in newspapers, mailboxes, 1-800, website, radio.

16 (day 28): Reefer unit fails on a truck and leaks into storage yard; public concern over the stench gathers media attention. Task Force worker with conjunctivitis infected with H7N3 HPAI.

17 (Day 29): Processors indicate storage capacity has been reached at 10M kg, with 500K kg still coming

in. CFIA to weekly test five birds/premise in the surveillance region. Since there was no prior accreditation by CFIA for AI testing during an outbreak by the local BCMAFF veterinary lab (despite proven capability including RT-PCR), all samples are required to be sent to NCFAD causing serious delays in depopulation. Testing delays causes so much virus to be generated in these heavily stocked barns that mortality sometimes reaches >95%.

18 (Day 30): Depopulation of second IP complete (nine days after testing positive by PCR). Depopulation techniques are questionable due to the use of an outside portable electrical stunning chute designed for spent fowl and reports of significant feather fly. Suspected compromise of biocontainment during the depopulation process. Cooked and processed product allowed to move beyond the control area.

19 (Day 31): A third broiler breeder farm in the HRR is suspected positive by PCR. The poultry industry offers a proposal to CFIA (via teleconference with Ottawa) that they would voluntarily depopulate all the commercial barns within the HRR at industry expense (est. $500,000). CFIA agreement is required since they would issue the movement permits. Considered and refused by CFIA since the outbreak is considered to be under control.

23 (Day 35): Four additional flocks within the HRR test positive, including the province's only Turkey Breeder farm. Total number of Infected Premises (IP) is seven, all commercial poultry.

24 (Day 36): Informational tele-seminar for industry with 200 moderated phone-in lines. CFIA agrees to depopulate all poultry in the HRR (16 commercial, 34 backyards). Public Health requires vaccination of all workers at least two weeks in advance as well as taking anti-viral Tamiflu; this severely restricts the available workforce. Processors start laying off workers.

25 (Day 37): The third IP is depopulated (11 days following PCR detection).

28 (Day 40): The only backyard flock in the HRR from which any AI was isolated becomes clinical three days after the commercial farm (IP #3) across the road was depopulated. This flock had tested negative twice in the previous two weeks. This indicates lack of biocontainment in the commercial depopulation process, with potential airborne spread.

From Feb 16, the Vet Lab tests all poultry submitted (61 farms) for AI, representing >1 million birds at risk. Retrospective serology of banked sera going back 3 months also does not reveal the prior presence of AI in the broiler breeder population. We still do not know the original source of LPAI.

April

1 (Day 44): A commercial layer farm near the Abbotsford International Airport tests positive for AI (well outside the HRR). A Poultry Industry liaison is invited into the EOC.

2 (Day 45): Federal Minister of Agriculture and the Chief Veterinary Officer for Canada meet with BCMAFF and Industry. Industry requests HRR to expand to encompass the entire Fraser Valley and offers to empty the commercial barns. Specific positive farm info cannot be released to industry due to confidentiality. Producers feed and egg trucks still don't know which farms are positive.

4 (Day 47): 20 positive commercial farms (15 within HRR). Human flu-like illness 15 cases, H7N3 recovered from two cases of conjunctivitis. Five testing protocols: Depop (high priority), Dead Bird Surveillance in enhanced zone (five-bird pools; two tests/barn; three times weekly), regular surveillance (2X week), msc calls & sick bird calls and pre-slaughter (25 birds/barn in pools of five). Weybridge confirms HPAI

5 (Day 48): CFIA announces total depopulation of the entire Fraser Valley involving the systematic depopulation of all estimated 19M birds, including backyard flocks, over a six week period. CFIA charters plane for nightly runs to NCFAD. NCFAD shares AI testing protocols with Vet Lab and is satisfied with the completion of a proficiency panel. Vet Lab to test the pre-slaughter samples (<36 hours to slaughter). Province issues Ministerial order under the Emergency Programs Act to activate the **Provincial Emergency Plan (PEP), bringing the "incident command system" into play.** The Ministerial order also enforces the use of local incinerators and landfills.

Three key events have now occurred that will expedite control of the burgeoning outbreak:

1. The poultry industry will work together and either process, render or compost over 13.5 million negative commercial birds and by emptying almost 400 farms there will be a lack of susceptible birds for the virus to spread to. Depopulation deadline is 6 weeks.
2. The expertise of PEP will more efficiently manage the logistics of the emergency.
3. CFIA can now focus on identification and depopulation of positive farms.

7 (Day 50): Depopulation of the HRR is completed (14 commercial farms, 34 backyard flocks. The Netherlands is contacted for advice in handling the outbreak.

12 (Day 55): 25 IP.

13 (Day 56): 28 IP.

16 (Day 59): 31 IP. Farm stress line established. Central composting facility for negative birds developed due to backlog of carcasses. Min security prisoners brought in as disposal workforce. Processors need 2M kg/week to supply market; table eggs imported to keep grader working. Hatcheries stop setting eggs.

20 (Day 63): 34 IP. 15.9M birds remain. Pull-back of complete Fraser Valley cull. Backyard flocks within 3 km of an IP are ordered destroyed.

22 (Day 65): 36 IP.

26 (Day 69): 40 IP.

May

6 (Day 79): 14M depopulated (12.8M negative, 1.2M positive); 84% processed, 18% composted/incinerated

19 (Day 92): The last IP identified, total 42 commercial premises. The 21 day clock starts. All commercial poultry barns in the Abbotsford area are waiting empty.

June

Ongoing: Cleaning and disinfection of all positive premises with CFIA sign off of last positive farm June 18 (Day 123). The second 21 day clock starts. The C&D costs the industry $3 million dollars.

July

9 (Day 143): Official restocking date. Industry repopulates negative barns but allows IP's to remain empty to pass the 60 days mark to avoid the CFIA surveillance requirement.

August

18 (Day 182): CFIA announces the Fraser Valley AI Response concluded. Control area movement restrictions are lifted. It is estimated that the BC poultry industry will not experience full production recovery for at least 18 months.

CONCLUSION

The last positive farm was detected May 19, 2004, just over 92 days after initial detection of HPAI, with a final total of 42 commercial premises and 11 backyard flocks being identified as positive in this outbreak.

From the outset no one could have predicted the direction this outbreak would take or the obstacles that would arise to challenge an unprepared industry and the unpracticed disease response plans of the governmental agencies responsible for disease eradication. A comprehensive review of the outbreak dynamics is vital to modeling future approaches to effective and efficient disease outbreak management. The recovery aspect of this outbreak began even before the barns had been emptied. Teams comprised of members from industry, provincial and federal governments worked together to address the issues of greatest importance: compensation, restocking strategy, enhanced biosecurity and modification of current disease response plans based on the valuable lessons learned from this outbreak.

An outbreak such as this will happen again, somewhere soon, someplace else, and we need to clearly relay the lessons we've learned so no other livestock industry or agricultural community has to endure this type of fear, frustration and loss. It should be about learning from a unique experience, sharing these lessons and advancing effort in early disease detection, local disease response plans, disease containment strategies and expedited recovery. Hindsight is a precious gift.

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[1]On February 18, 2004, the CFIA confirmed that the diagnostic sample provided to them by the BCMAFF-Animal Health Centre (Vet Lab) contained an AI virus and issued a quarantine of the index farm. This day has been considered the official Day 1 of the outbreak although clinical signs in the birds preceded this date (represented in the negative).

CHARACTERIZATION OF H7N3 INFLUENZA VIRUSES ISOLATED DURING THE FRASER VALLEY OUTBREAK IN 2004

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SUMMARY

In February 2004 a highly pathogenic avian influenza (HPAI) outbreak erupted in the Fraser Valley of British Columbia. Our investigations indicated that the responsible HPAI H7N3 virus emerged suddenly from a low pathogenic avian influenza (LPAI) H7N3 virus precursor. Analysis of the hemagglutinin (HA) genes of the LPAI and HPAI viruses isolated from the index farm revealed the only difference to be the presence of a 21 nucleotide insert at the HA cleavage site of the HPAI virus. We deduced that this insert most likely arose as a result of non-homologous recombination between the HA and matrix (M1) genes of the same virus. Over the course of the outbreak, a total of 37 isolates with, and three isolates without inserts were characterized. Isolates with inserts could be further categorized into seven variants based on amino acid substitutions which occurred within or adjacent to the insert. Intravenous pathogenicity indices ranged from 2.17 to 3.00 depending on the isolate, and the lesions in four- to six-week old and adult layer Leghorn chickens were consistent with those expected for a highly pathogenic virus. The events described here appear very similar to those which occurred in Chile in 2002 where the virulence shift of another H7N3 virus was attributed to non-homologous recombination between the HA and nucleoprotein gene.

(The full length article will be published in the *Journal of General Virology*.)

HUMAN ILLNESSES DUE TO AVIAN INFLUENZA H7N3 IN BRITISH COLUMBIA, CANADA

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ABSTRACT

Highly pathogenic avian influenza (HPAI) viruses have recently caused human illness ranging from mild to severe with substantial mortality. Our objective was to detect and describe human illness following exposure to infected poultry during a 2004 HPAI H7N3 outbreak in British Columbia.

Enhanced surveillance for conjunctivitis and/or influenza-like illness (ILI) was implemented among individuals exposed to poultry during the outbreak. Respiratory & serum specimens and detailed symptom and exposure histories were collected from symptomatic individuals and their close contacts.

Enhanced surveillance identified 57 people meeting the suspect or confirmed avian influenza case definition. The most common conjunctivitis symptoms were itchy, red and burning eye(s) and the most common ILI symptoms were cough, sore throat and coryza. Influenza H7N3 virus was isolated from two persons, both of whom had direct conjunctival contact with infected poultry. Both developed conjunctivitis, one also developed headache and the other coryza. No secondary cases were identified.

This study reports the first confirmation of influenza A H7N3 infection in humans after direct contact with HPAI infected poultry. Associated illness was localized and mild, and there was no further person-to-person transmission. Personal protective measures and close monitoring of human contacts may have prevented further illness and adaptation of a new influenza virus to the human host.

THE HISTORY OF *EIMERIA MIVATI*

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SUMMARY

In 1959 *E. mivati* was isolated from Florida following persistent and unusual outbreaks of intestinal coccidiosis in commercial chickens. The organisms were collected and the unknown *Eimeria* were labeled "X" and "Y" from Florida. Following extensive work, single oocyst isolations, immunization and cross-immunization trials with previously named species such as *E. mitis*, *E. acervulina* and *E. praecox*, the X and Y organisms were named *Eimeria mivati* ("wondering" coccidia). During the 1960s to 1980s *E. mivati* was an accepted species. Since the mid 80s, its validity has been questioned by some researchers.

Coccidiosis in chickens is primarily a disease of the intestine and ceca. There are pathological signs and lesions that are characteristic for each of the *Eimeria* species. There is a notion that there are seven recognized species; however, in most of the literature, nine species of coccidia have been described for the chicken. By 1929 four *Eimeria* species were identified and described: *E. mitis*, *E. acervulina*, *E. tenella*, and *E. maxima*. Between 1930 and 1945 *E. brunetti*, *E. praecox*, *E. necatrix* and *E. hagani* were named and described.

In 1964 *E. mivati* was added to the list of coccidia that affected chickens. This species was first recovered from the Hoerig's chicken farm in Zephyr Hill, Florida. After the naming and description of this new species researchers described finding this parasite throughout the world. Long and Tanielian (7), reported *E. mivati* in Lebanon; Reid *et al.* (10) reported *E. mivati* in Great Britain, Germany, Holland, France, and Canada. In 1967 Long stated that *E. mivati* can be confused with *E. acervulina* and *E. mitis* because all three species have small round oocysts and parasitize the same region of the small intestine. Long (6) suggested that *E. mivati* should be considered *E. acervulina* var mivati. Shirley (11) used a starch gel electrophoresis for a biochemical identification of some of the species of chicken *Eimeria*. The electrophoresis methodology showed that the Houghton *E. acervulina* and the *E. acervulina* var mivati were closely related.

For many years, *E. mitis* received little attention as a parasite of importance for the chicken. However, in 1977 Fitz-Coy and Edgar at Auburn University (AU) secured two isolates of *E. mitis* from commercial broiler farms from Mississippi and Georgia (MS thesis). Fitz-Coy and Edgar re-described the strains of *E. mitis* (biology, pathology and pathogenicity – PhD dissertation). In 1979 aliquots of the two strains of AU *E. mitis* were sent to Ruff at Beltsville, MD. Also, in 1979 Shirley stated that the *E. mivati* being used at Houghton Poultry Research Station (HPRS) was contaminated with *E. acervulina*. Norton and Joyner (8, 9) stated that *E. acervulina* can be easily differentiated from *E. mivati* and *E. mitis*, but *E. mivati* from *E. mitis* are more difficult to differentiate. During the early 1980s, Long from Houghton Poultry Research Station requested an aliquot of the AU strain of *E. mitis*. The GA strain of *E. mitis* was sent to Houghton Poultry Research Center. The purpose was to evaluate the Auburn strain of *E. mitis* via the electrophoreses methodology used by Shirley at the Houghton facility. The results were that the AU *E. mitis* was quite different from the isolates being used in the U.K. laboratory. It was accepted, *E. mitis* was different from the other species.

Some time later, Long requested a sample of *E. mivati* from AU. An aliquot of a recent field isolate from the AU coccidiosis repository, which was considered a good candidate for *E. mivati,* was sent to the UK. The recommendations to the UK group were to conduct single oocyst isolations to develop a pure line for the electrophoresis evaluations. In 1984, after receiving the coccidia, Shirley *et al*. reported that the sample received from AU was probably a combination of *E. acervulina* and *E. mitis*. The recommendation by the European research group was to consider *E. mivati* nomina dubia.

Tyzzer (14) described an outline for isolation and identification of new *Eimeria* species from gallinaceous birds. Tyzzer's methodology used by Edgar follows: Immunization and cross-immunization studies were conducted with isolates of *E. acervulina* and *E. mitis* from the laboratories of Drs. Moorehouse, Stroud, and Dickinson along with isolates of the same species from AU. Previous studies by Edgar has shown that *E. mivati* were fairly prevalent; in fact, 50% of samples were positive for these parasites. Data from Edgar's files show that chickens immunized with *E. mivati* and challenged with *E. acervulina* (Moorehouse or Auburn strains) were not protected from challenge (Table 1). Chickens that were immunized against *E. acervulina* (Moorehouse) and challenged with *E. acervulina* (Auburn and Moorehouse) showed a high

degree of protection; but when challenged with the *E. mivati* isolate, there was no protection (Table 2). In another trial, chickens immunized against *E. acervulina* (Auburn) and challenged with *E. mitis* (Stroud) or *E. mivati* showed no protection (Table 3).

During 1988 to 1990, three isolates thought to be *E. mivati* were isolated from commercial broiler farms. Preliminary immunization and cross-immunization trials with one of the isolates showed some similarities to those mentioned earlier in Edgar's work. Chickens that were immunized with *E. acervulina* and challenged with *E. mivati* were not protected, but when immunized with *E. mivati* and challenged with *E. mivati*, were protected.

Based on the writer's observations from field isolates and routine necropsy evaluations of chickens from commercial broiler farms, organisms that morphologically resemble those of *E. mivati* are seen. These organisms will appear in great abundance and found many times throughout the entire small intestines. Based on the writer's evaluations, the prevalence of these organisms could be as high as 20%. The oocysts of *E. mivati* are smaller than *E. acervulina* and *E. mitis* and are broadly ovoid.

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Table 1. Chickens immunized with *E. mivati* and challenged with *E. acervulina* (Auburn or Moorehouse strains).

Immunized	Challenged	Upper Duodenum	Lower Duodenum	Yolk Stalk	Ileum	Ileo-Cecal Junct	Rectum
E. miv	E. ac	++++	++++	Neg	Neg	Neg	Neg
No	E. ac	++++	++++	Neg	Neg	Neg	Neg
E. miv	E. ac/pr	++++	++++	Neg	Neg	Neg	Neg
No	E. ac/pr	++++	++++	Neg	Neg	Neg	Neg
E. miv	E. ac(M)	++++	+++	Neg	Neg	Neg	Neg
No	E.ac(M)	++++	++	Neg	Neg	Neg	Neg

From the files of .S.A. Edgar

Table 2. Chickens immunized with *E. acervulina* (Moorehouse) and challenged the homologous strain *E. acervulina* (Auburn) or *E. mivati*.

Immunized	Challenged	Upper Duodenum	Lower Duodenum	Yolk Stalk	Ileum	Ileo-Cecal Junct	Rectum
No	E. ac (M)	++++	Neg	Neg	Neg	Neg	Neg
Yes (M)	E. ac (M)	Neg	Neg	Neg	Neg	Neg	Neg
No	E. ac (A)	++++	Neg	Neg	Neg	Neg	Neg
Yes (M)	E. ac (A)	Neg	Neg	Neg	Neg	Neg	Neg
No	E. mic	++++	Neg	Neg	Neg	Neg	Neg
Yes (M)	E.miv	+++	Neg	++++	+++	+++	+

From the files of S.A.Edgar

Table 3. Chickens immunized with *E. acervulina* (Auburn) and challenged the homologous species, *E. mitis* or *E. mivati*.

Immunized	Challenged	Upper Duodenum	Lower Duodenum	Yolk Stalk	Ileum	Ileo-Cecal Junct	Rectum
E. a	No	Neg	Neg	Neg	Neg	Neg	Neg
E. a	E. miv	++++	+++	++	++	++	++
E. a	E. mit	+	+	+++	++++	+++	++++
No	E. a	++++	++++	Neg	Neg	Neg	Neg
No	E. miv	++++	+++	+++	+++	++	+
No	E.mit	Neg	++	+++	++++	++++	+++

From the files of S. A. Edgar

Table 4. Chickens immunized with *E. acervulina* or *E. mivati* and challenged with homologous or heterologous organisms.

Immunized/ Challenged	% Gain	F/G
Control/-----	100	1.88
Control/*E.mivati*	66	2.44
Control/*E. acerv*	51	3.00
E. acervulina/----	99	1.88
E. acerv/E. mivati	61	2.60
E. acerv/E. acerv	98	1.82
E. mivati/-----	100	1.90
E. mivati/E. miv	90	1.86
E. mivati/E. acerv	72	2.15

THE PATHOLOGY AND PATHOGENICITY OF RECENT ISOLATES OF *EIMERIA MIVATI*

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SUMMARY

Since 1991 and 2001, several field isolates of *Eimeria* that fit the description of Edgar's *E. mivati* have been isolated. Three of these isolates have been purified and the pathogenicity studied. These organisms, as were those described by Edgar, are highly pathogenic; causing morbidity and mortality, weight and feed conversion impairment. Pathological findings were hyperemia, mucus production, hemorrhage into the lumen, and slight to moderate swelling of the intestinal tissue.

In 1959, *E. mivati* was isolated in Zephyr Hill, Florida following persistent and unusual outbreaks of

intestinal coccidiosis in commercial chickens. The organisms were collected, and the unknown *Eimeria* were labeled "X" and "Y" from Florida. Since 1991 and 2001, several field isolates of *Eimeria* that fit the description of Edgar's *E. mivati* have been isolated. Three of these isolates have been purified and the pathogenicity evaluated. *E. mivati* is moderately pathogenic for chickens and on some occasions has caused mortality. Several probable *E. mivati* isolates have also been retained from field samples. In one such study, a mortality rate of 50% occurred in a group of naïve chickens; however, there was no pathology in the hyper-immunized hatch mates. Growth rate of infected chickens can be severely affected by *E. mivati*. Severe infections with these parasites may cause a negative growth pattern in young broiler chickens (Tables 1 and 2).

The signs and lesions with *E. mivati* are: listlessness, anorexia, ruffled feathers, watery droppings, followed by viscid and mucoid droppings. A tinge of blood may be seen in the droppings and a fetid odor from the feces may be detected. In the early infection, the mucosal surface of the upper third of the intestines may appear hyperemic; there may be a noticeable swelling of the affected portion of the intestine. As the infection progresses there may be a trace of blood in the lumen of the intestines, eventually white spots will appear. Microscopic findings are: engorged blood vessels, edema, eosinophilia, and many gametocytes and oocysts in the affected areas. Young broiler chickens inoculated with *E. mivati*, lesions were seen throughout the entire small intestines.

In recent years, routine necropsy evaluation of commercial broiler chickens has revealed organisms that morphologically resemble the organisms described as *E. mivati*. These organisms appeared to be in great abundance and are found many times in the entire small intestines. The oocysts of *E. mivati* are smaller than *E. acervulina* and are broadly ovoid.

Based on the writer's data, the prevalence of these organisms could be approximately 20% and is pathogenic for chickens.

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2. From the files of the late Dr. S. A. Edgar.

Table 1. Growth rate and mortality pattern of chickens inoculated with *E. mivati*.

Treatments	Gain/bird @ 7 days pi	Gain/bird@ 14 days pi	% Mortality
1	137	271	0
2	48	155	0
3	-4	132	0
4	-38	110	10

Oocysts inoculated per bird
1 = none; 2 = 750, 000; 3 = 5,000,000; 4 = 5,000,000 plus 5,000,000 at two days pi
After SAE 1964

Table 2. Percent growth and mortality pattern.

Treatments	% Gain	% Mortality
1	100	0
2	73	0

Oocysts inoculated per bird
1 = none, 2 = 500,000

COCCIDIOSIS SENSITIVITY TO CHEMICAL COMPOUNDS IN THE SOUTHEASTERN UNITED STATES

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INTRODUCTION

Resistance to synthetic chemical anticoccidial products has been a well recognized phenomenon under sustained field usage. Tamas, *et al*. (1991) reported that resistance to these products developed after sustained use. To gain a better understanding of current patterns of resistance a preliminary series of sensitivity studies was carried out to explore the sensitivity of a number of chemical anticoccidial compounds. In the fall of 2003 litter samples were collected from three different commercial operations in the Southeastern U.S. Among the comparisons made in this preliminary test were amprolium, amprolium + ethopabate, diclazuril, nicarbazine (Nicarb) and nicarbazin and narasin (Maxiban). A response was seen to the two compounds, Nicarb and Maxiban that had been reported by Bafundo and Jeffers (1990). Because differences were noted in this pilot study, additional sensitivity tests were carried out in 2004 with 12 additional isolates to further elucidate differences that could exist between Nicarb and Maxiban in critical coccidiosis control parameters.

MATERIALS AND METHODS

Collection of field samples. In addition to the three initial isolates, litter samples were collected from 12 broiler complexes in the summer of 2004 representing a cross section of commercial companies in the southeastern 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 and put on ice.

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 birds 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 12 battery tests and was conducted from September through December 2004. 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 (1,080 birds). Treatments consisted of chicks fed diets containing either Nicarb (113 g/ton) or Maxiban (72 g/ton), or a non-medicated control. Batteries were in an environmental structure with even illumination and a stocking density of 0.63 $ft.^2$/ 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 75,000 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 (1970). Lesion scores were determined for upper, middle, and cecal regions of the intestine and scores reported as average of all regions.

RESULTS

The 15 isolates were combined and are reported as follows:

Weight gain. Birds receiving the non-medicated control diet gained the least weight ($P < 0.05$) compared to those receiving either Nicarb or Maxiban. There was a trend for broilers receiving Nicarb to have greater weight gain ($P = 0.10$) than those receiving Maxiban.

Feed efficiency. Birds fed the non-medicated control diet had the least favorable feed efficiency compared to broilers fed Nicarb or Maxiban ($P < 0.05$). Birds receiving Nicarb had a six point, non-significant advantage in feed efficiency compared to those receiving Maxiban.

Lesion score. Broilers receiving the non-medicated diet had the highest average intestinal lesion

score ($P < 0.001$) followed by Maxiban. Birds receiving Nicarb had significantly lower lesion score compared to those fed either the non-medicated control or Maxiban diets ($P < 0.001$).

CONCLUSION

Overall results were consistently better for birds fed Nicarb compared to Maxiban. The 15 litter samples collected for this study contained oocysts that were more sensitive to Nicarb than Maxiban as evaluated by weight gain, feed efficiency, and lesion score.

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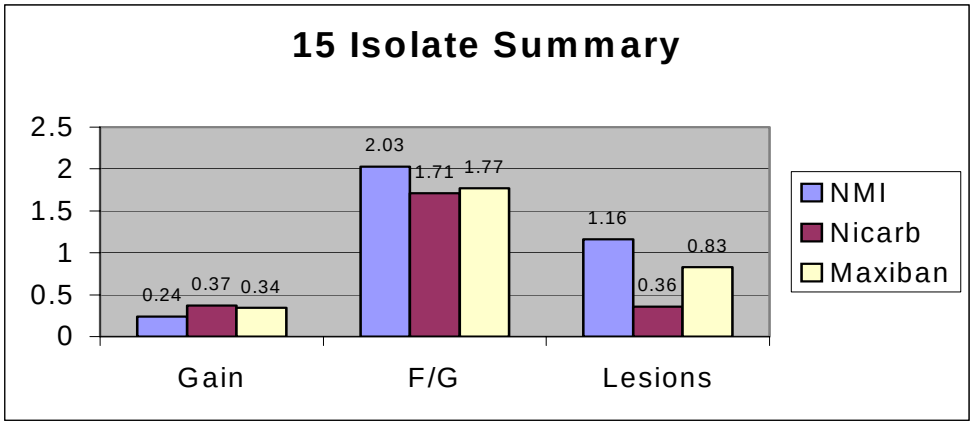
Figure 1. Summary of isolates.



UNIFORM DELIVERY OF INOVOCOX™ COCCIDIOSIS VACCINE OOCYSTS THROUGH THE INOVOJECT™ AUTOMATED *IN OVO* INJECTION SYSTEM

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SUMMARY

Inovocox™ is a live coccidiosis vaccine under development for the prevention of coccidiosis disease in broiler chicks. A series of trials were conducted to evaluate consistent and uniform administration of the coccidiosis vaccine oocysts by automated delivery through the Inovoject™ system. Both the McMaster's counting method and the Coulter Multisizer counter were used to enumerate delivered oocysts. Studies were performed with different doses of vaccine in the presence and absence of cell-associated Marek's disease vaccine. Results showed that all embryonated eggs will receive an equivalent dose of vaccine and the same ratio of vaccine components when delivered using the Inovoject system.

INTRODUCTION

Broiler *in ovo* vaccination is in wide practice today, particularly in the United States, where hatcheries have routinely incorporated this procedure in their daily operations. Commercial *in ovo* vaccination against Marek's disease began in the US in 1992 and is presently occurring in hatcheries in more than 40 countries.

Inovocox is a live oocyst coccidiosis vaccine under development by Embrex for administration *in*

ovo. Previous research has demonstrated the feasibility of achieving immunity against coccidiosis via an *in ovo* delivered live vaccine (1,4,5). Automated *in ovo* delivery of coccidiosis may be more advantageous than other methods of vaccine administration as it provides convenient and uniform dosing to every chick in the flock. It is anticipated that coccidiosis vaccine would be administered simultaneously with other *in ovo* vaccines such as Marek's disease viral vaccine or infectious bursal disease virus vaccine. Coadminstration of Inovocox with either Marek's or bursal vaccines has been shown to not interfere with efficacy of either vaccine (2,3).

The objectives of these experiments were to demonstrate uniform oocyst delivery in individually dispensed doses over the timeframe of typical vaccine administration via the Inovoject system and the relative ratio of each *Eimeria* species delivered at these time points.

MATERIALS AND METHODS

As cross-protective immunity does not occur between *Eimeria* species, a commercial vaccine must provide protection against multiple species of *Eimeria*. The test formulation of Inovocox vaccine was comprised of *E. acervulina, E. tenella,* and two strains of *E. maxima*. Experiments were conducted using a 1X Inovocox concentration with or without cell-associated Marek's disease vaccine.

Inovoject and vaccine preparation. The Inovoject M-series egg injection system was used to deliver 50µL of the vaccine preparation to each egg in the egg flats. There are several different Inovoject models currently used in commercial hatcheries and therefore both a 36-injector and a 150-injector model were utilized in these experiments. Every effort was made to mimic standard hatchery conditions. The Inovoject system was sanitized, primed, and set to dispense 50 µL through the injector heads. A stock solution of the appropriate oocyst species was prepared and injected into Select's Marek's diluent. In designated experiments, the Marek's disease vaccine (HVT/SB-1) was also added to the diluent bag at a concentration of one dose per 50 µL. The bag was mixed thoroughly initially, but not mixed during the experimental run. Initial oocyst concentrations in the diluent bag were determined. Dispensed samples were either pooled, representing all 36 toolings or collected individually from representative toolings (5). Samples were collected at various times throughout the experiment. At the completion of each experiment, approximately 500mL of sterile saline was forced through the Inovoject and collected as a sample.

Sample analyses. Dispensed vaccine samples were enumerated by McMaster's counting method or by automated counting. As an effective vaccine needs to contain the appropriate ratio of each species in each dose, samples were analyzed by the Coulter counter. Coulter technology is based on the disruption of an electric field by particles in suspension. The technology allowed both the enumeration of particles in suspension as well as estimation of the size of each particle counted during the course of vaccine delivery.

RESULTS

Both the 36-tooling or 150-tooling Inovoject system provided consistent delivery of oocysts during the injection run time (Figure 1). The concentration of total oocysts/mL being dispensed from the individual injector toolings increased to expected concentrations at the end of the priming period. Percent expected concentration was calculated based on the initial oocyst sample taken directly from the vaccine diluent bag. Oocyst concentration plateau around the expected concentration after the priming phase of injection, typically before five minutes. The plateau remained steady throughout the remainder of the experiment indicating consistent delivery of the same number of oocysts throughout the vaccination run. Excluding the priming period, the concentration of oocysts being delivered individually by the selected/individual injector toolings did not significantly differ from each other. Data will be presented showing that the distribution of *Eimeria* oocysts in the mixed species vaccine did not vary between toolings or vary as the vaccine was delivered over time.

DISCUSSION

The experimental results demonstrate that Inovocox can be precisely and uniformly delivered through the commercial Inovoject system. The presence of Marek's disease virus vaccine does not interfere with vaccine delivery. Continuous mixing of the vaccine is not required for uniform delivery as the distribution of oocysts in a multiple species vaccine remains consistent over time. The accurate number of oocysts/dose is distributed to all parts of the injection system platform and the distribution of all *Eimeria* species exists at the beginning, middle, and end of the injection run. The Inovoject system has been proven to be a convenient method for broiler vaccination *in ovo*. The data presented here indicate that this method of vaccine administration can be readily adapted by the industry for Inovocox with each egg receiving a uniform and accurate dose.

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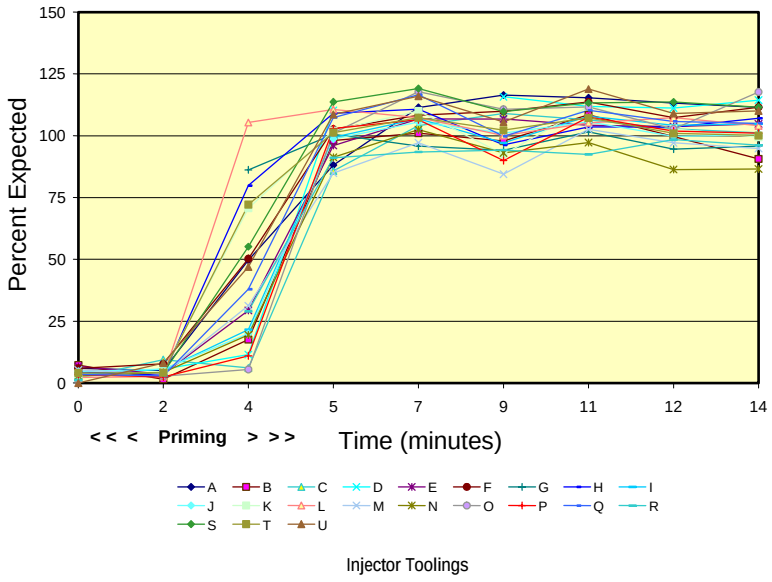
Figure 1. Monitoring consistent delivery of oocysts from 150-injector Inovoject system via Coulter method.



CURRENT AMPROLIUM ANTICOCCIDIAL SENSITIVITY STATUS OF TURKEY AND CHICKEN COCCIDIAL FIELD ISOLATES

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SUMMARY

A series of anticoccidial sensitivity tests were designed to determine amprolium's current anticoccidial sensitivity both in turkey and chicken coccidial isolates. Amprol and Amprol Plus proved to be very effective against chicken isolates that were predominately *E. tenella*, with lesion scores averaging below 0.5. Slightly less control of *E. maxima*, followed by only moderate control of *E. acervulina* was observed. Less control of *E. acervulina* was probably more of an efficacy issue than a resistance issue. Anticoccidial control by Amprol 125 ppm to turkey coccidial isolates was similar to Coban 73 ppm, Avatec 89 ppm, and Clinacox 1 ppm, except where

Clinacox had not been used before. This shows that currently amprolium is a highly effective anticoccidial, especially against *E. tenella*, with a low degree of resistance.

INTRODUCTION

While amprolium has been consistently used in the turkey industry, today there is renewed interest in older anticoccidials in broiler chickens. Amprolium was approved in the USA in 1960 as a feed additive at a use range of 80-250 ppm. Amprol Plus has added ethopabate 4 ppm to increase the species spectrum of activity. Due to water solubility, availability of other anticoccidials, and safety, amprolium has mostly been used as a water treatment medication in broiler chickens. Thus as a feed additive, amprolium has not been extensively used in broiler chickens. This is not the case with turkeys. A limited number of anticoccidials are available, and as with clearances with most feed additives, amprolium has been extensively used. The objective was to determine the current amprolium anticoccidial sensitivity status of broiler chicken and turkey coccidial field isolates.

MATERIAL AND METHODS

Commercial male broiler chicks or poults were used in all anticoccidial sensitivity tests. At the hatchery, the birds were sexed and received routine vaccinations. Upon arrival from the hatchery, birds were raised in Petersime battery cages. Even illumination was provided. Feed and water were given *ad libitum*. A complete randomized block design was used. Cages were blocked by location in the battery with block size equal to treatments. The floor space was 0.51 ft^2 (470 cm^2) per bird. The feeder space was 10 birds per 43 cm x 6.8 cm feeder. At twelve days of age (Day of Test (DOT) 0), the chicks or poults were grouped into sets of 10, group weighed, and then allocated to the experimental cages. Only healthy birds were selected. No birds (including cull or dead birds) were replaced during the course of the study. Each cage contained 10 male birds. Treatment feeds were issued. The treatments for the sensitivity tests for the broiler chickens coccidial isolates were: nonmedicated, noninfected (NMU); nonmedicated, infected (NMI);Amprol 125 ppm; Amprol Plus (125 ppm +4 ppm); Nicarb 125 ppm; Maxiban 79.2 ppm; and Clinacox 1 ppm. The treatments for the sensitivity tests for the turkey coccidial isolates were: nonmedicated, noninfected; nonmedicated, infected; Amprol 125 ppm; Coban 73 ppm; Avatec 89 ppm; and Clinacox 1 ppm.

The coccidial inoculums were made from coccidia isolated directly from broiler or turkey production houses. The poultry house litter served as the original source of coccidial oocysts. Coccidial oocyst inoculations were given on DOT 2. When the birds were 14 days of age, each NMU bird received 1 mL of distilled water by oral pipette (po). Birds in the other treatment groups received the coccidial inoculum diluted to a 1 mL volume (po). All inoculums contained approximately 100,000 sporulated oocysts/ mL.

The trial ended six days post inoculation (DOT 8) when the birds were 20 days of age. All birds were humanely sacrificed by cervical dislocation and weighed by cage. After weighing, the intestinal tracts of all birds from each cage were examined for coccidiosis lesions and given a numerical score. For the chicken coccidial isolates, depending on the species of *Eimeria*, either the upper (*E. acervulina*), middle (*E. maxima*), or cecal (*E. tenella*) regions of the intestine were scored using the system of Johnson and Reid (1970) (1), wherein 0 is normal and 1, 2, 3, or 4 indicate increasing severity of infection. For the turkey coccidial isolates, depending on the species of *Eimeria*, either the middle (*E. meleagrimitis*) or lower (*E. gallopavonis*) regions of the intestine were scored using a similar numerical scoring system.

RESULTS

Three coccidial isolates from broiler chicken houses (GA, AL, and MS) were tested. The GA isolate was predominately *E. acervulina*. The isolate caused a moderate infection with 18 % weight reduction and average lesion score of 2.4 in the NMI birds. All anticoccidials provided significant protection as seen by significantly lower percentage weight reductions (all less than 5%) and lesion scores all below 1.5 compared to the NMI. Amprol Plus reduced the *E. acervulina* lesions more than Amprol. Nicarb had only a lesion score of 0.5. Amprol showed similar control to Maxiban. Some lesions were observed in the Clinacox medicated birds. The AL isolate was a mixture of *E. acervulina*, *E. maxima*, and *E. tenella*. The isolate caused a significant infection with over 50% weight reduction and average lesion score of 3.0 *E. acervulina*, 2.1 *E. maxima*, and 3.5 *E. tenella* in the NMI birds. All anticoccidials provided protection as seen by significantly lower percentage weight reductions (all 30% less than NMI). Variation among results between anticoccidials was observed. Nicarb controlled all species with very low lesion scores. Amprol and Amprol Plus significantly reduced *E. maxima*, and to a greater extent, *E. tenella* lesions. Both Maxiban and Clinacox had similar coccidial control with only a moderate reduction in lesion scores. The MS isolate was a mixture of *E. acervulina* and *E. tenella*. The isolate caused a significant infection with over 35% weight reduction and average lesion score of 2.8 *E.*

acervulina and 2.5 *E. tenella* in the NMI birds. All anticoccidials provided protection as seen by significantly lower percentage weight reductions (all 30% less than NMI). Variation among results between anticoccidials was observed. Amprol and Amprol Plus significantly reduced *E. tenella* lesions with no visible lesions observed. Some *E. acervulina* lesions were observed in the Amprol and Amprol Plus medicated birds. Amprol Plus did reduce the *E. acervulina* lesions more than Amprol. Nicarb controlled both species with only an average lesion score of 0.1. Both Maxiban and Clinacox had again only moderate but similar coccidial control, with over 10% weight reduction and average lesion scores above 1.0.

Two turkey coccidial isolates (VA and MN) were tested. The VA isolate was predominately *E. meleagrimitis*. The isolate caused a moderate infection with 22 % weight reduction and average lesion score of 1.8 in the NMI birds. All anticoccidials provided significant protection as seen by significantly lower percentage weight reductions (all less than 10%) and lesion scores all below 1.4 compared to the NMI. Amprol, Coban, and Avatec showed similar control. Very low lesions and no weight reduction were observed in the Clinacox medicated birds. The MN isolate was predominately *E. gallopavonis*. The isolate caused a significant infection with 48 % weight reduction and average lesion score of 2.9 in the NMI birds. All anticoccidials provided significant protection as seen by significantly lower percentage weight reductions (all less than 25%) and lesion scores all below 2.0 compared to the NMI. Amprol, Coban, Avatec, and Clinacox showed similar control.

DISCUSSION

The *E. tenella* isolates tested were very sensitive to Amprol and Amprol Plus. The *E. maxima* isolate was controlled by Amprol and Amprol Plus. Amprol and Amprol Plus did show only moderate activity against *E. acervulina*. Amprol Plus had stronger *E. acervulina* anticoccidial activity than just Amprol. This reduced control by Amprol is due to species efficacy instead of resistance. Thus due to limited recent usage, resistance to Amprol and Amprol Plus is not a concern at this time. Resistance can develop to all anticoccidials. Sensitivity needs to be monitored for any anticoccidial program as demonstrated by the observed sensitivity of these isolates to Clinacox. All of the chicken coccidial isolates came from farms that had extensively used Clinacox. The appearance of moderate lesions and weight reduction indicated that all of these coccidial isolates were at least partially resistant to Clinacox. The turkey complex from where the VA coccidia were isolated had never used Clinacox, which explains its significant control. The turkey complex from where the MN coccidia were isolated had used Clinacox, and some partial resistance was observed. Coban and Avatec had been used at both of the turkey complexes.

Even though amprolium has been used for many years, these isolates showed comparable control by amprolium with the other available anticoccidials. This study was limited in the number of isolates tested and the absence of *E. adenoeides*. These results do agree with the literature that amprolium appears to have a broad species spectrum of anticoccidial activity against turkey coccidia (2).

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EFFECT OF MONENSIN ON THE INTESTINAL CARRIAGE OF *SALMONELLA* IN TURKEYS

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INTRODUCTION

The objective of this study was to evaluate the intestinal carriage of *Salmonella* in turkeys post monensin treatment.

MATERIALS AND METHODS

Five-week-old turkeys were divided into four groups and placed in isolation. Each group totaled 25 birds and was replicated four times for a total of 400 birds used in the experiment. One week post

acclimation to isolation rooms, birds in groups 1 and 2 were treated with monensin at 60 and 90 g / ton of feed respectively. Birds in groups 3 and 4 were not treated with monensin. At three days post monensin treatment birds in groups 1, 2, and 3 were orally inoculated with nalidixic acid resistant *Salmonella senftenberg* at the rate of 1 x 10^6 cfu/bird. Birds in group 4 were kept as no-monensin and no-*S. senftenberg* inoculated controls. At days 2, 7, 12 and 17 days post *S. senftenberg* infection, five birds from each group were sacrificed with CO_2 inhalation and liver, spleen, ceca, and a small area of small intestine were collected. A 10% homogenate was prepared in phosphate-buffered saline (PBS) with each sample and serial dilutions were prepared in PBS for quantitation of *S. senftenberg*. 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} dilutions of each sample were plated on Brilliant Green agar (BGA) containing 150ug/mL of nalidixic acid. Plates were incubated at 37^0C for 24 to 48 hrs and the *S. senftenberg* colonies were counted and tabulated. The data were analyzed for statistical significance. Statistical analysis of the data was performed using analysis of variance (ANOVA).

CONCLUSIONS

There was no significant difference between no-monensin and monensin at the rate of 60 g per ton of feed, in the reduction of *S. senftenberg* in infected turkeys.

There was significant difference ($P < 0.05$) between no-monensin and monensin at the rate of 90 g per ton of feed, in the reduction of *S. senftenberg* in infected birds. However this difference was not significant at the level of $P < 0.01$.

The reduction of *Salmonella* varied greatly between tissues. 90 g of monensin per ton of feed more effectively reduced the number of organisms in ceca than in small intestine. (Cecal carriage was less.)

The difference between no-monensin and monensin at the rate of 90 g per ton of feed in reducing organisms in ceca was more pronounced at two and seven days post infection compared to 12 and 17 days post infection.

Table 1. Reisolation and quantitation of *S. senftenberg* from tissues collected from infected birds with and without monensin supplementation.

DPI	90g/ton Monensin 10^6 *Salmonella*		60g/ton Monensin 10^6 *Salmonella*		No Monensin 10^6 *Salmonella*		No Monensin No *Salmonella*	
	Ceca (Cfu/g)	Small intestine (Cfu/g)	Ceca (Cfu/g)	Small intestine (Cfu/g)	Ceca (Cfu/g)	Small intestine (Cfu/g)	Ceca (Cfu/g)	Small intestine (Cfu/g)
2	7×10^1	4.52×10^2	1.8×10^3	8.83×10^2	1.85×10^3	9.57×10^2	0	0
7	4.88×10^1	1.6×10^1	7.5×10^2	1.53×10^2	7.55×10^2	4.25×10^2	0	0
12	8.8×10^1	1.05×10^1	8.9×10^1	1.0	1.45×10^2	2.0	0	0
17	1.0	1.0	2.0	1.0	6.0	2.0	0	0

DPI-Days post infection. No *S. senftenberg* could be isolated from liver and spleen in any of the four groups mentioned above.

AVIAN INFLUENZA CONTROL IN HONG KONG IN THE FACE OF ASIAN OUTBREAKS OF H5N1 AVIAN INFLUENZA

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INTRODUCTION

The 1997 highly pathogenic avian influenza (HPAI) H5N1outbreaks in Hong Kong caused high mortality in chicken farms and 18 human cases with 6 deaths, which was the first report of lethal HPAI H5N1 infection in humans. In the past eight 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) in commercial chickens as an additional measure to control H5N1 HPAI in Hong Kong (5). Since February 2003, there has been no outbreak in commercial poultry farms for almost two years, and only five isolated cases of H5N1 virus-infected wild birds were found in various non-commercial poultry areas (4). Since Dec 2003, various outbreaks of avian influenza viruses in both avian species and humans have been reported in the neighboring Asian countries (H5N1 subtype in South Korea , Japan, Thailand, Cambodia, Laos, Indonesia, mainland China, and Malaysia; H5N2 subtype in Taiwan and South Korea) (6). Thirty human cases with 22 deaths in Vietnam and 17 human cases with 12 deaths in Thailand have been reported (3). In the face of the increased human cases and potential pandemic threat of AI in Asia, further stepped-up control and surveillance measures have been introduced in Hong Kong from the perspectives of veterinary and human public health. The following describes the major features of stepped-up control and preventive measures against incursion of HPAI that have been or will be introduced in Hong Kong over different phases.

INITIAL ENHANCED CONTROL MEASURES (5)

- Biosecurity measures based on Hazard Analysis Critical Control Point (HACCP) principles have been further enhanced. The major goals are to stop any HPAI viruses from entering local poultry populations, and if incursion does happen, to minimize the spread of the disease through rapid diagnosis, control movement, safe disposal, vaccination, placing sentinel birds as an incursion indicator, thorough disinfection and others. The biosecurity plan applies to local farms, live bird markets (retail and wholesale) and the points of poultry import and transport.
- Vaccination with Nobilis IA inac® (H5N2 inactivated oil emulsion vaccine) was first introduced to 22 chicken farms in Pak Sha area in April 2002 and then gradually extended to all local chicken farms by June 2003. The vaccination program aims to increase chickens' resistance to the disease and if infected, to reduce their shedding and dissemination of the virus. Two vaccinations are given to local chickens which are usually raised up to 80 - 100 days for market sale. The first vaccination is given at around eight days of age and the second at around four weeks after.
- Routine serological surveillance and accounting for the 60 sentinel birds is done in every single batch of pre-sale local poultry. Apart from assuring desirable vaccine antibody levels have been achieved in the vaccinated bird, hemagglutination inhibition (HI) tests are used on the sentinels to detect exposure to H5 AI viruses (particularly LPAI) or unintended vaccination in sentinels (1). If sentinels are seropositive or all sentinels are not accounted for real-time RT-PCR test for detecting viral genomes, virus culture and clinical investigations are conducted on the batch of birds. Only the poultry that have passed all laboratory and field surveillance tests imposed by the government are provided with an approval code and allowed to be released to markets for sale.
- Dead bird monitoring forms an important part of the overall AI surveillance program. All unexplainable dead poultry from farms, retail markets and wholesale markets are investi-gated for AI involvement. Dead wild or captive birds are also subjected to postmortem investigation for cause of death and AI exclusion testing.
- Continuous education and communication with all poultry workers during farm

inspections has been an important factor in maintaining the enhanced avian influenza biosecurity program on farms.
- Improvement of the farm premises and management practice is encouraged by the government.

FURTHER STEPPED-UP CONTROL MEASURES

- Compulsory AI vaccination of all imported live birds had been gradually put in place by the mid January of 2004. Since then, the need for every batch of poultry coming into Hong Kong to achieve a satisfactory level of vaccine antibody responses against H5 AI viruses has become mandatory. This measure is supported by the recent FAO report advocating the proper use of vaccination as one of the main measures available to control HPAI (2).
- The requirement for strict compliance with mandatory biosecurity measures in local farms has been further reinforced through the government's regulation and licensing systems. Any farm that does not fulfill the requirements is subject to the possibility of penalty and revocation of farm license.
- Frequency of routine inspection of local farms has been stepped up and any unexplainable dead birds found require immediate pathological and laboratory investigations. This system facilitates the early detection of incursion of HPAI viruses and alerts the authority to necessary precautionary measures especially when infected wild birds are detected.
- Control movement of poultry. The policy of suspending import of live poultry and processed poultry meat from infected regions has played an important role in strengthening the concept of poultry movement control as a measure against incursion of HPAI. In this regard, different poultry species are raised and processed in segregated farm areas. Some local farmers in Hong Kong have now raised their own breeder birds for producing day-old chicks instead of purchasing from other farms in Hong Kong or from other regions as before.
- Formal liaison system with Mainland China has been established and depends on mutual cooperation. Regular communication is maintained to share and update the latest epidemiological information on HPAI.
- Public health measures. Through regulation and legislation, AI H5 was listed as one of the statutory notifiable diseases under the Quarantine and Prevention of Disease Ordinance (Cap. 141) on Jan 30, 2004. Report on human cases involving influenza-like symptoms by human medical profession, monitoring of patients who have traveled to HPAI-infected regions or people who have recently contacted with HPAI-infected birds, are also part of the control and preventive programs. Enhanced AI surveillance programs targeting pet birds and wild birds (as free-flying in wetlands and captive in various local parks) have been introduced. Other HA subtypes of AI viruses (e.g. H9 and H7) are being closely monitored on routine basis and are reported to the government.
- Enhanced public education for improving public awareness. Besides the continuous education of poultry farmers through seminars and other channels, various kinds of media such as publications, web media, TV and radio programs, educational leaflets and posters in different languages have been used to target the general public including foreign residents, visitors and domestic helpers. The publicity is also delivered to the public at different locations such as live bird markets, hospitals and ports of entry.
- Strengthened external communication, information exchange and regional cooperation. HPAI is now a global public health issue, and it is very difficult to control the spread of HPAI in free-flying wild birds. The recent spread of H5N1 HPAI across national boundaries again demonstrates that the disease must be managed as a transboundary animal disease (TAD) with the cooperation of countries in the concerned region (2). The HPAI control experience in Hong Kong has been communicated to different Asian countries, and it is hoped that the regional communication, coordination and harmonization of approaches would help monitor and understand the continuing evolution of the H5N1 virus and facilitate the control of the disease. Awareness and access to information are needed at all levels, including policy makers, administrators, veterinary officers, para-veterinary workers, poultry farmers...etc (7).

FUTURE CONTROL MEASURES

The Hong Kong government's overall objectives are to maintain a zero infection rate of HPAI in humans and minimize the contact between humans and live

poultry. Public consultation on segregating humans and live poultry at the retail market level was completed in 2004. Both central slaughtering and regional slaughtering have been discussed with the following two basic concerns in mind.

- Central slaughtering is considered for supply of poultry using a cold chain approach.
- Regional slaughtering is considered for supply of both chilled and fresh chicken to meet with the culinary requirements of the traditional culture.

OUTCOMES AND CONCLUSION

The above control program and preventative measures have been developed from a combination of technical input from veterinary professionals and virologists and risk assessment measures based on the local poultry industry and imported live poultry supply structure. The program has been introduced through the great support and cooperation of regulator, legislator, administrator, farmer, veterinarian, medical doctor, poultry trader, and the poultry farmers. In the face of the Asian HPAI outbreaks, no outbreaks occurred in Hong Kong and the poultry price reached its highest level since 1997 during the Chinese New Year days of 2004 indicating that the public had confidence in the HPAI control programs in Hong Kong.

ACKNOWLEDGMENTS

The authors would like to thank the staff of Tai Lung Veterinary Laboratory, Livestock Farm Division, Import and Export Division of AFCD, FEHD and CHP for providing highly competent services and efforts to enforce the HPAI control and monitoring programs in the region.

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A STUDY OF BIOSECURITY IN THE GLOBAL VILLAGE: THE HUMAN FACTOR

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ABSTRACT

This presentation proposes that the design for a biosecurity system for zoonotic diseases in the urban environment requires a consideration of the "human factor." The modern city is a complex of geological-biological-zoological-noetic- infrastructural systems. Where aspects of the country, including intensive agricultural production, are included within city limits, the containment and management of zoonotic diseases demands the recognition of an ecology of human players, as well as the ecology of the disease factors. The multiple perspectives of individual, industry groups, bio-experts and three levels of government across national and international jurisdictions, make the human factors involved highly complex. In addition to the multiple perspectives, a multiplicity of human values influences motivational factors in each human interest system. This brings into play a plurality of human system boundaries, structures and relationships and creates endless opportunities for disconnections and breaches of any bio-security system design. The presentation asks if all the players use the same management strategy for avian flu and what are the value assumptions influencing the biosecurity

strategies of the poultry industry. Suggestions are made to design effective avian flu management strategies that encompass multiple human values, motivations, roles, relationships, responsibilities, and accountabilities. Lessons are derived from the avian influenza experience in 2004 in the Central Fraser Valley, BC. Suggestions are reviewed for addressing the human factor through education, industry leadership, animal-human health expertise and systems development.

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A RATIONAL BASIS FOR BIOSECURITY PROGRAMS: A SIMPLIFIED THEORY OF DISEASE TRANSMISSION RISK

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Which is more important in a disease prevention or control program: a truck wash station or an employee shower? Gated entrances or locked doors? Boots and coveralls or a no visitor policy?

Risk (the chance that something will go wrong) is a function of hazard and exposure. Greatest risk, like beauty, seems to be in the eye of the beholder; and when veterinarians are queried they often reply that everything is high risk or very high risk. Dollars are limited and inadequate to prevent all risks so how do we justify spending on biosecurity? Which events, practices, or circumstances are most risky?

Every possible event cannot be predicted. Just stop and think of some of the anecdotes you've heard about introductions of disease. However, there are general hazard categories that can be identified. What are those categories? Movement of animals, movement of manure, movement of people and equipment, etc. are readily listed. Identifying hazards is relatively straight forward, but when it comes to determining exposure one must assess likelihood of an event happening. And what is the likelihood of disease exposure as a result of any event? Is it 1%? 50%? 95%?

Hypothesis: Risk of disease is related to amount of microbial load (hazard) and its proximity to susceptible animals (exposure).

Using this approach risk can be crudely calculated by converting the hazard to microbial load divided by a distance factor. Amount of microbial load is related to the mass of contaminant, numbers of microbes per unit of mass in the contaminant, the portion of the contaminant that is available to be spread to susceptible animals, and the age and half-life of disease agent (under given conditions). Proximity of susceptible animals to the hazard is related to the square of the distance or area, since the greater the distance from the source of contagion the more area that contagion will be spread over.

Risk then equals microbial load of the hazard divided by the proximity squared times pi. For convenience one can convert this to a log number.

Simple examples of the application of this formula are presented in Table 1. One hundred-fold differences in mass of the contaminant and one hundred-fold differences in distance are presented. The results of these calculations can be referred to as "relative risk."

SUMMARY

Disease transmission risk can be estimated using the hazard and exposure model. By dividing the microbial load by a proximity factor one is left with a relative risk number that can be used to quantify

events, practices and circumstances in poultry production. From this relative risk number attempts to prevent or control disease can be focused appropriately. Biosecurity programs and disease control measures can be directed at those areas of greatest risk of spreading disease.

Table 1. Relative risk* calculated for different hypothetical hazards to poultry production.

Hazard	Microbial load Mass (avail.)	Distance (meters) 0.01	1.0	100
Manure	10 tons (1%)	NA	5	1
Dead birds	100 kg (1%)	NA	3	-1
50 sparrows	1 kg (1%)	5	1	-3
Poultry dust	10 gm (100%)	5	1	-3
Hands	100 mg (100%)	3	-1	-5

*Log_{10} Mass of contaminant/(pi)(distance^2)
NA = Not applicable

POULTRY PRODUCERS SURVEY: CONTACTS WITH POULTRY AND BETWEEN POULTRY FARMS

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ABSTRACT

Theoretical simulation models of highly pathogenic avian influenza (HPAI) introduction and spread are useful tools in predicting and preventing the impact of outbreaks. However, information regarding the level of human exposure to affected birds and the potential for secondary spread by human movement is lacking. In this study, questionnaires were sent to poultry producers of two counties (low and high poultry density) to estimate the frequency and nature of human contacts with poultry and the movement between poultry farms.

Five poultry companies with farms in the selected regions were contacted and agreed to participate in the study. Survey questions regarding poultry personnel and producers' visits and management were sent to commercial broiler and breeder chicken producers. Responses were used to estimate contact rates and patterns and were compared between the two regions. Producer participation in the survey was 60.6%. In both counties, feed trucks, service persons and managers were the most frequent visitors.

There were no significant differences in the number of farms owned by a producer, chicken houses on a farm, and other physical characteristics between the two counties. In the high density county, externally contracted clean-out services were utilized by 40.3% of the producers and 9.6% of them admitted to loaning equipment to other growers. However, in the low density county only 3.4% utilized externally contracted clean-out services and 3.4% stated that they loaned equipment to other producers. Approximately 48% of the producers in the high density county reported that they had one or more contacts with other poultry growers within the previous seven days, and 30.7% of them contacted growers who contracted with different integrator. The number of interactions between producers was also high in the low density county (62%); however, just 6.9% of the producers contacted growers from other companies.

Data obtained in this study will be used to assess the potential differences in the contact rates within counties of different poultry densities and will aid in the development of an effective simulation model for poultry disease spread under conditions of different population pressures.

(The full-length article will be published.)

LESSONS FROM THE BIOSECURITY FIELD REPORT OF COMMERCIAL EGG LAYER PREMISES IN THE CALIFORNIA 2002-2003 EXOTIC NEWCASTLE DISEASE OUTBREAK

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INTRODUCTION

An outbreak of Exotic Newcastle Disease (END) during 2002-2003 resulted in the death and destruction of approximately three million commercial egg layers in southern California. Preventing another such event is a goal that will require ongoing assessment of biosecurity for premises, companies, and the industry as a whole.

What follows is the result of reports collected by members of the END Task Force working in the Commercial Planning Unit, at Colton, CA. Data is presented in grouped or aggregate form and are intended to give an overview of biosecurity of the southern California layer industry during a specific time period. Changes to biosecurity at premises must be addressed on an individual basis, keeping in mind that risks will be unique at any premises at any given time. Over 1000 biosecurity issues were identified in 214 field reports, were recorded in relation to 80 premises owned by 33 companies, and 56 exit interview questionnaires were used to score individual premises. This report is intended to provide an overall "baseline" of biosecurity practices recorded as well as changes made at southern California commercial layer premises from January to August 2003.

METHODS

Premises were assessed using a biosecurity report that included the following information: CCP (California Commercial Poultry) number; name of submitter; date of investigation; demographic information; premises security; animal activity; poultry movement; cleaning and disinfection; personnel biosecurity; mortality, trash and manure; shared equipment; egg equipment and movement; flock health; production; mortality; feed consumption; and recommendations.

For each report, recommendations were listed and given to the owner of birds at each premises. The format of the questionnaire included both open-ended (e.g. state, list, etc.) and close-ended (e.g. Yes/No) questions. Quality of questionnaire administration and responses varied to some degree due to submitter turnover and experience, owner compliance, access to premises, and variable exposure time at ranch. There was a range of one to six reports completed per premises. Sources of additional information included epidemiological investigations, production related information from egg processing audits, and direct observation.

RESULTS

A total of 1040 biosecurity issues were identified in completed reports for southern California layer premises. Three quarters (75%) of the biosecurity risks identified by company liaison veterinarians dealt with personnel, premises security, wild or domestic animal activity on or adjacent to the premises, and cleaning and disinfection procedures. Personnel issues include use of protective clothing and footwear, change rooms (showers when appropriate), biosecurity training and movement restrictions on and between ranches. Premises security includes use of gates, visitor logbook, adequate fencing, signs, retail outlets, and movement/access control. Animal activity includes presence of wild and domestic animals on or adjacent to the premises not including presence of loose fowl. Cleaning and disinfection issues include adequate use, quantity and placement of sprayers, footbaths and disinfectants. Equipment shared among premises includes poultry racks and trucks, manure machines and trucks, dead haul trucks, and other assorted equipment. In addition, some vehicles were used for more than one purpose such as hauling dead birds and carrying egg racks/flats. Egg handling issues include dedicated, clean racks, mixing nest run and processed eggs from a common loading dock, and use of returned egg flats from retail customers. Proximity to poultry includes loose or penned poultry evident on or adjacent to the premises. Mortality biosecurity issues include placement, security, and handling of mortality such as transport of mortality from one premises to another. Trash biosecurity issues include placement, security, and handling of trash. Manure handling issues include storage and direct farm-gate sales.

As a percentage of premises with biosecurity risks, 88% of premises shared risks related to premises

security and animal activity in or around their premises and 83% shared risks related to personnel. Although high-pressure sprayers were issued during the outbreak, 63% of premises shared risks related to improper placement or use of equipment and disinfectants. Issues related to equipment and proximity to loose poultry at 58% and 49% of layer premises. Biosecurity risks related to egg handling were identified at 43% of premises. Risks related to trash, manure, mortality, and health issues were noted less frequently. It is important to note that these percentages represent an overview of all premises, and prioritization of specific risks for each premises are derived from individual premises reports.

It is interesting to compare these risks with those factors identified with the introduction and spread of END into the commercial layer industry in southern California. Primary sources of infection included employees as the primary entry point of the virus into the industry and proximity to infected neighboring backyard birds as an important external source. Secondary associations with spread within the industry include direct marketing links among the majority of affected commercial premises through shared egg racks, flats, and trucks. Marketing links created similar patterns to what was seen due to Low Pathogenic Avian Influenza H6N2 during the preceding three year period. Two out of three ranches (14/21) positive for END were previously positive for AI.

At the conclusion of the Exotic Newcastle Disease Task Force, a total of 33 exit interviews were conducted during July and August 2003 to determine responses to recommendations made for 56 premises. Exit interviews were conducted in person at a neutral site and included verbal as well as written feedback from producers with reference to five questions. The questions and a summary of responses follow.

Specifically, what have you done to increase biosecurity in your operation since the beginning of the END outbreak in Southern California? The most common measures taken at premises were improving cleaning and disinfection methods, providing employees with dedicated clothing and footwear, improving use of footbaths, and compartmentalizing traffic on the premises. Interestingly, only 5% of premises reported providing employee training when the actual figure was much higher. This may reflect recall bias in an open-ended question or it may reflect owner perception or attitude regarding the importance of employee training as a fundamental component of biosecurity. Further evaluation of this information is needed.

Of the increases in biosecurity you have implemented, which ones will you continue after USDA and CDFA finish their work on the END outbreak? Eighty-four percent (84%) of responders indicated they intend to maintain ALL biosecurity measures indicated in the first question. The remainder indicated that they would maintain only selected items.

What additional biosecurity actions do you plan to implement before USDA and CDFA finish their work on the END outbreak? Respondents indicated that they would make additional changes in egg flat management, improve fencing, compost dead birds, and providing change facilities for employees before Task Force activities ended. Forty-five percent of responses indicated that no further measures would be taken in the same time frame.

What biosecurity actions recommended by USDA and CDFA are you unwilling to implement? Briefly explain why. The biosecurity strategy least likely to be adopted is use of a visitor logbook, mainly because there were not enough visitors to justify its use. Fencing improvements and changing traffic patterns were also not likely to be acted upon. Nineteen (19) premises indicated they would implement all recommendations made by USDA and CDFA while the remainder (42) did not answer this question.

If you have not participated in the END Task Force biosecurity training for your employees would you be willing to have this training done in Spanish or English before USDA and CDFA finish their work on the END outbreak? Owners/managers of 71% of premises indicated that had or would participate in employee training. A large proportion of those who responded positively said that they would like to maintain regular employee training beyond the duration of the Task Force.

All premises, positive premises, and negative premises were compared with respect to biosecurity scores calculated by dividing the number of remedial measures taken by the total number of biosecurity issues identified on an individual premises basis. Due to the high variability of scores, the median score was selected as a useful measure of determining the "average" score for each group. The overall median score for all premises was 35% and previously positive premises had a median score of 25% while negative premises had a median score of 45%. This means that the middle score for all premises, positive premises, and negative premises was 35%, 25%, and 45% respectively. The median score for positive premises was lower than the median score for negative premises, but the difference between these scores was not statistically significant; however, several positive premises did not complete the exit interview (Median Test, $X\ 2 = 0.38$ (0.17, 2.69)). Interestingly, 10 of 56 premises (18%) took remedial measures equal to or greater than 100% of initial issues identified by Task Force personnel. Of the 56 premises exit interview responses, 21/56 premises (37.6%) addressed greater

than 50% of biosecurity issues identified while 35/56 Premises (62.5%) had addressed fewer than 50% of biosecurity issues identified.

DISCUSSION

This report has presented summary "baseline" data reflecting biosecurity preparedness of southern California egg producing premises immediately following the 2002-2003 Exotic Newcastle Disease outbreak. Every ranch is different, and it is neither possible nor advisable to extend the findings of this report to individual premises. Measures taken that weren't recorded as commonly can still be very important at individual premises. The likelihood and means of exposure to avian pathogens will vary from premises to premises as well as over time, and this report does not consider risk of exposure for each premises. Prioritizing which biosecurity risks are most important or most likely lead to disease introduction at individual premises remains challenging for these reasons.

As the layer industry moves forward with plans to promote biosecurity, it is recommended that each premises be evaluated as unique with specific challenges. Baseline reports for individual premises and for each company are still available for this purpose. Promoting biosecurity will require ongoing efforts that will require validation through ongoing third-party audits that will be helpful in measuring individual as well as industry progress. Specifically, this report recommends sharing results with the southern California layer industry, utilization of individual and aggregate biosecurity scores from this report as a baseline for future improvement of biosecurity in the southern California layer industry, and development of an individualized assessment method to periodically evaluate the biosecurity for all southern California layer premises and companies.

ACKNOWLEDGEMENTS

Information gathered in this report is a result of the cooperation, involvement and support of the egg producers of Southern California and consulting veterinarians Gregg Cutler, Mark Bland, and Marion Hammarlund. University of California Cooperative Extension faculty and farm advisors Carol Cardona, Doug Kuney, Ralph Ernst, and Francine Bradley are acknowledged gratefully for their support. Acknowledgement is due to all members of the Commercial Planning Unit, especially veterinarians John Hahn, Keith Hand, David Kerr, Andrea Mikolon, and Layton Smith who lead company liaison veterinarians during a challenging period. The commitment of all of these parties has contributed to the goal of preventing future outbreaks.

EFFICACY OF INOVAPURE 222 ADMINISTERED IN THE FEED FOR THE CONTROL OF NECROTIC ENTERITIS CAUSED BY *CLOSTRIDIUM PERFRINGENS* IN BROILER CHICKENS

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SUMMARY

The objective of the study was to evaluate the anticlostridial efficacy of broiler chickens fed Inovapure 222 in floor pens under conditions simulating commercial broiler production. Inovapure 222's (200 ppm) anticlostridial efficacy was compared to the commercially available feed additive, virginiamycin 15 g/ton. A significant necrotic enteritis infection developed in the nonmedicated, infected birds, with over 33% weight reduction and 17 % higher feed conversion (compared to the nonmedicated noninfected control) and 14.5 % necrotic enteritis mortality. Virginiamycin reduced effects of necrotic enteritis with 11.9 % weight reduction, 4 % higher feed conversion and only 1.5 % necrotic enteritis related mortality. Inovapure 222 also proved to significantly reduce the level of necrotic enteritis with 19.4 % weight reduction, 7 % higher feed conversion and 7.2 % necrotic enteritis related mortality. Inovapure 222 proved to significantly reduce the level of necrotic enteritis caused by *Clostridium perfringens.*

INTRODUCTION

Necrotic enteritis is a common poultry disease caused by *C. perfringens* type A or C (1). Reductions in feed efficiency, lower weight gain, and mortality are associated with this disease. *Clostridium perfringens* is commonly found in poultry litter and feces. Being opportunistic, *C. perfringens* rapidly grows when disturbances in the intestinal microflora or damage to the intestinal mucosa occur. An example of damage to the intestinal mucosa occurs with coccidiosis.

A common method of control of necrotic enteritis is use of antibiotics. Antibiotics such as bacitracin and virginiamycin (2) have inhibitory action against *C. perfringens*. Other methods of control are use of ionophorous anticoccidials (5), dietary manipulation, improved coccidia control, intestinal acidification, pre- and probiotics (3), and enzymes (4). One such enzyme is lysozyme (6). Lysozyme is a food preservative derived for egg whites. It has been demonstrated that lysozyme has some inhibitory activity against *Listeria monocytogenes*, *Clostridium botulinum*, and other Gram positive bacteria. Studies have also shown that lysozyme significantly inhibits toxin production by *C. perfringens.*

The objective of this study was to evaluate the anticlostridial efficacy of lysozyme (Inovapure 222) in floor pens under conditions simulating commercial broiler production. Anticlostridial efficacy was compared to the commercially available growth promoting antibiotic virginiamycin.

MATERIALS AND METHODS

The experiment consisted of 24 pens starting with fifty male broiler chickens. The treatments were replicated in six blocks, randomized within blocks of four pens each. The treatments were Nonmedicated, noninfected; Nonmedicated, infected; virginiamycin (16.55 ppm), infected; and Inovapure 222 (200 ppm), infected. No birds were replaced during the course of the study. Bird weights (kg) by pen were recorded at study initiation (day 21) and termination (day 42). All pens had approximately four inches (10 cm) of built up litter with a coating of fresh pine shavings.

Broiler feeds were corn/soy diets representative of local formulations and calculated analyses met or exceeded NRC standards. Birds received feed appropriate to the treatment from day 0 to day 42. A change from starter to grower occurred on day 21. A change from grower to finisher occurred on day 35. At each feed change, non-consumed feed was weighed by pen.

All birds were challenged with a mixture of *Eimeria acervulina* and *E. maxima* on day 14. The challenge level was titrated to allow a light coccidiosis infection to develop. The built up litter also was a source of coccidial oocysts. To induce necrotic enteritis, on days 18, 19, and 20 all birds except Treatment 1 were dosed with a broth culture of *C. perfringens*. A field isolate of *C. perfringens* known to cause NE and originating from a commercial broiler operation was utilized as the challenge organism. Fresh inoculum was used each day. The titration levels were approximately 1.0 X 10^{8-9}. No concomitant drug therapy was used during the study.

Pens were checked daily for mortality. A bird was culled only to relieve suffering. When a bird was culled or found dead, the date and removal weight (kg) was recorded. A gross necropsy was performed on all dead or culled birds to determine the sex and probable cause of death. Signs of necrotic enteritis were noted.

RESULTS AND DISCUSSION

Using commercial broilers on built up litter and exposed to both coccidia and *C. perfringens* simulated natural conditions occurring within a poultry facility. A mild coccidiosis infection damaged the intestinal mucosa of the birds which enabled the *C. perfringens* to proliferate and clinical necrotic enteritis to develop.

A significant necrotic enteritis infection developed in the nonmedicated, infected birds, with over 33% weight reduction and 17 % higher feed conversion (both compared to the nonmedicated noninfected control) with 14.5 % of the birds dying from necrotic enteritis. The antibiotic virginiamycin reduced effects of necrotic enteritis with 11.9 % weight reduction, 4 % higher feed conversion, and only 1.5 % necrotic enteritis related mortality. The *C. perfringens* isolate used in this study was directly isolated from a field case of necrotic enteritis. Laboratory analysis showed this isolate to be sensitive to virginiamycin. Some performance loss occurred in the virginiamycin fed birds; this was probably due to the methodology of the *Clostridium* challenge, which gave a broth culture that contained not only live bacteria but also large amounts of the alpha toxin.

The Inovapure 222 also proved to significantly reduce the level of necrotic enteritis with 19.4 % weight reduction, 7 % higher feed conversion and 7.2 % necrotic enteritis related mortality. Inovapure 222 proved to significantly reduce the level of necrotic enteritis caused by *Clostridium perfringens.* As seen with virginiamycin, some of the observed effects of the necrotic enteritis are probably due to the alpha toxin in the challenge broth. Under commercial conditions, the level of *C. perfringens* proliferation would be kept lower by the Inovapure or virginiamycin so the level of toxin would be less. Thus, performance and necrotic enteritis control associated with virginiamycin or Inovapure should be improved.

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HIGH YIELD OF PARASITES AND PROLONGED *IN VITRO* CULTURE OF *HISTOMONAS MELEAGRIDIS*

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INTRODUCTION

Numerous media for the culture of *Histomonas meleagridis* have been described. A frequently used medium is Dwyer's medium which consists of M199, 10% heat-inactivated horse serum, 5% chicken embryo extract and 10 to 12 mg of rice starch per 12.5 mL of medium. The amount of rice starch is considered to be critical as addition of larger or smaller amounts resulted in decreased yield of histomonads (2). However, no data supporting this observation have been published, and no other factors like particle size of the grounded rice starch were considered. Therefore, the effect of adding larger quantities of rice starch to the culture medium as well as the influence of the size of the rice particles on growth of *H. meleagridis* was examined in the present study.

MATERIALS AND METHODS

Several *in vitro* culture experiments with varying amounts of rice starch were performed. Tissue culture flasks with 12.5 mL Dwyer's medium with either 0, 10, 20, 40, 60, 80, 100, 150 or 200 mg (± 0.1 mg) of rice starch were inoculated with 1 mL *H. meleagridis* culture (using standard Dwyer's medium). Histomonads were counted with a Bürker-Türk hemocytometer three times daily at a four hours interval. The cultures were supplemented with the same amount of starch as used initially every 48 hours (no sub-culturing). In other experiments, the effect of rice starch particle was examined by supplementing Dwyer's with either 12 or 100 mg of rice starch of five different particle sizes (standard (not sieved), >250 µm, <250 µm, 100-250 µm, or <100 µm) obtained after sieving the starch through a 0.25 mm steel wire sieve and a 100 µm nylon sieve.

RESULTS

Increasing the amount of rice starch from the standard amount of 10-12 mg to 50-100 mg per 12.5 mL of medium resulted in approximately a 10-fold increase of parasites ($\geq 10^7$ histomonads/mL culture medium). Larger quantities of rice starch did not give better yields. *H. meleagridis* cultures could be prolonged from approximately four days to at least two weeks without sub-culturing by supplementing the culture medium with rice starch only.

The effect of different rice particle sizes on the histomonads concentrations found during culturing was relatively small in comparison to the effect of the amount of rice starch. Only the cultures with the largest particle sizes (>250 µm) tend to yield lower histomonad concentrations.

DISCUSSION

High yield of histomonads may prove useful if large quantities of antigen are needed when it comes to developing laboratory tools for diagnostic purposes like ELISA.

Rice starch is an essential constituent of Dwyer's culture medium (2). If it is omitted from the medium no multiplication of histomonads is observed (5). Histomonads probably feed directly on the rice starch, since many of the parasites show rice particle inclusions at microscopy (7,4). But the rice starch probably also provides food for specific bacteria that serve as food for histomonads (7,5).

Normally, the numbers of parasites during *in vitro* culturing reach a maximum at two or three days after inoculation of fresh medium with a stock culture of *H. meleagridis* (2). Then, probably due to lack of nutrients, populations of histomonads decrease rapidly and die out after approximately five days. In the present study we found that *H. meleagridis* cultures remain viable to at least 21 days without sub-culturing when only a small amount (10 to 12 mg) of rice starch was added. It was not possible to extend the life of *H. meleagridis* cultures based on more than 12 mg of rice starch by adding extra rice starch at regular intervals. Such cultures died out rapidly, and no organisms were found anymore. The histomonads grew abundantly in the first two days and probably other nutrients than rice starch quickly became exhausted.

Typically, during the culture of *H. meleagridis* concentrations ranging from $10^{5.5}$ to 10^{6} histomonads per mL have been found as maximum yields (1,3,6). The same yields were also obtained in the present study when the standard 10 to 12 mg of rice starch was used. However, 50 to 100 mg of rice starch per 12.5 mL of medium gave significantly higher yields of parasites, concentrations sometimes exceeding 10^{7} parasites per mL. Higher amounts of rice starch were not beneficial.

The influence of rice starch particle size on the yield of histomonads proved relatively unimportant. When only large rice starch particles (> 250 µm) were used, the yield of parasites was slightly less.

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HISTOMONAS MELEAGRIDIS: THE SITUATION AFTER THE BAN OF THE LAST AVAILABLE DRUG IN THE EU

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SUMMARY

Blackhead or histomoniasis is a protozoan disease, primarily of turkeys. However several other species like chickens, quail, and peafowl are also susceptible. The disease in turkeys is characterized by listlessness, anorexia, droopy wings, and yellow sulphur-colored feces. Infections with blackhead in turkeys are often fatal, with high morbidity in survivors. It is the most fatal parasitic disease of turkeys, capable of causing mortality up to 100%.

Histomonas as a parasite depends on complicated interactions with cecal worms (6), earthworms, intestinal microflora, and coccidia in order to spread from flock to flock and develop full pathogenicity (13).

The causative organism, *Histomonas meleagridis*, represents a class of protozoa distinctly different from coccidia (13). It is closely related to *Entamoeba*, *Giardia,* and *Trichomonas*, of humans and other animals (5). As a class, these organisms react to

distinctly different types of chemotherapeutic agents than other protozoa (12).

For more than 30 years histomoniasis has been very well controlled with Dimetridazole. The ban of Dimetridazole as a treatment in 1995 and then as a feed additive made the poultry industry and farmers very anxious, but the disease was controlled relatively successfully with Nifursol as a feed additive. Until March 2003, Nifursol, a nitrofuran, was authorized for use as a feed additive for the prevention of the disease in turkeys. The marketing authorization was given for a period of ten years, without a re-evaluation. During the years 1990 -1995, both the joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Committee for Veterinary Medicinal Products (CVMP) gave opinions on the use of veterinary medicinal products (VMPs) in food-producing animals of the group of substances known as nitrofurans. They concluded that it was impossible – because of the genotoxicity and carcinogenicity of the substances – to identify acceptable daily intakes. Accordingly, it was not possible to set Maximum Residue Levels (MRL) for these substances. All nitrofurans were then inserted into Annex IV to Council Regulation (EEC) No 2377/90 (1) with the effect of prohibiting the administration of these substances as veterinary medicinal products to food-producing animals throughout the European Community (4).

The European Commission asked the Scientific Committee on Animal Nutrition (SCAN) to make a new scientific risk assessment for Nifursol. On 11 October 2001, the SCAN adopted its opinion, which concluded that on the basis of the studies on mutagenicity, genotoxicity and carcinogenicity as provided by the holder of the marketing authorisation, and because of the lack of data on developmental toxicity, it was not possible to derive an acceptable daily intake for the consumers. On 18 April 2002, after having examined complementary data provided by the holder of the authorisation, the SCAN confirmed its opinion. As a consequence of this and in the absence of a favourable opinion of the Standing Committee on the Food Chain and Animal Health, the Commission concluded that the use of nitrofurans for food producing animals should be prohibited. The Council Regulation (EC) No 1756/2002 (2) that views upon this matter now applies from 31 March 2003 (4). The holder of the authorisation for marketing of nitrofursol decided to discontinue further product development because of an expected low and uncertain return on investment.

Because an effective product against blackhead was available, not very much research into the prevention and treatment of the disease has been done in the last decades. Currently, no drugs are available for treatment, and immunization does not appear to be a practical approach to control. Anticoccidials and antibiotics have little or no effect on the course of infection in turkeys (10). In addition, in recent years blackhead has emerged as a serious disease in layer flocks coinciding with an increase in free range flocks (3,7) in Europe. Furthermore, several outbreaks in turkey flocks in Europe were observed (8,9,11,14).

Since March 2003 several histomoniasis outbreaks were observed in commercial turkey farms all over Germany. Ten outbreaks in 10 farms with totally 144,000 birds are known to us. In all cases the birds were aged between 4.5 and 11 weeks. Clinical signs included depression, lethargy, anorexia, diarrhea, loss of weight, and death. Lesions were characterized by severe enlargement of the liver with numerous white nodules, enlargement of the ceca with caseous cores, and a few birds with white nodules in the kidneys. The diagnosis was confirmed using PCR from liver and ceca as described by Hafez *et al*. (6).

In all cases known to us only toms were affected and mostly only one pen, while female birds kept at the same farm didn't show any signs. Treatment trials using natural products or changing the litter directly after the onset of clinical signs were not effective in reducing the mortality. The mortality rates in affected farms rose within one week to about 40% and more. The source of the infection and the reason of the unusual pathogenicity of *H. meleagridis* causing such high mortality are not known. Since histomoniasis is neither a reportable nor a notifiable disease and there is no legislation for its control, in all cases the male birds on the affected farms were voluntary culled. In total 66,000 birds were culled. For culling of the birds on the farms several methods were used, namely electrified water bath in six cases, injection in two cases, CO_2-culling in closed containers in one case, and neck dislocation in one outbreak. Although only the routine cleaning and disinfection was carried out after the outbreaks, until now in all farms the disease was not observed in the same houses in the newly restocked flocks.

Currently the control of histomoniasis is based on good hygienic measures at farm level and using drugs that reduce the presence of cecal worms. For the future, intensive research for the development of new drugs to prevent and treat histomoniasis is necessary due to economic as well as animal welfare reasons. A scientific evaluation of so-called "alternative" products under experimental condition is essential and in progress.

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THE INCIDENCE OF *BLASTOCYSTIS* IN THE INTESTINE OF TURKEYS

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Blastocystis, first described in 1911, is a water-borne, ubiquitous parasite that can be found in the intestinal tract of various animals including mammals (humans, primates, rodents, cattle, pigs, etc.), birds, reptiles, fishes, and amphibians. *Blastocystis* has also been isolated from insects. Despite its ubiquitous nature, not much is known about the taxonomy, life cycle, transmission, host range, and pathogenicity of *Blastocystis*.

B. hominis in humans is becoming increasingly associated with gastrointestinal disease. Among birds, *Blastocystis* has been described in chickens (*B. galli*), turkeys (*M. gallopava*), geese (*B. anseri*), ducks (*B. anatis*), guinea fowl (*B. numidae*), pheasants, quail, red-legged partridge, grey partridge, peafowl, and

ostriches. Nucleotide sequence and RFLP comparison of the ssRNA genes of numerous *Blastocystis* isolates have indicated that is not host specific and may be cross infective among various animal hosts. According to a recently revised classification, *Blastocystis* is not a protozoon as previously assumed. Taxonomically it has been placed in the Kingdom Chromista, Subphylum Opalinata and Class Blastocystea. Whether *Blastocystis* is a pathogen, commensal or an opportunistic organism in mammals and birds is not known. However, experimental infections of chickens and red-legged partridges failed to cause any clinical signs.

Blastocystis is a pleomorphic organism. Isolates from humans and animals can range in size from 3 - 200 μm and appear in various forms including vacuolar, granular and cystic. Other forms such as avacuolar, multivacuolar and cells containing filament-like inclusions occur less frequently. *Blastocystis* ranges from round and oval to ellipsoid and ameboid. Reproduction is by binary fission, but other modes of replication have been suggested.

Blastocystis can be isolated from feces in various media and can be identified in wet smears of feces, using bright field or differential interference contrast optics. Air-dried or methanol-fixed fecal smears stained with Giemsa, PAS, Wright's, trichrome, Loeffler's methylene blue or iron hematoxylin are useful in confirming *Blastocystis*. Transmission electron microscopy also can be helpful in identifying the organism. Interestingly, *Blastocystis* has not been described in the intestinal lumen of birds or animals on hematoxylin and eosin (H & E) stained sections of the intestine.

Occurrence of *Blastocystis* in birds has been reported mainly from Europe, Japan and Australia. There have been no reports of *Blastocystis* in birds from North America. Infection rates in chickens as high as 95 % have been reported. The infection rate of *Blastocystis* in turkeys is not known. In this study the incidence of *Blastocystis* in turkeys was determined by wet and Wright's stained smears of cecal contents. Prospective and retrospective histopathologic studies of sections of ceca stained by H&E were also undertaken.

Blastocystis was detected in five out of 12 cases by wet and Wright's stained smears of cecal contents. Most of the positive cases had large numbers of *Blastocystis* in the feces. A prospective histopathologic study of ceca stained with H & E detected *Blastocystis* in 18 out of 28 cases. Retrospective histopathologic studies of ceca stained with H & E of 37 cases selected for the presence of undetermined protozoa were positive for *Blastocystis.* The age of the turkeys studied ranged from two days to 15 weeks.

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MODIFICATIONS OF THE OFFICIAL REAL-TIME REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RRT-PCR) ASSAY FOR THE DETECTION OF VIRULENT NEWCASTLE DISEASE IN CLINICAL SPECIMENS

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ABSTRACT

Virulent Newcastle disease (vND) is a serious disease of poultry that has caused severe economic losses in many countries, including the United States. In October of 2002 an outbreak of vND occurred in game fowl in southern California. The disease eventually spread to 21 commercial poultry operations as well as game fowl in Nevada and Arizona. During the outbreak, a Real-Time Reverse Transcriptase Polymerase Chain Reaction (RRT-PCR) assay was developed (Wise *et al*., *J. Clin. Micro*. 2004) and validated to facilitate eradication of vND. Following the outbreak modifications were made to the CalMex (virulent) primers to improve diagnostic sensitivity of the assay. In addition, the Qiagen® RNA extraction procedure was compared to the Ambion® 96-well magnetic bead procedure for efficiency of RNA isolation and high throughput specimen processing. The volume of swab medium and number of swabs per tube were evaluated as well. The diagnostic sensitivity and specificity of the modified vND assay was shown to be 91.26% and 97.5%, respectively. Primer modifications resulted in a significant improvement in diagnostic sensitivity. The CalMex vND assay had been shown to have a diagnostic sensitivity and specificity of 85.1% and 99.0% during initial assay validation. The Ambion 96-well magnetic bead extraction system was shown to be more adapt to high throughput specimen processing and compared favorably to the Qiagen RNeasy RNA extraction kit for the isolation of RNA from cloacal and oralpharyngeal swab specimens. The modified v-NDV assay has replaced the CalMex assay for the detection of v-NDV in the official NVSL RRT-PCR protocol used for the surveillance of v-NDV currently being conducted by the National Animal Health Laboratory Network.

(The full-length article will be published in the *Journal of Clinical Microbiology*.)

POTENTIAL LIMITING FACTORS IN VACCINATION WITH A NEW SEED OF V4 NEWCASTLE DISEASE (ND) VACCINE VIRUS IN THE AUSTRALIAN ND ERADICATION PROGRAM

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SUMMARY

Australia developed a National ND Management Plan (NNDMP) in 2003 to attempt to eradicate and prevent the re-emergence of precursor and virulent ND virus (vNDV). Because of the potential for vNDV re-emergence, a vaccination strategy using the live lentogenic strain V4 and inactivated ND vaccines was developed to out-compete precursor viruses that have a closely related sequence to that of the vNDV. The extent to which vaccination can prevent the spread of precursor virus seems paramount to the success of the program. The results of a number of laboratory and field studies on the efficacy of an experimental live ND V4 vaccine have been discussed in relation to their

impact on the current ND eradication program in Australia. Evidence for reduced efficacy associated with ND Mab antibody (Mab) and when used in combination with infectious bronchitis (IB) vaccines raised concerns about the likely outcomes from the eradication program. Whilst some additional studies could assist in further evaluation of the now preferred day-old spray vaccination method, several alternate vaccination programs could be adopted in the interim.

INTRODUCTION

Australia would appear to have experienced a rather unique event in terms of the mutation of vNDV from an endemic lentogenic strain (8). The factors that encourage precursor and vNDVs to emerge are not well understood, although flock immunosuppression due to concurrent MDV, IBDV or CAV infection has been suggested to alter selection pressure during virus propagation through the flock, assisting evolution to virulence. Alternatively, Westbury (9) has suggested that vNDV may have emerged slowly in accordance with the quasi-species concept (2) with evolutionary selection pressures on a heterogeneous population of NDVs causing those sub-populations to emerge that are best adapted to the changing poultry growing environment.

Because of the potential for vNDV re-emergence, a vaccination strategy was developed that aimed to out-compete precursor viruses that have a fusion protein gene sequence similar to that of the virulent virus. The only live vaccine permitted is the lentogenic V4 strain (7). This paper describes laboratory and field trials undertaken to evaluate a new seed of V4 ND vaccine virus in terms of its likely impact on the national ND eradication program.

REGULATORY VACCINATION REQUIREMENTS

All Australian states have or will shortly (in 2005) require all commercial poultry to follow mandatory vaccination programs as described in a set of Standard Operating Procedures (SOPs) developed by the ND Management Group (NDMG). The SOP for ND vaccination of broiler chickens recommends that chickens be preferably vaccinated with live V4 strain ND vaccine at 7-14 days of age. However, the SOP also allows farmers the option of vaccinating at day-old provided they can show evidence of equivalence to the preferred program. Adequacy of the response to vaccination is defined as the mean haemagglutination inhibition (HI) titer of the flock being at least log 2^3 with at least 66% of the individual samples reaching a HI titer of 2^3 by 35 days of age. The latter program retains control of ND vaccination in hands of the hatchery where it has been claimed that improved uniformity (coarse-aerosol spray vaccination) and lower cost can be achieved than through administration by drinking water undertaken by broiler growers. Commercial layer and breeding flocks are required to be vaccinated with inactivated ND vaccine after initial priming with live V4 vaccine.

INTERFERENCE WITH EFFECTIVE VACCINATION

Vaccination of chickens with live ND vaccines, including ND V4, in the presence of ND Mab has been contra-indicated by a number of studies (1, 10) based upon the poorer development of active antibody following use of live lentogenic vaccines. It was found that Mab HI titers above 2^3 would delay and depress the active antibody response to live V4 vaccine (10). However, it was also generally concluded that young chickens vaccinated by the oro-tracheal/spray routes were more resistant to challenge than non-vaccinated or parenteral vaccinated chickens due to a combination of passive antibody and local/cell-mediated immunity (CMI). Stimulation of local immunity has been shown (3) to bypass interference by Mab antibody and whilst CMI may play a role in protection in the face of ND Mab, it also required neutralising antibody to be present. Further studies (3) have shown that local secretory antibody at the mucosal surface provided protection when chickens with high levels of Mab antibody were challenged with virulent virus. Hence, protection may be obtained in the absence of significant levels of active ND antibody.

In Australia, hatcheries are also required to spray infectious bronchitis (IB) vaccines concurrently with live ND vaccines at day-old. In many countries, concurrent administration of these two vaccines has remained controversial despite the marketing and wide sale of combined IB and ND vaccines. Vaccine manufacturers have generally followed recommendations by Winterfield (1984) to increase the ND vaccine component some 2 to 3 $\log_{10}$ over the titer of the IB vaccine component.

STUDIES ON A NEW V4 ND VACCINE VIRUS

Over the past two years, as part of the application for registration, Bioproperties Pty Ltd (BPL) has undertaken a series of laboratory and field studies on the safety and efficacy of a new seed of the V4 vaccine virus in chickens. It has used the existing registered live ND V4 vaccine as a benchmark in many of the trials. Whilst the trials were designed towards satisfying regulatory requirements, they also touched on many of the variables described above that can

impact on the success or failure of live ND V4 vaccine relative to SOP requirements.

The following laboratory studies have been undertaken on the ND antibody response to BPL ND V4 vaccine in:

1. SPF chickens vaccinated at 14 days of age.
2. SPF chickens vaccinated at 14, 42 and 70 days of age.
3. SPF chickens vaccinated at day-old.
4. ND Mab negative broilers vaccinated at day-old.
5. ND Mab positive broilers vaccinated at day-old.
6. Mab positive commercial broilers vaccinated at either 7, 12 or 17 days of age
7. Commercial broilers vaccinated at day-old and 17 days of age compared to vaccination at 17 days of age only.
8. SPF chickens vaccinated at day-old with combined BPL ND V4 and IB vaccine compared to ND V4 and IB vaccines alone.
9. SPF chickens vaccinated 3 weeks after vaccination with infectious bursal disease virus (Strain V877).

In addition, field trials of ND V4 vaccines have been completed in 2.2 million commercial broiler chickens in two states.

SUMMARY OF RESULTS FROM THE STUDIES

SPF and Mab negative broiler chickens responded rapidly (within 14 days) to the BPL ND V4 vaccine and exceeded the SOP titer requirements by a wide margin (>2.0 Log_2 HI units). However, the ND antibody response in Mab positive broilers was delayed and inferior to Mab negative broilers. Antibody levels declined with age to approximate the SOP minimum level where they remained before increasing marginally by 35 days of age. Unvaccinated in-contact chickens exceeded the SOP minimum level by day 28 post-vaccination. Commercial broiler chickens responded more rapidly and exceeded the SOP minimum requirements when Mab titer levels were lower at the time of vaccination. Mab levels above 2^3 were associated with delayed and lower active antibody responses. A single vaccination at 17 days of age gave a superior active antibody response to a day-old vaccination followed by vaccination at 17 day of age. The ND antibody response in SPF chickens following a combined ND V4 and IB vaccine exceeded the SOP minimum titer by a wide margin. However, the antibody response was lower than that of ND V4 given alone. The ND antibody response in SPF chickens that had been previously vaccinated with an IBD vaccine exceeded the SOP minimum titer by a wide margin. In field trials involving some 36 broiler flocks (2.2 million birds), the experimental BPL ND V4 vaccine provided active antibody levels that exceeded the minimum SOP titer in 91% of flocks compared to 64% following the administration of the reference ND V4 vaccine. Broiler flocks were vaccinated at 10 days of age when the mean Mab titer was 2^2.

POTENTIAL IMPACT ON ND VACCINATION IN AUSTRALIA

The results of studies undertaken by BPL were consistent with those previously obtained on the efficacy of live ND V4 vaccines. Interference by ND Mab was clearly evident on response to ND V4 vaccine particularly when administered in the face of high levels ($\geq 2^3$ HI units) of ND Mab. The delayed and lower humoral response observed in these studies due to the presence of high Mab levels suggests the possibility that field viruses more virulent than V4 may have a greater opportunity for replication in these flocks if they are able to replicate in the presence of higher levels of Mab than the V4-strain vaccine. Concurrent administration of IB vaccine with ND V4 vaccine at day-old could further reduce the active antibody response. This could be particularly so when arbitrary proportions of the two vaccines are chosen rather than use of a correctly formulated combined vaccine.

As vaccination at day-old has now become the currently preferred program by most poultry companies, the results described above should raise concerns as to the overall efficacy of this type of program. Whereas, broiler chickens with low levels of ND Mab could respond adequately and possibly seed the broiler shed, a high level of Mab transfer from breeder flocks could well lead to inadequate humoral antibody responses. In the absence of adequate humoral antibody responses from day-old vaccination, dependence on local antibody and CMI for life-long broiler protection is contrary to accepted recommendations (MAFF 1974). These programs normally recommend a second vaccination at about 18-21 days of age to stimulate high levels of neutralising antibody.

The control of endemic virulent and precursor viruses in Australia following the 1998-2002 outbreaks is partly dependent on the objective of the ND V4 vaccine out-competing those viruses. Although there is evidence that ND V4 vaccine will reduce the excretion of precursor viruses, the level and frequency of challenge from such viruses is not well understood. The continued application of sub-optimal programs could well encourage the evolution of further precursor or virulent endemic strains.

RECOMMENDATIONS

The NNDMP for Australia should be re-evaluated as to whether the currently recommended SOPs meet the overall objectives of the eradication program. Additional information on the response to vaccination under field conditions is urgently needed. Evidence of protection following day-old vaccination should be obtained through challenge studies or though evaluation of CMI/ local immune responses to ND V4 vaccine. In the interim, a number of alternate programs could be considered, as follows:

a) Delay primary vaccination until mean flock ND Mab levels fall below 2^3

b) Following day-old spray vaccination, revaccinate at about 18-21 days of age

c) Reduce ND Mab levels in broilers by reducing the administration of inactivated vaccines to breeding stock.

d) Optimize the application of ND V4 and IB vaccines when administered together.

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COMPARISON OF SUSCEPTIBILITY AND IMMUNITY FOLLOWING VACCINATION TO THE 2002-03 CALIFORNIA VIRULENT NEWCASTLE DISEASE VIRUS (vNDV) BETWEEN COMMERCIAL AND SPF TURKEYS

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ABSTRACT

Newcastle disease vaccination is widely practiced in the USA with the majority of commercial chickens and turkeys receiving multiple vaccinations during their lifetime. The objectives of the present study were to extend the knowledge of susceptibility and protection by live and inactivated Newcastle disease virus (NDV) B_1 vaccines in commercial and specific-pathogen-free (SPF) turkeys following lethal challenge with the California 2002-03 virulent NDV (vNDV). Unvaccinated commercial turkeys were less susceptible to mortality following vNDV challenge than unvaccinated SPF turkeys. Vaccination with live or inactivated NDV B_1 provided complete protection of SPF turkeys, but only increased protection of commercial turkeys against morbidity and mortality following lethal challenge. Cloacal and oropharyngeal swabs were collected and tested by virus isolation and RRT-PCR. The results indicate vaccination could not prevent virus shed up to 14 days post-challenge in both commercial and SPF turkeys.

INTRODUCTION

During October 2002, vNDV was isolated from back yard game chickens in southern California (Los Angeles county), which preceded vNDV isolation from commercial poultry in December 2002 (6-9). Before the last positive isolation from commercial poultry was made on March 26th, 2003, more than 19,000 premises would be quarantined in five states, including California, Nevada, Arizona, Texas and New Mexico. The outbreak was deemed eradicated with the last quarantine lifted during September, 2003. More than 3 million birds, including approximately 150,000 backyard flocks and 806 commercial sites, were depopulated. Final cost of the outbreak is estimated to be in excess of $200 million.

Current vaccination programs for NDV include the use of low-virulent, live-virus and inactivated vaccines designed to control against endemic, low virulence field strains. Although the efficacy of currently available NDV vaccines against vNDV is widely accepted (2-5, 10, 11), the recent outbreak of vNDV in California underscores the need for continued evaluation of NDV vaccines and vaccination programs. For vNDV outbreak situations, reducing the shed of virus from infected birds is also critical to controlling spread of disease. The objectives of the present study were to extend the knowledge of protection against vNDV by live and inactivated NDV vaccines, including duration and amount of viral shedding, in commercial and SPF turkeys following lethal challenge with a California 2002 vNDV isolate.

MATERIALS AND METHODS

Turkeys. One-day-old SPF Beltsville White turkey poults were obtained from our flock at the Southeast Poultry Research Laboratory. Two-day-old commercial turkeys, Ridgeway Broad Breasted Whites, were received from Ridgeway Hatcheries (LaRue, OH). Birds were housed in Horsfall isolation units under negative pressure in a biosafety level 3 agriculture facility and received feed and water *ad libitum* (1).

Viruses. Lentogenic NDV vaccine viruses utilized during this study included commercial type B_1 inactivated and live virus (Lohmann Animal Health International, (LAHI), Gainesville, GA). A velogenic strain of vNDV, California 2002 (CA02; game chicken/US(CA)/S0212676/02), was used for challenge. This isolate was responsible for a recent epizootic outbreak in the southwestern United States recovered from a game bird in California during October 2002 (6). NDV was propagated and titrated in 9-11day-old SPF chicken embryos via the chorioallantoic sac route.

Experiment I. Forty-eight two-day-old commercial turkeys were arbitrarily divided into 4 groups of 12 birds. Birds in groups 1 and 2 received 100 µL of phosphate-buffered saline (PBS, pH 7.4) via intranasal (IN;50 µL) and eye drop (ED;50 µL) routes at 10 days-of-age. Birds in group 3 received a commercial live-virus B_1 vaccine (LAHI) via ED and IN route according to the manufacturer's recommend-dations at 10 days-of-age. Birds in group 4 received 100 µL of inactivated oil-emulsion B_1 vaccine (LAHI) injected subcutaneously in the neck, according to the manufacturers recommendations at 10 days-of-age. Two weeks post-vaccination (day 24), birds in group 2, 3, and 4 were challenged via ED and IN route with $10^{5.9}$ embryo infectious dose $_{50}$ (EID_{50}) / bird CA02. Negative control birds were sham-challenged with 100 µL PBS via ED/IN route. Following challenge, birds were monitored daily for overt clinical signs of disease and mortality. Turkeys displaying severe clinical signs of disease were euthanized by overdose of sodium pentobarbital. Serum samples were taken by wing bleed at -14, 0, and 14 days post-challenge (pc). Oropharyngeal and cloacal swabs were collected into 2 mL brain-heart infusion (BHI) broth with antibiotics (1000 units/mL penicillin G, 200 µg/mL gentamicin sulfate, and 4 µg/mL amphotericin B; Sigma Chemical Company, St. Louis, MO) from each bird on day 0, 2, 4, 6, 10, and 14 days pc for virus isolation.

Experiment II. Sixty one-day-old SPF turkeys were arbitrarily divided into four groups of 15 birds, as in experiment I. Birds were vaccinated as described above at twenty-one days of age. At thirty-five days of age, birds in group 2, 3, and 4 were challenged via ED and IN route with $10^{5.9}$ EID_{50} / bird CA02. Birds in group 1 were kept as unchallenged controls. Following challenge, birds were monitored daily for clinical signs of disease and mortality, with serum and swabs processed as described above.

RESULTS AND CONCLUSIONS

Unvaccinated commercial turkeys were less susceptible to mortality following vNDV challenge than unvaccinated SPF turkeys. Although the commercial birds displayed clinical signs of disease following challenge, most (67%) recovered following challenge. In contrast, SPF turkeys displayed 100 % mortality by day 6 pc.

Vaccination with live or inactivated B_1 NDV increased protection of commercial turkeys (75-91%); however, vaccinated SPF turkeys were completely protected against morbidity and mortality following lethal challenge.

Serum samples taken prior to vaccination (day 10) from commercial poults indicated the presence of maternal anti-NDV antibodies. Vaccination with either

live or inactivated vaccine resulted in uneven seroconversion at day of challenge. By day 14 pc, all surviving birds displayed positive antibody titers against NDV. As expected, serum samples taken from SPF poults were negative prior to vaccination. All vaccinated birds displayed positive antibody titers against NDV at day of challenge, which increased following challenge.

Cloacal and oropharyngeal swabs were collected and tested by virus isolation. The results indicated vaccination could not prevent virus shed up to 14 days post-challenge in both commercial and SPF turkeys. In addition, vaccination with the live virus vaccine resulted in generally lower viral titers from both the oral and cloacal sites compared to the inactivated vaccine.

In conclusion, although NDV-vaccinated turkeys were protected against vNDV, they continued to shed virus in the absence of clinical signs up to14 days pc. Following an outbreak situation this condition may prevent diagnosis of an infected flock and result in further spread of disease. It is evident that testing both commercial and SPF vaccinated turkeys is critical to measuring vaccine protection and designing new vaccination strategies. The development of improved vaccines and vaccination strategies to induce protection against infection and inhibit shed of virus are needed to limit the duration of the next vNDV outbreak in the US.

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FIELD AND EXPERIMENTAL EVIDENCES OF AVIAN PNEUMOVIRUS VACCINE REVERSION TO VIRULENCE

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ABSTRACT

Avian pneumovirus causes an upper respiratory tract infection in turkeys leading to turkey rhinotracheitis (TRT) and in some other avian species, including chickens where it is also involved, in the etiology of multifactorial diseases such as swollen head syndrome. Since the 1980s a number of live attenuated subtype A and B APV vaccines have been developed in Europe and these have generally afforded good protection. All have been produced by repeated passages of field virus in a laboratory culture system to achieve various degrees of attenuation. They are widely used in commercial growing turkeys and to prime future layers and breeders. When tested under experimental conditions, these empirically derived vaccines were shown to be fully protective whilst not causing detectable disease themselves. However, they have not performed as well when used in the field and unstable attenuation has been considered to be a possible factor. Since pneumoviruses are single-stranded RNA viruses their relatively high mutation rates have been thought to be the underlying reason for instances of reversion to virulence observed in experimental conditions (1). This paper describes the first evidence of reversion of an APV vaccine in the field. First, systematic evidence of extended vaccine virus persistence in the field arose from a longitudinal study performed in the UK in 1995. Secondly, from an outbreak of TRT in an Italian turkey flock previously vaccinated at day old, we isolated an APV which proved to be a vaccine derivative. Finally, in order to determine whether vaccine virus or a derivative of increased virulence had been isolated, the virus was applied to one-day-old poults in secure isolation conditions. The vaccine derivative virus was shown to be able to cause clinical disease.

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

IMMUNOSUPPRESSION OF AVIAN PNEUMOVIRUS INFECTED TURKEYS

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ABSTRACT

A laboratory experiment was designed to evaluate the persistence of APV in birds post-infection. Two-week-old APV-antibody-free commercial turkey poults were divided into two equal groups. At two weeks of age, poults in one group were infected with APV. Poults in the second group were sham inoculated with virus free cell culture fluid and were maintained as APV non-infected controls. At three weeks post-experimental APV infection, poults in the infected group were further divided into two subgroups and housed separately. Poults from one subgroup were immunosuppressed by treating with subcutaneous injection of dexamethasone (2mg / kg body weight) and cyclophosphamide (50mg / kg body weight) to induce immunosuppression (Jones *et al*., 1992). Following treatment with immunosuppressants, tracheal and choanal swabs were collected on week 1, 4, 8, 12 and 16 post treatment for virus detection and isolation. We also collected blood samples from all

birds at weekly intervals to examine seroconversion to APV. Flow cytometry analysis was done to analyze the T and B lymphocyte counts using anti-CD_4, anti-CD_8, and anti-IgM markers. Birds showed severe clinical signs in the form of copious nasal discharge, swollen sinus, conjunctivitis and depression from day 4 PI to 12 day PI. Viral RNA could be detected for 14 days post infection from nasal turbinate and for 9 days from trachea. The birds showed seroconversion as detected by APV-ELISA. Choanal swabs tested by RT-PCR for the presence of APV RNA were negative from week one through 15 post treatment with immuno-suppressants. In conclusion, our experimental studies did not appear to reactivate virus if any, post treatment with immunosuppressants.

INTRODUCTION

Avian pneumovirus (APV) causes an acute upper respiratory tract infection in turkeys (2,3,4,5,15). It is considered to be involved in the etiology of swollen head syndrome in chickens. The disease caused by APV in turkeys is characterized by coughing, sneezing, nasal discharge, tracheal rales, foamy conjunctivitis, and swollen sinuses (2,3,4,5). Uncomplicated cases have low mortality (2 to 5%) but infections accompanied by concurrent secondary bacterial infections can result in up to 25% mortality. In breeder turkey flocks, there is a transient drop in egg production and also paleness of the egg shell (15).

Avian pneumovirus belongs to the genus *Metapneumovirus* of the *Paramyxoviridae* family (8). It is a negative-sense single-stranded RNA virus. Avian pneumovirus is classified in the genus *Metapneumovirus.* There are four subtypes of APV identified as, A, B, C, and D. Subtypes A, B and D were identified in Europe (1,5). Various studies suggested that APV isolates from turkeys in the United States were antigenically and genetically different from the European A and B subtypes. So the U.S. isolates were classified as subgroup C (7,9,10,11,12,13,14).

MATERIALS AND METHODS

Virus. A Vero-cell-propagated, Minnesota isolate of APV (APV/MN19/2003) with a titer of 10^5 $TCID_{50}$ was used in the study. The virus was isolated from the nasal turbinates of eight-week-old turkeys with acute upper respiratory tract infection. A 20% homogenate of nasal turbinate tissue was prepared in Hank's balanced salt solution. This was centrifuged at 3000 X g for 10 min and the supernatant was passaged on chicken embryo fibroblasts (CEF). Six passages were performed on CEF followed by six passages on Vero cells. After three rounds of freezing and thawing, the cell suspension was centrifuged at 3000 X g for 10 min. The supernatant was used as the inoculum after determining the titer of the virus on Vero cells.

Experimental design. A laboratory experiment was designed to evaluate the persistence of APV in individual birds. Two-week-old turkeys were experimentally infected with APV and individually monitored for the persistence of the virus until the age of marketing (22 weeks). Specifically, two-week-old APV-antibody-free commercial turkey poults were divided into two equal groups. At two weeks of age, poults in one group were infected with APV. Fifty microliters of APV (APV/MN19/2003) infected cell culture fluid ($TCID_{50}$ 10^5/mL) was instilled into each conjunctival space and nostril (total of 200 uL/poult). This dose and the route of exposure were successfully used in our experiments in the past (4). Poults in the second group were maintained as APV non-infected group.

At three weeks post-experimental infection, poults in the infected group were further divided into two subgroups and housed separately. Poults from one subgroup were treated with subcutaneous injection of dexamethasone (2mg / kg body weight) and cyclophosphamide (50mg / kg body weight) to induce immunosuppression. Following treatment with immunosuppressants, choanal swabs were collected on week 1, 4, 8, 12, and 16 post treatment for virus detection and isolation. (Immunosuppressants dexamethasone and cyclophosphamide were injected at three weeks PI because of the fact that it has been very difficult to isolate APV from birds three weeks post infection from a clinical outbreak in the field.) Whether the virus excretion ceases to take place after three weeks or excreted at levels undetectable with methods used today are not known. We also collected blood samples from all birds at weekly intervals to examine seroconversion to APV. In this experiment we monitored the same birds for virus detection post infection *with and without* immunosuppression. This laboratory investigation was designed to simulate where other stressful conditions may exist.

RESULTS AND DISCUSSION

The main clinical signs showed by infected turkeys included unilateral and/or bilateral nasal discharge, swelling of infraorbital sinus, conjunctivitis, open mouth breathing, and depression. Birds inoculated with APV started showing clinical signs from 4 day PI and continued up to 12 day PI. However there were no clinical signs after 12 days PI. Birds in the sham inoculated group did not show any clinical signs.

Birds were positive for APV RNA when choanal swabs were tested by RT-PCR in the first two weeks of infection. All the birds in the sham inoculated group

were negative for the presence of APV at any time of sample collection. We could not detect viral RNA in the immunosuppressed and non-immunosuppressed birds from three weeks PI until 20 weeks PI.

Birds in the APV infected group seroconverted by one week post infection. Birds were positive for APV antibodies when tested by APV until 18 weeks PI. There was a gradual increase in antibody titers from one week post infection up to six weeks PI, and then it gradually declined.

In this study we investigated the persistence of APV in infected birds under normal and immunosuppressed conditions. The experiment was designed to simulate the real world situation. We have not looked at the mechanisms of persistence. However it is well known that viruses with high mutation rates, especially RNA viruses persist by generating attenuated variants or altering epitopes in response to immune pressure. Khehra and Jones (1999) treated APV infected poults and chicks with the T-cell suppressor, cyclosporine A to look at the re-excretion and persistence of APV but did not recover any virus beyond nine days from poults and five days from chicks. In our study we could not detect APV in birds beyond two weeks post infection both in immunosuppressed and non-immunosuppressed birds.

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PATHOGENESIS OF AVIAN PNEUMOVIRUS INFECTION IN TWO-WEEK-OLD TURKEYS

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ABSTRACT

Two-week-old turkeys were experimentally infected with a most recent (2003) isolate of avian pneumovirus (APV). Seventy turkey poults were divided equally into two groups. One group was inoculated with APV (MN 19) with a titer of $10^{5.5}$TCID$_{50}$ oculonasally. Birds in the second group were maintained as sham inoculated controls. Samples such as nasal turbinates, trachea, conjunctiva, lungs and liver were collected at 1, 3, 5, 7, 9, 11, and 14 days post-inoculation. The birds were monitored daily for clinical signs and samples were analyzed by histopathology, immunohistochemistry, quantitative RT-PCR and *in situ* hybridization. Birds showed severe clinical signs in the form of copious nasal discharge, swollen sinus, conjunctivitis, and depression from 4 day PI to 12 day PI. Viral RNA could be detected for 14 days post infection from nasal turbinate and for 9 days from trachea. Histopathological lesions were prominent in nasal turbinate and were seen from 3-11 day PI. Immunohistochemistry revealed the presence of APV from 3-9 day PI in nasal turbinate and trachea. Immunohistochemistry also showed the presence of APV in lungs in two infected birds. *In situ* hybridization demonstrated the presence of APV from 1-11 day PI in nasal turbinates and 3-9 days PI in the trachea. Quantitative real time PCR data showed the presence of maximum amount of virus at 3 day PI in nasal turbinate and trachea.

Avian pneumovirus (APV) is the causative agent of an acute upper respiratory tract infection in turkeys (5, 15). The disease caused by APV in turkeys is characterized by coughing, sneezing, nasal discharge, tracheal rales, foamy conjunctivitis, and swollen sinuses (2, 5). Avian pneumovirus was first detected in South Africa in 1978. In the United States, APV appeared for the first time in 1996 in Colorado. APV was detected in 1997 in Minnesota where the incidence of the disease has increased over the past eight years (2, 3, 6, 7).

Avian pneumovirus belongs to the genus *Metapneumovirus* of the *Paramyxoviridae* family (8). It is a negative-sense single-stranded RNA virus. Avian pneumovirus is classified in the genus *Metapneumovirus*, whereas their mammalian counterparts belong to the genus *Pneumovirus*. There are four subtypes of APV identified as, A, B, C, and D. Subtypes A and B were identified in Europe. Various studies suggested that APV isolates from turkeys in the United States were antigenically and genetically different from the European A and B subtypes (6,9,10, 11,12,13,14). So the US isolates were classified as subgroup C. Subtype D was recognized from France by a retrospective sequence analysis of two French isolates obtained in 1985 (1).

The objectives of the present study were to investigate the pathogenesis of recent isolates (2003) of APV in two week old turkeys in an experimental infection. Another objective of this study was to evaluate the quantitative distribution of the virus in various tissues during the course of infection. This was not done in previous studies.

MATERIALS AND METHODS

Virus. A Vero-cell-propagated, recent Minnesota isolate of APV (APV/MN19/2003) with a titer of 10^5 TCID$_{50}$ was used in the study. The virus was isolated from the nasal turbinates of 8 week-old turkeys with acute upper respiratory tract infection.

Experimental design. Seventy, two-week-old turkey poults that tested negative for APV antibodies, were used in this study. The turkey poults were divided into two groups. One group was inoculated with the most recent Minnesota isolate of APV (APV/MN19/2003) with a titer of $10^{5.5}$ TCID$_{50}$. Fifty microliters of the virus suspension was instilled in each eye and nostril. Birds in the second group were used as sham inoculated controls. Five birds from each group were sacrificed at day 1, 3, 5, 7, 9, 11, 14 post infection. Samples of nasal turbinates, trachea, conjunctiva, lungs, and liver were collected for analysis. The birds were monitored daily for clinical signs. The samples were analyzed by histopathology, immunohistochemistry, quantitative RT-PCR, and *in situ* hybridization.

RT-PCR. The viral RNA from the tissue samples was extracted using a commercial viral RNA extraction kit (Qiagen, Valencia, CA). RT-PCR was performed using the one step RT-PCR kit (Qiagen). The primers used in this assay for the detection of the APV genome were those designed based on the matrix (M) gene of APV (GenBank Accession No. AF 187151).

Histopathology. The hematoxylin and eosin (H&E) staining technique was used for histopathological examination. Tissues including the nasal turbinate, trachea, and lungs were fixed in 10% buffered neutral formalin. Paraffin embedded tissues were sectioned at a thickness of 3-4 μm and stained with H&E.

Immunohistochemistry (IHC). Tissues includeing the nasal turbinate, trachea and lungs were subjected to IHC. Formalin fixed tissue sections were analyzed by an immunoperoxidase procedure developed to detect APV antigen in tissues.[8]

***In situ* hybridization.** A 137 bp segment of the M gene of APV was amplified using M1 and M2 primers. The product of RT-PCR was purified using a PCR purification kit (Qiagen). The DNA fragment was then labeled with digoxigenin-dUTP by random priming using the DIG DNA labeling and detection kit (Roche Diagnostics GmbH, Manheim, Germany). *In situ* hybridization was performed as per Masot *et al*. (2000) with modifications.

Real time quantitative PCR. Viral RNA was extracted from tissue samples using QIA amp viral RNA mini kit (Qiagen). Oligonucleotide primer probes were designed based on the M gene sequence of APV. The probes having nucleotides with a 5' reporter dye 6-carboxy fluorescein and a 3' quencher dye 6-carboxytetramethylrhodamine were ordered and purchased from Applied Biosystems (Foster City, CA). Real-time quantitative PCR was performed in an ABI Prism 7700 sequence detection system (Applied BioSystems).

RESULTS

Clinical signs. Birds inoculated with APV started showing clinical signs from 4 day PI and continued up to 12 day PI. The initial sign was a watery nasal discharge which became a thick mucus discharge within a few days. In most of the cases the clinical signs were unilateral. Swelling of the infra orbital sinus was noticed 6 days PI and was mainly unilateral. The clinical signs also included conjunctivitis, open mouth breathing and depression. In infected birds, the clinical score increased gradually from 4 days PI, reached its peak by 6 days PI and declined gradually over the next 6 days. Birds in the sham inoculated group did not show any clinical signs.

RT-PCR. Viral RNA could be detected in the nasal turbinate from 1 day PI until 14 days PI by RT-PCR. The trachea showed the presence of viral RNA from 3 days PI and remained positive up to 9 days PI. A high percentage of birds were positive for APV in the first week following infection (60-100%). About 40% of the birds had the virus in the nasal turbinates when tested by RT-PCR in the second week following infection whereas only 20% of the birds had virus in their trachea on 9 days PI. Viral RNA could not be detected in all other tissues tested. This included the lungs, conjunctiva, infra orbital sinus, harderian gland, liver and spleen. All the birds in the sham inoculated group were negative for the presence of APV at any time of sample collection in all tissue samples.

Histopathology. Histopathological lesions were evident in nasal turbinates by 3 days PI. There was an increased infiltration of inflammatory cells such as lymphocytes, macrophages and a few heterophils in the lamina propria. A few of the mucosal glands were dilated. Squamous metaplasia of the epithelial lining, individual necrotic cells in the epithelial lining, and a multifocal loss of cilia also were observed. Histopathological lesions were prominent in the nasal turbinate by 5 days PI and were seen over a period from 3 to 11 days PI. The main lesions in the trachea consisted of an increased infiltration of lymphocytes, macrophages and heterophils in lamina propria. Tracheal lesions were observed on days 5, 7, and 9 PI. Lung tissues showed prominent lymphoid aggregates around airways on days 3, 5, 9, and 11 PI. The nasal turbinate, trachea and lung tissues from sham inoculated birds did not show any histopathological lesions.

Immunohistochemistry. Immunohistochemistry revealed the presence of the APV antigen from 3-9 days PI in the nasal turbinate and the trachea. Viral antigen could be detected in the apical surface of the ciliated epithelium of the nasal turbinate. About 80% of the birds showed the presence of the APV antigen in the turbinate on day 5 PI. In the case of the trachea, 60% of the birds were positive for the presence of the viral antigen. Viral antigen could be detected in the mucosal epithelium of the trachea. Immunohistochemistry also showed the presence of the APV antigen in the lungs on days 7 and 9 PI in two infected birds. Birds in the sham inoculated group were negative for the presence of APV antigen by immunohistochemistry.

***In situ* hybridization.** *In situ* hybridization demonstrated the presence of APV from 1-11 days PI in the nasal turbinates. The staining was mainly confined to the apical surface of the ciliated epithelium of the nasal turbinate. About 80% of the birds showed the presence of viral RNA in the turbinate at 3 days PI. In the case of the trachea, the mucosal epithelium

showed the presence of virus from 3 to 9 days PI. About 60% of the birds were positive for the presence of virus in the trachea on days 5 and 7 PI. All the samples from sham inoculated control birds were negative for the presence of APV RNA.

Quantitative real-time RT-PCR. Quantitative real time RT-PCR detected APV RNA as early as 1 day PI and as late as 14 days PI in the nasal turbinate. In the trachea, APV RNA could be detected from 3 to 11 days PI. The data showed the presence of the maximum amount of virus at 3 days PI in the nasal turbinate and the trachea. Viral RNA could not be identified from lung tissues collected at any time period from the infected birds. All the birds from the sham inoculated group showed the absence of viral RNA.

DISCUSSION

In an experimental infection with APV subtype C, Jirjis *et al.* (4) noticed nasal discharge, swelling of the infraorbital sinuses, and frothy ocular discharge from 2 to 11 days PI. In a natural outbreak, Shin *et al.* (13) reported that infected turkeys showed respiratory disease manifested by coughing, swollen sinuses, and nasal discharge. An experimental infection of broiler chicks with APV showed mild clinical signs, including coughing, sneezing, nasal discharge and watery eyes from 2-8 days PI. In the present study, the major clinical signs observed were nasal discharge, swelling of the infraorbital sinus, conjunctivitis and open mouth breathing. The nasal discharge was watery in the beginning and later became thick mucus. Birds started showing clinical signs from day 4 to 12 days PI.

Jirjis et al. (4) noticed mild increase in the number of lymphocytes and macrophages in the mucosa and submucosa of the nasal turbinates and infraorbital sinuses on 2 days PI. In the case of trachea, there was a small increase in the number of lympocytes macrophages and a few heterophils between days 4 and 10 PI. In the present study, we noticed histopathological changes in nasal turbinates by 3 days PI. There was an increased infiltration of lymphocytes, macrophages and a few heterophils in the lamina propria. A few of the mucosal glands were dilated. We detected squamous metaplasia of the epithelial lining, individual necrotic cells in the epithelial lining, and a multifocal loss of cilia. Loss of cilia was not observed in previous studies with US subtype of APV. Previous studies did not reveal any major lesions in the lung tissues. We observed prominent lymphoid aggregates around airways on days 3, 5, 9, and 11 PI.

Apical surface of the ciliated epithelium of the nasal turbinates and infraorbital sinuses showed the presence of APV in infected birds from 2-8 days PI upon immunohistochemical staining. No APV antigen could be detected in the lung sections. The present study revealed the presence of APV antigen in the lung tissues on days 7 and 9 PI in two infected birds. This could be a chance finding or we can also speculate that alveolar macrophages may be playing a role in carrying the viral antigen. We detected viral antigen from 3-9 days PI in the nasal turbinate and the trachea. Viral antigen could be detected in the apical surface of the ciliated epithelium of the nasal turbinate and in the mucosal epithelium of the trachea. In the present study, we demonstrated the presence of APV in nasal turbinate and trachea by *in situ* hybridization from 1-11 days PI and 3-9 days PI, respectively. Our quantitative real-time RT-PCR data revealed the presence of the maximum amount of virus at 3 days PI in the nasal turbinate and the trachea. This is the first report of a quantitative evaluation of the distribution of APV subtype C in tissues of infected turkeys.

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PRODUCTION DROPS IN BROILER BREEDERS DUE TO AVIAN ENCEPHALOMYELITIS INFECTION

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SUMMARY

Two broiler breeder flocks in two geographically distinct regions suffered severe production drops due to avian encephalomyelitis (AE) infection in the summer of 2004. In both flocks, diagnosis was confirmed in broiler progeny by histopathology and clinical picture. History and presentation of each flock will be described separately.

Flock 1. A broiler breeder flock at 29 weeks of age suffered a dramatic production drop coming into production. Production never returned to normal levels. The flock scheduled for vaccination by drinking water for AE at 10 weeks of age. Serology at 16 weeks suggested poor seroconversion to AE after vaccination, but no attempt to revaccinate the flock was made. Diagnosis was based upon clinical signs and histopathology in broiler progeny. Broilers exhibited signs of tremors and lateral recumbency and mortality ranging from 3-6%/week for the first 3 weeks.

Flock 2. A broiler breeder flock at 41 weeks of age suffered a production drop of almost 10% for 18 days. The eggs set during this period suffered from very poor hatches (60%) despite excellent fertility (98.1%) with very high amounts of late dead mortality (30%) in the unhatched residue breakout. This was similar to findings from Taylor *et al.* (1). Broilers from these hatches also exhibited signs consistent with AE infection. The hens were scheduled for AE vaccination at 10 weeks of age by the drinking water. These birds were raised on new litter in the pullet house with an excellent house sanitation program.

The most likely reason for AE infection in these hen flocks is improper pullet vaccination in the drinking water for AE. AE virus is considered to be very hardy (2); therefore it may be considered unlikely that the vaccine could be mishandled in such a way that the virus could be harmed. Additionally, since the virus is shed in the feces after vaccination, very few of the birds would have had to be vaccinated for flock seroconversion to be adequate. It is suspected that the vaccine was either grossly mishandled or that the vaccination was simply forgotten. Since no other reports of vaccine failure occurred within this serial, it is unlikely that vaccine titer or embryo adaptation failures occurred.

Another point to consider is that Flock 1 was vaccinated for fowlpox at the same time as the 10-week AE vaccination. In this instance it seems logical to combine these vaccinations in a wing-web administered bi-valent AE/pox combination since the birds are already handled. In Flock 2, there was no fowlpox challenge/vaccination and thus water

administration of the vaccine may be indicated. A discussion of various techniques and cost considerations for vaccination and serological monitoring for AE will be made at the time of presentation.

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GAIT SCORING IN COMMERCIAL BROILER PRODUCTION: A MEASURE OF POULTRY WELFARE

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ABSTRACT

Now that poultry welfare has become part of the purchasing decision by some U.S. poultry merchandisers, non-performance measures of welfare are being sought by commercial broiler producers. Gait scoring is one such measure that has been studied and reported from controlled university facilities. Immobility, that is the inability of the bird to move to eat and drink, has been reported as over 7% of the population at 49 days in research facilities (4).

Commercial broiler producers presumed that commercial facilities produced less lameness based on anecdotal observations and final flock performance numbers. To confirm this estimation, two hot weather (May/June and August) mobility observation data sets were collected in southern Mississippi. The mobility of broiler chickens was evaluated just prior to marketing to determine a percentage of the chickens with impaired gaits. The chickens were divided into two different populations, one with an age of approximately 50 days and 6 lbs. live weight, the other approximately 60 days and 8 lbs. live weight. Twenty-five chickens were caught and evaluated at four separate locations in the house to remove potential in-house environmental differences.

In accordance with a previously reported study (1), chicken gaits were scored as Normal (0), Reluctant (2), and Impaired (3). In the 60 day old group, 95.3% scored "0", with 4.3% and 0.3%, scoring 1 and 2 respectively. In the 50 day old group, 98.9% scored "0", with 0.92% scored 1 and 0.1% scored 2. Overall, very few birds, 0.22%, were found to be non-ambulatory (Score 2). The low occurrence of gait abnormality was not a result of rigorous culling or premature mortality since the cull rate and total mortality averaged 0.91% and 3.11%, for the 60 day old group respectively, and 0.71% and 2.69% for the 50 day old group. Plant condemnation for whole birds and parts was less the 0.5% for both groups. Stocking densities were at industry standards.

As hot weather produces greater mortality in the southern U.S., two hot weather observation periods were considered to look at the worse possible scenario. Even with such a bias, commercial broilers appeared to fare better in at least one welfare measure than their university housed counter-parts. Gait scoring commercial broilers confirmed their producers' assumption that well performing animals are in a state of good animal welfare.

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THE CHANGING FACE OF THE VACCINE INDUSTRY

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The recent press coverage on both the shortage of and the reason for it, of human influenza vaccine is most pertinent to this paper. Both the human and veterinary vaccine industries have come under increasing pressure when compared to the pharmaceutical side of the business. The premise is, that humans (or animals) use pharmaceutical compounds on multiple occasions (often daily) compared to the limited use of vaccines. In the view of the present, economically driven, leadership the decision as to where to invest is clear. To illustrate my point, ONE flea control product for dogs had sales TEN TIMES the TOTAL avian vaccine market within three years of launch! In the biologics business one "molecule" does not cover all scenarios, and multiple presentations of short shelf life vaccines are needed. Regulations have also changed over this time and, unlike the 70s it is nearly as difficult to license a veterinary product as a human one with FAR less return.

The past 20 years has experienced on an accelerating basis, acquisition, mergers, withdrawals and a complete change of focus within the industry. The avian sector, due to lower margins, has been under even more pressure in the competition for internal funding. Financial returns have been drastically altered over this period. Human products have, of course, always been number one followed by cattle. Avian products which were in the 80s number three, have fallen to number five. This is not only behind dogs but, since 2000, avian lag even behind the cat market.

In the 1970s and 1980s in the USA 13 companies were in the avian market. All but one was US owned. The situation now in 2005 is that only six remain, only two of which have US ownership. The key focus for the majority of them is both human and pharmaceutical or chemical. Many well known companies have either disappeared from the scene, or market in combination with companies who used to be fierce competitors. This has altered the amount of investment dollars both for research and for the overall efforts at market penetration.

When the market sectors are evaluated, a *pharmaceutical* compound has high initial R&D costs BUT few compounds, strong patents, high value products with high margins, and world wide application.

A *biologic*, ESPECIALLY avian, has relatively low R&D investment BUT many variations (strains), patents difficult to enforce and are controversial, relatively low value, and are often regional at best. To illustrate my point, if we consider a "new" Bronchitis, such a virus will at first be *local*, then *regional*, possibly in a few years, *country wide*, and VERY rarely *world wide*.

The biologics industry must justify key opportunities, have the commercial poultry industry both need and give full support for the use of a product, and must use newer technology for rapid response. (We still use 60s technology in many areas and have an "Achilles heel" in the requirement for SPF eggs.)

Therefore, it is clear that both the vaccine industry AND the commercial poultry industry MUST recognize that the whole scenario of lower margins and lower returns in the poultry section is *unhealthy*. We do NOT want to have a situation when only one or two companies are viable in this critical area for food producing animals.

The intense competition within the pharmaceutical industry, especially for research and development dollars, is a major factor. Human and companion animal products have potentially larger returns and, so far, it is only the expansion of the commercial poultry industry world wide that has retained the viable interest that exists today. It is VITAL that ALL sectors of our business recognize the changes (many not positive) that have occurred. We MUST *work together* to ensure that we continue to enjoy the MAJOR benefits the vaccine industry has brought about. Without a continuous supply of modern high quality vaccines, serious and unwelcome consequences could result.

ISOLATION AND IDENTIFICATION OF RETICULOENDOTHELIOSIS VIRUSES FROM CHICKENS AND CHICKEN VACCINES

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ABSTRACT

Reticuloendotheliosis (REV) infection is common but not ubiquitous. In order to understand the REV infection status, antibody determination and virus isolation were performed for chickens in Taiwan. The results showed that REV infection is quite common in chickens. Two REV viruses were isolated from infected chickens and one from a fowl poxvirus vaccine. The phylogenetic analysis of those REV isolates will be presented.

INTRODUCTION

Reticuloendotheliosis viruses (REVs) belong to the Gammaretrovirus genus and have a genomic structure consisting of the group-specific antigen (gag), protease (pro), polymerase (pol), and envelope (env) regions flanked by long terminal repeats (LTRs). Birds infected with REV after hatching, a transient viremia followed by antibody formation. Infection of older birds rarely results in clinical diseases (9). The prevalence of seropositive flocks and the proportion of seropositive birds in a flocks increase with birds' ages. In certain instances, some infected chickens show chronic lymphomas and an immunosuppressive runting disease. Two types of tumors are found in infected chickens, bursal and non-bursal. The bursal type tumor originates in the cloacal bursa, and later spreads to liver and other organs in 17-43 weeks after inoculation with nondefective chick syncytial or T strains of REV (11). A second type of chronic tumor, nonbursal lymphoma, in chickens occurs by six weeks after inoculation with spleen necrosis or chick syncytial strains of nondefective REV (10). Some birds show an immunosuppressive runting disease; this developed by three to five weeks after infection (5).

Although REV infection is common, natural outbreaks of this disease in commercial poultry are rare. One possibility is from contaminated vaccines, especial fowl poxvirus or Marek's disease virus vaccines (2, 3). Those vaccines have been used extensively in chickens. The impact of vaccination and the relationship between infection and vaccination are not clear for the present.

This paper describes the REV infection status in chickens and compares the REVs from infected chickens and vaccines.

MATERIALS AND METHODS

Case history. Case 3122/03 was from a breeder farm in Yilan with two kinds of breed, breed A and breed B, 3 flocks each. The ages and the breed of those chickens in this farm were 10, 38, 66 weeks old of breed A and 13, 39, 63 weeks old of breed B in December 2003. Besides ordinary vaccination for commercial broiler breeder chickens, those chickens were vaccinated with live herpes virus of turkey and live SB1 strain of Marek's disease virus vaccines at one day of age and vaccinated two times with live fowl poxvirus vaccines at the age of one and 13 weeks, respectively. Those chickens were considered to be normal because their weekly mortality ranged from 0 to 0.15%. Blood samples were taken from those chickens for REV antibody determination and virus isolation.

Case 3295/04 was from a color breeder farm with 15,000 chickens. The weekly mortality of this farm was less than 0.2%, considered to be normal. The blood samples were taken for REV antibody determination and virus isolation.

REV antibody determination. Plasma samples from different flocks were tested for the presence of anti-REV antibody by using a commercial ELISA kit (Idexx, Westbrook, ME) according to the manufacturer's instructions.

REV virus isolation from chickens. Blood samples were taken from whole blood, collected with heparin (Becton Dickinson, Franklin Lakes, NJ), and put on ice immediately. Buffy coat from whole blood was co-cultured with DF1 cells (ATCC CRL-12203, American Type Culture Collection, Manassas, VA) and incubated at 39^{o}C for seven days each passage. The presence of virus was examined by RT-PCR in supernatant with LTR primers for long terminal repeat

(1). Virus isolation was attempted three passages in cell culture before considering negative.

REV gene detection in vaccines by PCR. Eighteen commercial fowl poxvirus vaccines and 18 Marek's disease vaccines sent for inspection at Animal Health Research Institute, Taiwan were collected for REV gene detection in 2003 and 2004, respectively. The REV LTR gene in fowl poxvirus vaccines was with primer, TR-1/TR-2 (7) and that in Marek's disease vaccines was detected by polymerase chain reaction by using LTR primer (1).

REV virus isolation from vaccines. All the collected vaccines were re-suspended with PBS and co-cultured with DF1 cells. After changing medium at the second day, those cells were culture for one week in one passage. The presence of virus was examined by RT-PCR in supernatant with LTR primers for long terminal repeat (1). Virus isolation was attempted three passages in cell culture before considering negative.

REV env gene sequencing. The env genes of those REV isolates were amplified by RT-PCR with env5/env6 primer (new designed), amplifying the whole env gene and cloned into yT&A vector (Yeastern Biotech, Taipei, Taiwan) and sequenced.

RESULTS

Seropositive prevalence and virus isolation in the broiler breeder farm. The seropositive prevalence and the ages of case 3122/03 farm in breed A were 0% (0/16) at the age of 10 weeks, 24% (4/17) at the age of 38 weeks, and 93% (15/16) at the age of 66 weeks; and those in breed B were 0% (0/13) at the age of 13 weeks, 92% (12/13) at the age of 39 and 100% at the age of 63 weeks. The seropositive prevalence increased with ages but not immediately after Marek's disease or fowl pox vaccinations. A REV, 3122/03, was isolated from a 31-week-old female broiler breeder chicken of breed A from that farm. The total virus isolation rate from those chickens was 1% (1/88).

Seropositive prevalence and virus isolation in the color breeder farm. The seropositive prevalence and the ages of case 3259/04 farm were 0% (0/15) at the age of 12 weeks, 93% (13/14) at the age of 25 weeks, 67% (10/15) at the age of 26 weeks, 86% (12/14) at the age of 28, and 100% at the age of 50 weeks. One REV virus, 3295/04, was isolated from a 12-week-old chicken.

Detection and virus isolation of REV in vaccines. Seven of the tested 18 fowl poxvirus vaccines had approximate 290 bp PCR product. However, only one REV was isolated from those vaccines. The vaccine with positive virus isolation had not this PCR band. No PCR product was found in Marek's disease vaccines with the primer used. After two passages of virus isolation from fowl poxvirus vaccines in DF1 cells, a faint band was found. After 13 times of passage, the PCR became clearer and finally a REV, 3134/03, was isolated from that vaccine.

REV env gene analysis. The identity of the env genes from the two chicken REV isolates, 3122/03 and 3259/04, was 99.7%. The REV isolate, 3134/03, from fowl poxvirus vaccine showed 461 n.t. deletion in its env gene when compared with the chicken isolates. Thus, the identities of this REV and those two chicken isolates were 67% and 67.1% respectively. Another clone from the same virus will be performed for this deletion. The identities of a reference strain, FPV-REV (AF246698) and these chicken isolates were 99.7% and 99.8%, respectively. The identity of strain A REV with the present two chicken isolates (X01455) was 95.5%.

DISCUSSION

Although REV infection is common, it is not ubiquitous (8). Data presented in this paper document the presence of REV in the field is quite common. Almost one-hundred percent of chickens get infection at old ages. However, those chickens showed neither clinical signs nor REV-induced tumors. Like avian leucosis virus infection, chicken showed resistant to REV infection after one week of age (8). From the late seroconversion of those flocks (after 16 weeks old), those chickens were infected at the later ages. This means those chickens established their resistance but not immune tolerance getting infection before hatching.

Infection of those chickens with REV was not caused by contaminated vaccines since the antibody didn't appear immediately after Marek's disease and fowl pox vaccinations. In addition, the low prevalence at the beginning of seroconversion (at the age 20 weeks, seven weeks after the second fowl pox vaccination) revealed some chickens got infection at the beginning because vaccination likely caused almost the whole seroconversion but not only some.

More than 1/3 of fowl poxvirus vaccines contained small part of REV LTR gene and failed to give virus in culture. This means a small LTR fragment remains in the vaccine genes but fail to produce intact virion. REV LTR incorporated in the genomes of fowl poxvirus vaccines without indication of the presence of infectious REV (4). Large part of the REV provirus may be lost from host DNA over time, whereas LTRs remaining (6).

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ACKNOWLEDGMENT

We gratefully express our appreciation to Dr. Henry D. Hunt at the ADOL, ARS-USDA for his help and advice. This study is funded by Council of Agriculture and National Science Council, Taiwan.

FIELD EVALUATION OF *IN OVO* VS SUBCUTANEOUS APPLICATION OF MAREK'S AND INFECTIOUS BURSAL DISEASE VACCINES IN BROILERS

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ABSTRACT

The effect of the route of application (subcutaneous application at day one of age, and *in ovo* at 19 days of incubation) of the vaccine against Marek's and the infectious bursal disease was evaluated in a series of paired comparison trials. Standard commercial production parameters were used to compare the effect of the *in ovo* vaccination vs. the traditional system in which the chickens are vaccinated subcutaneously after they hatch. The tests included approximately 600,000 eggs per treatment for the evaluation of hatchability (20 replicates of approximately 30,000 eggs) and approximately 270,000 chickens per treatment placed in the field for evaluations across 14 replicates. The same breeder flocks were incorporated into the two treatments. The two treated groups (*in ovo* and subcutaneous) received a full dose of Marek's disease + infectious bursal disease vaccines (Merial Select, Gainesville, GA). The vaccine contained herpes virus of turkeys (7,000 plaque forming units (PFU) of HVT) and the ST-14 strain of infectious bursal disease virus. Both groups were vaccinated via spray against infectious bronchitis (H-120) and the Newcastle disease (B1B1) (Merial Select, Gainesville, GA) before delivery to the farms. The chickens were grown in commercial chicken houses for a period of 51 days. The chicken houses were equally divided in two parts (one half with chickens vaccinated *in ovo* and the other half with chickens vaccinated subcutaneously).

Hatchability was not significantly affected by the route of vaccination (*in ovo*, subcutaneous). The data collected in the field included feed consumption, final body weight, seven-day mortality, total mortality (viability), and age of processing. The *in ovo* treatment resulted in better feed conversion (1.938 vs. 1.950), lower total mortality (2.49% vs. 2.70%) and a higher average body weight at the end of growing period (2.795 grams vs. 2.779 grams).

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UPDATE ON EMERGING IBD VIRUSES IN THE UNITED STATES AND HOW COMMERCIAL INACTIVATED IBDV/REO VACCINES PROTECT AGAINST THEM

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INTRODUCTION

Infectious bursal disease virus (IBDV) infections before two weeks of age can cause profound and long-lasting immune suppression in chickens. For the past 15 years, hyperimmunization of broiler breeders using two separate injections of bursal tissue origin (BTO) inactivated vaccines has provided the best levels of progeny protection. However, IBDV is notorious for its ability to antigenically change over time in response to a constant pressure – namely, all conventional BTO vaccines contain Delaware E as their primary immunogen. As a result, there has been an emergence of New-type IBDVs that are genetically distinct from the Delaware viruses (3,4,5,6).

According to the phylogenetic analysis of over forty U.S. field isolates from recent surveys, about half of the samples fell into a separate branch from the Delaware family (6). Some of these New-type IBD viruses, such as T1 and AL-2, have demonstrated the ability to overcome higher levels of conventional BTO derived antibodies, resulting in lower protection rates in 7-14 day broiler challenge models (4,9).

In the progeny studies summarized here, the ability of three variant viruses to infect broilers at two weeks of age was compared.

MATERIALS AND METHODS

Broilers came from prime-age breeders from three distinct geographic locations. Flock A/B and Flock C/D were using a mixed program (two different killed IBD vaccines) while Flock D/D was giving two shots of the same killed product. Each letter represents a different conventional BTO vaccine. All broilers were vaccinated with Marek's and IBD vaccines in the hatchery and were housed after hatch in Horsfal isolator units at 20 per group. At 14 days of age, groups were challenge by oral gavage with 3.5 log10 of one of the following: Delaware E, AL-2 or Ark-6. Ark-6 is a Group 6 virus that was isolated from a farm with a history of various disease problems, poor performance and lymphocytic proventriculitis. For each flock, there was also an unchallenged control group. At 21 days of age birds were sacrificed and weighed and bursas were

weighed and placed in formalin for microscopic evaluation using computer imaging analysis.

Gross bursal protection was calculated based on the number of challenged birds whose bursa to body weight ratio (B:BW) was higher than the cut-off standard (mean B:BW minus two standard deviations) of the respective control group. Microscopic bursal protection was calculated based on the number of birds meeting a set imaging score that had been established by previous challenge studies.

RESULTS

The percent protection scores for each flock are summarized below so that the gross protection score is listed first followed by the microscopic protection score:

Flock A/B→ 58/53 (Del-E), 70/45 (AL-2) and 47/26 (Ark-6).
Flock C/D→ 90/79 (Del-E), 45/21 (AL-2) and 50/45 (Ark-6).
Flock D/D→ 50/40 (Del-E), 61/0 (AL-2) and 52/40 (Ark-6).
3-flock avg.→ 66/57 (E), 59/22 (AL2), 52/40 (Ark-6).

Body weights of all challenge groups were lower than the non-challenged. The average body weight suppression for the three flocks against each challenge virus was as follows:

Flock A/B→ 6.5% (Del-E), 6.7% (AL-2) and 17.8%* (Ark-6).
Flock C/D→ 9%* (Del-E), 14.9%* (AL-2) and 15.4%* (Ark-6).
Flock D/D→ 14%* (Del-E), 1.2% (AL-2) and 12.5%* (Ark-6).
3-flock avg.→ 9.8 (E), 7.6 (AL2) and 15.2 (Ark-6).

*Indicates a statistical difference ($p<0.05$) from the non-challenged controls using Duncan's Multiple Range Test.

DISCUSSION

The use of IBD vaccines mixed with Marek's will tend to enhance the protection levels in commercial broilers. This has recently been demonstrated when given either at day of age (1,7) or *in ovo* (2,8). Thus, flocks in this study with protection rates below 30% should be considered especially susceptible to challenge. Using this criterion, all flocks appeared reasonably protected based on B:BW analysis, although Del-E protection stood out noticeably in Flock C/D (90% vs. 45% and 50%). Based on imaging analysis, however, the differences between challenge viruses became more apparent. In Flock A/B Ark-6 protection was quite low (26%), while in Flocks C/D and D/D AL-2 protection was low (21%) to absent (0%).

It is interesting that there was good agreement between gross and microscopic protection scoring with Del-E challenge, but microscopic protection was lower in all flocks with the AL-2 challenge virus and in one of the three flocks with the Ark-6 virus. Two factors that can contribute to the discrepancy between gross and microscopic scoring are 1) the difference in onset of lesions after infection (damage is seen sooner microscopically) and bursal edema (if an IBD virus causes subtle edema, acutely infected bursas may actually make the B:BW cut-off despite the loss of lymphocytes). Thus, subtle edema could have made some AL-2 challenged bursas look grossly intact when they were in fact significantly depleted.

Based on imaging analysis, broilers from breeders receiving two conventional commercial BTO vaccines were consistently protected against Del-E challenge. Regarding the heterologous challenge viruses (they each have a different amino acid sequence from Del-E in hydrophilic Peak B), protection was much lower against Ark-6 in Flocks A/B and C/D and against AL-2 in Flocks C/D and D/D. Overall, AL-2 resulted in the fewest protected birds (22%), followed by Ark-6 (40%) and finally Del-E (57%).

All three viruses caused some degree of body weight suppression. The most variable weight suppression was seen from AL-2 challenge (1.2% to 14.9%), while the most consistently high weight suppression was seen from Ark-6 challenge (12.5% to 17.8%). A significant degree of body weight suppression was seen in one flock after AL-2 challenge, in two flocks after Del-E challenge and in all three flocks after Ark-6 challenge. It is interesting to note that the Ark-6 isolate came from a farm with reported lymphocytic proventriculitis. In fact, this virus caused a mild proventriculitis in these studies while the other viruses did not.

To summarize, AL-2 and Ark-6 viruses were capable of infecting broilers at a higher rate than Delaware E in progeny challenge studies. This demonstrates the capability of certain New-type viruses to infect flocks at an earlier age and, thus, potentially cause more IBD-related problems.

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CASE REPORT: BLINDNESS IN 7 TO 14 DAY OLD BROILER CHICKS

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ABSTRACT

In a flock of 35,000 broiler chickens, housed in two barns, blindness was noted between the age of seven and 14 days in approximately 0.5% of the flock. Chicks were of the same breed but from three different source breeder flocks. Clinically affected chicks walked aimlessly or stood with head hanging and were unable to find feed and water. The eyes of six chicks examined did not respond to pupillary light reflexes or the menace response. Post mortem of three chicks revealed no significant gross abnormalities. Laboratory diagnostics including molecular diagnostics, bacteriology, virology, parasitology, and serology revealed unremarkable results. Blood glucose level was within normal avian range. Microscopic examination of retina indicated necrosis/apoptosis and loss of photoreceptors (both rods and cones) which explains the bilateral blindness in affected chicks. As the chicks are of the same breed and the incidence level is low, a genetic etiology is suspected as the cause of blindness in this flock. The remainder of the flock completed the growing cycle with above industry average production results.

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

TRANSIT-INDUCED HIGH MORTALITY IN LAYER PULLETS ASSOCIATED WITH DEHYDRATION AND RENAL FAILURE

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ABSTRACT

During the last week of December 2004, 70,000 17-week-old Lohman layer pullets were trucked from Missouri to a lay ranch in southern California. Five trucks were involved, each carrying approximately the same number of pullets and arriving over a period of two days. Transit time was approximately 36 hours for each truck. On arrival, approximately 6,000 were dead: 2,500 on truck 1; 1,880 on truck 2; 1,462 on truck 3; 47 on truck 4; and 17 on truck 5. The live pullets were placed in two similar open-style California cage houses, approximately one-half in each house. Truckloads 1 and 2 and one-half of load 3 went to house A, and the remaining pullets to house B.

After placement, daily mortality was: 465, 391, 74, 6, 28, 11, and 9 in house A; and 74, 65, 167, 17, 26, 8, and 4 in house B. By day 7 after placement, total mortality was 7,251. Necropsies were performed at the ranch on approximately 30 pullets that died on day 2 after placement. Common findings were pale pink kidneys with white specks scattered throughout and visceral gout. Some had small spleens, possibly related to dehydration, and others had gizzard erosions with submucosal edema and red foci in proventricular mucosa.

Both houses had similar water drinkers and the same feed ration supplemented with 200 grams of Aureomycin per ton plus additional vitamins.

An additional 19 pullets that died on day 3 after placement were submitted for laboratory diagnostic evaluation. Common gross abnormalities were marked dehydration (19/19), pale pink kidneys (17/19) with multiple punctate white foci (10/19), small spleens (18/19), and dark brown gizzard contents associated with yellow-tan friable changes in the koilen of the proximal gizzard (12/19). Some had swollen kidneys (3/19) and visceral gout (5/19). Common histopathologic lesions were acute nephrosis (18/19) with widespread necrosis of tubules many of which were distended with urate crystals, and acute heterophilic exudative ventriculitis with degeneration and loss of koilen (14/19). Occasional pullets had coagulation necrosis of the glands and calcification of the mucosa of the proventriculus. Tracheal swab pools were negative for influenza virus by Directigen™ and pharyngeal-cloacal swab pools were negative for influenza virus and exotic Newcastle disease virus by RRT-PCR. Tissue pools were positive for reovirus by virus isolation.

The high prevalence of lesions of severe gouty nephrosis clearly indicates that the mortality in this case was due to renal failure. The strong association of these lesions with marked dehydration suggests that deprivation of water, perhaps enhanced by over-heating and poor ventilation during transit, was the major primary causative factor. The temporal association of the mortality with prolonged transit as well as the rapid decline in mortality after placement supports this suggestion. The significance of the isolation of reovirus from this case is doubtful because no clinical signs or gross lesions of tenosynovitis were seen.

AVIAN TUBERCULOSIS IN CHUKARS

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ABSTRACT

Four chukars were submitted for postmortem examination, with a history of a loss of 21 birds out of a breeder flock of 200 chukars. Other adjoining breeder flocks were not affected. The livers of three chukars were severely enlarged, hard in texture, and some areas were pale. The liver of one chukar was not enlarged, however, had numerous pale white areas. The spleens of all chukars were enlarged and contained numerous pale white foci. The cecal wall contained several raised focal areas on the serosal surface. The mesenteric attachments near the ceca and serosal surface of the rectum of one chukar contained several raised focal areas. The cecal wall was not enlarged in any of the birds.

The results of aerobic cultures performed on one liver and two individual spleens were negative. Results of *Salmonella* culture were negative on composite sample of all livers and intestines.

Composite tissues of liver and spleen were positive for *Mycobacterium avium* isolation. The isolated organism was also identified by PCR to be *M. avium*. The hepatocytes of all four liver sections were replaced by multifocal, small to large, occasionally coalescing aggregates of hypereosinophilic, granular material, surrounded by a rim of macrophages and multinucleated giant cells, bordered by dense fibrous tissue or collagen, or small foci of epitheloid macrophages, lymphocytes, and plasma cells. The hypereosinophilic material often contains large numbers of acid-fast bacilli (presumptive *Mycobacterium* spp.). In the adjacent hepatocellular cord, 75 to 95% of the hepatocytes were widely separated or replaced by large amounts of palely eosinophilic, homogenous to finely granular material (presumptive amyloid) that often obscures sinusoids. Remaining hepatocytes were mildly to moderately swollen and contained numerous, indistinct, intracytoplasmic vacuoles. The sinuses and lymphoid aggregates in the spleen were replaced by multifocal, large aggregates of cellular debris surrounded by macrophages, multinucleated giant cells, and fibrous tissue, similar to those described in the liver. Small to large, multifocal granulomas were present in the intestine, similar to those described in the liver and spleen replaced the lamina propria, as well as the tunica muscularis. Similar, smaller granulomas were adhered to the serosal surface, and widen the coelomic air sacs. Histologic diagnoses were of hepatitis, granulomatous, multifocal, severe with presence of intralesional *Mycobacterium* spp. Other lesions were of amyloidal splenitis, granulomatous, severe, with intralesional *Mycobacterium* spp. The intestine lesions were of enteritis, coelomitis, multifocal, severe, with intralesional *Mycobacterium* spp.

SARCOMAS IN WHITE LEGHORN LAYERS: A RE-EMERGING PROBLEM?

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ABSTRACT

Even though leukosis/sarcoma viruses have not been eradicated from chicken flocks, sarcomas are rarely observed in commercial chickens. In 2004 Ono *et al*. (1) reported on a case of subcutaneous tumors in Japan. In New York State we observed sarcomas in three flocks of a white Leghorn strain. To date, the problem has been observed only at two locations in Western New York State. Both farms belong to the same table-egg producing company. Three flocks have had the same type of tumors. The flocks hatched on March 2003, February, 2004 and May 2004, and were comprised of 24,000, 67,000 and 24,000 chickens, respectively.

The tumors were observed for the first time by the care-taker at around seven weeks of age in the 67,000-

chicken flock and thought to be abscesses. Most of the tumors observed during the growing period were present on the face and head of the chickens, but closer examination at 17 weeks of age revealed solid-mass tumors also on the wings and legs. Lacerations and hematomas were observed associated with some of the tumors. When cut, the tumors were subcutaneous, moderately well circumscribed, unencapsulated, yellowish in color, and varied from gelatinous to firm with abundant mucinous material easily squeezed from the tumors. Several tumors had central areas of mineralization and necrosis. Evidence of bursal and visceral involvement was not observed grossly.

Egg production and mortality rates were within normal limits in the affected flocks, however close to half of the daily mortality had tumors. Many of the birds with tumors appeared healthy otherwise, eating, drinking and laying normally. Mortality appeared to be due to lesions caused by cage on the tumors and elimination of affected chickens by the care-taker.

Histologically, all masses were composed of spindle-shaped cells that ranged from loosely arranged to more compact with small to abundant amounts of extracellular matrix that was typically basophilic and mucinous. Neoplastic cells had small to moderate amounts of cytoplasm and contained bland nuclei that had lightly stippled to slightly vesicular chromatin. Mitotic figures were not seen in the sections examined. Moderate numbers of plasma cells infiltrated the mass either diffusely or formed small aggregates, most often in a perivascular location. Most masses located on an exposed extremity were ulcerated and in the bed of the ulcerated tissue were mats of fibrin, heterophils, and clusters of bacteria. Following a thorough histological examination of visceral organs, lesions typical of Marek's disease or lymphoid leukosis were not observed.

Avian leukosis virus (ALV) was isolated from 12 out of 14 blood samples collected from clinically affected chickens. Seven of the samples consisted of serum and an additional seven consisted of plasma-rich white blood cells. ALV was isolated also from tumor material collected from the same affected chickens. Two new specially designed molecular tests confirmed the presence of replicating ALV in cultured cells infected with tumor material or blood from affected chickens, and molecular tests indicate that a subgroup A avian leukosis virus is involved. Reticuloendotheliosis virus was not detected by PCR. Initial contact with our colleagues in Japan indicates that there is a commonality in the breeder line.

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ACKNOWLEDGEMENTS

We wish to thank particularly thank Kurt Kreher, Ron Pritchett and Vern Stanton of Kreher's Poultry Farm for production records and detecting affected birds for us.

PHYLOGENETIC ANALYSIS OF FOWL ADENOVIRUSES ISOLATED FROM CHICKENS WITH INCLUSION BODY HEPATITIS IN CANADA

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Fowl adenoviruses (FAdV) are ubiquitous in domesticated fowl and have a worldwide distribution. FAdVs have often been isolated from asymptomatic chickens, but several FAdV serotypes/strains have been associated with outbreaks inclusion body hepatitis in broilers (IBH). Typically, the disease affects two- to six-week old broilers. Outbreaks of IBH, causing various degrees of morbidity and mortality, have been occurring yearly in Southern Ontario. In the past two years similar problems have been often reported in western Canadian provinces (Manitoba, Alberta and British Columbia). We have characterized a total of 114 fowl adenoviruses (FAdVs) isolated during outbreaks of IBH in Ontario and western Canadian provinces. The comparison was done by sequencing and phylogenetic analysis of the L1 loop of hexon protein amino acid (aa) sequences (1). Forty-eight field isolates appeared to be highly related to FAdV-8a strains TR-59 (100%) and T-8 (98.9-99.5%), while 41 viruses showed highest percentages of identity to FAdV-11, strain 380 (96.2%). Seventeen Canadian field isolates were 94.7-95.2% identical to FAdV-7, strain x11a.

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

OVERVIEW OF INCLUSION BODY HEPATITIS AND ITS CONTROL IN BREEDER AND BROILER CHICKENS

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INTRODUCTION

The first avian adenovirus (AAV) associated with clinical disease was isolated from an outbreak of respiratory disease in quail in 1950 (7). Since that time AAV have been found to be ubiquitous in chickens and numerous bird species. AAV has been isolated readily from both healthy and sick poultry. AAV have been isolated in cases of inclusion body hepatitis (IBH), hydropericardium syndrome (HPS), egg production drops, reduced growth rate and feed conversion, tenosynovitis and respiratory disease. In North America, IBH has produced the most adverse economic impact in commercial poultry. IBH was first described in the USA in 1963 (5). Subsequently, it was determined that IBH was associated with adenovirus infection by Fadly and Winterfield (3). Initially it was thought that IBH could occur only if the bird's immune system was first weakened by exposure to immunosuppressive agents such as infectious bursal disease (IBD) and chicken anemia virus (CAV). However, recent work has demonstrated that virulent strains can produce the disease alone (2,8).

ADENOVIRUS

Currently AAV are classified into three groups. Group I or conventional adenoviruses cause IBH, hydropericardium syndrome (reported in Asia, Central and South America, and Russia) and quail bronchitis. These are divided into five species and include 12 serotypes. IBH is commonly associated with European serotypes 8 and 4; however, nearly all serotypes have been reported to cause this condition. There has been some confusion regarding the identification of serotypes in Group I as two systems, European and American, have been used. The International Committee on Taxonomy of Viruses has proposed new criteria for classification of adenoviruses that should clarify serologic classification (1).

Group II includes hemorrhagic enteritis virus of turkeys and marble spleen disease of pheasants. Group III viruses are found in ducks and cause egg drop syndrome in chickens.

CLINICAL SIGNS AND LESIONS

IBH typically occurs in meat-type chickens under six weeks of age but can occur as early as six days and as late as 20 weeks. There is a sudden onset of mortality that usually ranges from 2 – 10% but in some cases may be over 40%. Mortality will vary depending on the pathogenicity of the virus, susceptibility of the chicks (level of maternal immunity) and infections with other infectious agents. Mortality generally peaks within three to four days and ceases within one week, although in some cases it may linger for several weeks. Morbidity is low and sick birds that do not die will recover. Affected birds appear depressed with ruffled feathers.

The primary lesion is an enlarged, pale, and friable liver. Small hemorrhages may be present in the liver and muscle. In some cases a straw-colored fluid is present in the sac surrounding the heart.

With HPS, the lesions are similar except the incidence of fluid in the heart sac is greater and the mortality is higher.

TRANSMISSION

Both vertical and horizontal transmission plays a role in IBH. Most outbreaks are initiated by transmission of the virus through the embryonated egg. Hens exposed during production will typically shed virus to their progeny for three to six weeks until development of immunity occurs. Vertically infected chicks that survive will begin to shed virus at three to four weeks of age. Horizontal spread occurs primarily from contact with infected feces. There is evidence that adenovirus infections can become latent and that

periods of stress, such as the onset of egg production, will reactivate viral shedding.

DIAGNOSIS

Diagnosis is made by submitting liver samples from affected birds for histopathological examination and virus isolation. It is critical to isolate the virus and perform serotyping and/or molecular analysis.

The most common serologic test is the immunodiffusion test that detects the group I antigen. The test is not sensitive so may miss positive birds and does not differentiate by serotype. A group I enzyme-linked immunosorbent assay (ELISA) is more sensitive but will not differentiate serotypes either. ELISAs can be developed for individual serotypes but may not detect the immunity to other serotypes. The serum neutralization test has been used to detect serotype-specific antibody, however, this test is labor intensive and expensive because twelve serotypes must be included.

Interpretation of serologic tests is difficult. Adenovirus is found in both healthy and diseased birds. Serologic surveys of commercial breeder flocks demonstrate that 75 – 100% of flocks are antibody positive against several serotypes (4, 6). Also, it has been documented that most flocks under commercial conditions (particularly those on multiple age farms and/or reared on built-up litter) are infected with multiple serotypes during the growing period, and develop antibodies before the onset of production. Therefore, serology is most useful in flocks that are expected to have little or no adenovirus exposure (such as SPF, primary breeder and commercial flocks reared in clean and disinfected, all in/all out farms). Furthermore, serological assays are used to evaluate the immune response of flocks after vaccination against specific serotypes.

CONTROL

As most cases of IBH are the result of vertical transmission, ensuring that breeder flocks have seroconverted prior to the onset of egg production can prevent the disease. Typically this occurs naturally as AAV are ubiquitous and relatively resistant to most disinfectants, heat and low pH. However, on newly constructed farms or those with exceptionally stringent biosecurity, seroconversion may only be achieved by vaccination. Live vaccines and inactivated vaccines produced from liver homogenates or produced in tissue culture have been used successfully for many years. Commercial vaccines are available in Mexico and several other countries. Since such products are not available in the USA, some companies use inactivated "autogenous" vaccines to induce immunity against specific serotypes before flocks reach sexual maturity. Another option is to expose a susceptible flock to contaminated bedding material from a seropositive flock. This practice represents a biosecurity risk as other pathogens may be inadvertently introduced into the flock. Progeny from vaccinated flocks will have maternal immunity that will improve resistance to horizontal exposure.

Frequently two or more serotypes can be isolated from an individual bird indicating that there is little cross-protection. Therefore, in cases of IBH it is important to isolate and serotype the virus or viruses. If multiple serotypes are identified then challenge studies need to be conducted to determine the potential virulence of each strain and to select those strains that may need to be incorporated into an inactivated vaccine. Also research is needed to determine if priming with live vaccine followed by administration of inactivated vaccine will produce better immunity or possibly broader protection against multiple serotypes.

Evaluation of post-vaccinal protection is difficult. Testing vaccinated flocks with serotype specific serology will provide an indirect measure of protection. Conducting progeny challenge studies can make a more accurate assessment of protection. This is accomplished by challenging day-old chicks in the laboratory with known virulent serotypes. Generally, chicks possessing maternal protection do not show any clinical symptoms after challenge.

Finally, as it is well known that IBH will be more severe in birds affected by immunosuppressive conditions, it is important to control infectious bursal disease (IBD), chicken anemia virus (CAV), nutritional problems and management factors that cause stress.

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ISSUES SURROUNDING THE USE OF VACCINES FOR ADENOVIRUSES

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Avian adenoviruses (Aviadenovirus) are a genus of viruses that are causative agents for several disease entities in poultry. The avian adenoviruses are separated into three distinct subgroups: Group I –Fowl Adenoviruses #1-11 and Quail Bronchitis; Group II – Hemorrhagic Enteritis Virus (HEV), Marble Spleen Disease Virus; Group III – Egg Drop Syndrome (EDS)(1). For this presentation, the focus will be on the Group 1 viruses – the viruses causing Inclusion Body Hepatitis and other diseases. Mention will be made to biologics available for the other groups of avian adenoviruses.

Adenoviruses are found in healthy poultry as well as a primary disease-causing agent. Commonly, adenoviruses are found as secondary or co-infections with other viruses or bacteria such as IBDV, CAV, IBV, and MG and MS. Clinical signs from infection with Group 1 adenoviruses range from egg production drops, inclusion body hepatitis, to respiratory diseases (1).

Important characteristics of adenoviruses that are critical to their transmission and control are their ability to be vertically transmitted and their resistance to inactivation (1). In terms of reducing egg transmission, seroconversion prior to onset of lay is critical to avoid progeny infections. This can be accomplished through natural exposure or vaccination. For the group II and III adenoviruses, many commercially available vaccines are found worldwide; however, for the group I adenoviruses, autogenous or local vaccines are the often the only solution. Cross protection does not exist between groups of adenoviruses, i.e. a group II vaccine will not protect against a group I challenge (2). Additionally, within group I viruses, there are 12 different serotypes, who share a common group antigen, but that antigen does not appear to elicit a cross-protective neutralizing antibody to all serotypes within Group I (1).

Since cross-protection may not exist with isolates available in commercial products, breeder flocks in areas experiencing challenges with adenoviruses are vaccinated with autogenous vaccines. Typically these programs involve one or two vaccinations of an inactivated adenovirus, given along with other breeder vaccinations for such agents as IBDV, reovirus, etc.

The production of autogenous biologics is carefully regulated by USDA, Center for Veterinary Biologics and is defined in Title 9, Code of Federal Regulations (9 CFR) 113.113. The microorganisms selected for addition to a vaccine must be isolated from sick or dead birds in the flock. The agent in question must be identified as the primary etiology of the disease in the flock. Microorganisms added to an autogenous vaccine must be inactivated and non-toxic. Autogenous biologics must be ordered under the direction of veterinarian who has a valid veterinarian-client-patient relationship with the flock in question. More than one organism from the same flock can be used in the seed (3).

Upon receipt of the isolates, quality control laboratories check the culture purity and identity. The isolate is then multiplied for production purposes and samples are frozen at –70 degrees F for future use. For adenoviruses, the production of the master seed stock takes six weeks to produce, due to special SPF embryo requirements and propagation methods.

When ordering an autogenous vaccine, there are several options for vaccine preparation. Both vaccines and bacterins can be made into a concentrated emulsion to deliver a 0.25 ml dose or a standard dose size, which is 0.5 ml. The type of emulsion is also an additional option, with most companies offering a

water/oil (WO), a water/oil/water (WOW), or novel emulsions by manufacturer.

Initial serial orders for adenovirus vaccines must allow for an extended production time period, up to four months to final product. The virus is best propagated in chick embryo livers from 15-day-old embryos, a tedious and time-consuming process. This method of virus growth has lower yields than other methods, adding extra time to build antigen quantity for production of a serial. Additionally, only highly trained biologics technicians are trusted to carefully extract the embryo livers, resulting in only two harvests of adenovirus antigen per week due to scheduling of other manufacturing. Release testing for adenovirus products takes additional time as well, taking four weeks to complete the testing. With traditional autogenous vaccines, the initial serial must only undergo sterility testing, and can be release in three days. Following the initial serial, repeat orders tend to take an additional six weeks. These serials are subject to testing under the guidelines for general inactivated products, which mandates testing for purity, safety and identification, 9 CFR 113.200 – Viral Testing.

Autogenous vaccines are intended for use in the flock where the microorganism was isolated; however, permission can be awarded to use the vaccine in adjacent flocks following specific guidelines set forth by CVB. Once permission is granted, the veterinarian must also notify the state veterinarian in writing that the vaccine is to be used in an adjacent flock.

The isolates used for autogenous production can only be maintained for 15 months from date of isolation, which is considered to begin on the date culture is taken from bird or 12 months after production of the first serial, whichever date is earlier (3). Vaccine companies are required by law to destroy organisms that exceed these limits and they cannot return isolates to the supervising veterinarian. A resubmission of same isolate is prohibited. Therefore, the company ordering the vaccine must submit new isolates annually. Notification is sent to the buyer three months prior to expiration, giving them 90 days to submit a new organism.

The submitting veterinarian can apply for an extension of expiration out to 24 months. To the CVB, the veterinarian must submit an assessment of continued involvement, stating that the original organism is still involved in the flock. The statement should be supported by a summary of the diagnostic work to prove existence of the original organism. The veterinarian must also provide proof of satisfactory protection from the autogenous product, which can be satisfied by supplying proof of a commercial product being either unavailable or ineffective. The applicant may be asked for additional information to support the request for extension (3).

In conclusion, commercial vaccines are only readily available in the U.S. for groups II and III adenoviruses. Due to the limited availability and lack of cross-protection among and within groups, autogenous vaccines are often the only choice to offer effective protection to challenge. Autogenous regulations offer additional challenges in producing the product for the end-user in a timely manner to stop a pathogenic challenge with adenovirus. Unfortunately, as with many poultry agents, due to the variety of adenoviruses and the diseases they cause, developing a vaccine for each serotype among the group I viruses would not be an economically feasible option for vaccine manufacturers. Therefore, autogenous biologics remain the answer to most group I adenovirus challenges.

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A COMPARISON OF REOVIRUS PROGENY PROTECTION IN BROILERS FROM SISTER FLOCKS USING DIFFERENT INACTIVATED IBDV/REO PROGRAMS

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INTRODUCTION

Broiler progeny were taken from two 60 week-old sister flocks on two different bursal derived vaccination programs. The goal was to see if the difference in reovirus titers between the two breeder programs would translate into different progeny protection levels against a controlled reovirus challenge. One flock (A/A) received the company's standard vaccination program, which includes 2 shots of Vaccine A. The other flock (B/A) received Vaccine B in the first slot and Vaccine A in the second slot. All broilers received Marek's vaccine plus a 1/6th dose of an attenuated reovirus vaccine. This company has remained on the same killed bursal program for several years. It has also been using live reovirus in the hatchery during the summer months for the past few years to help on issues potentially related to reovirus infection—low weights, leg problems and ruptured tendons.

MATERIALS AND METHODS

All broilers were housed in Horsfal isolator units. 20 chicks from each flock were bled and sacrificed at three days of age for reovirus serology (Idexx ELISA). Also at three days of age, 20 birds per flock were challenged by intratracheal (IT) gavage using malabsorption reo strain 2408. Two groups remained as non challenged controls; while at 10 days of age the remaining birds were challenged by foot pad (FP) inoculation with either saline, 2408 or tenosynovitis isolate S1133. All dosages were titrated at 4.0 $logs_{10}$ (chick ID50). At 14 days of age, the IT challenged birds and non-challenged controls were weighed. At 20 days of age all birds were weighed and sacrificed. FP inoculated birds were lesion scored using the following system: no swelling (Grade 0); mild→ half of FP swollen (Grade 1); moderate→ entire FP swollen (Grade 2); and severe→ swelling extends into the shank +/- the opposite FP (Grade 3). All birds were examined for lesions and bursas were weighed.

RESULTS

Day three reovirus serology revealed that Flock A/A broilers had a 1,216 geometric mean titer (GMT) and 70% coefficient of variation (CV), while Flock B/A broilers had higher (1,981 GMT) and more uniform (41% CV) titers.

After the three day IT challenge, Flock A/A broiler body weights were significantly lower than the negative controls at 14 days of age (29.5%) and 20 days of age (23.1%), while Flock B/A progeny did not differ (<1%) from their respective controls.

After the 10 day FP challenge, Flock A/A progeny had about two to three times lower protection rates against both challenge strains, depending on the whether mild or no swelling was acceptable, respectively (Figure 1).

DISCUSSION

Flock A/A broilers had a 39% lower geometric mean reovirus titer and a 71% higher coefficient of variation at three days of age. This was consistent with the differences seen between the two breeder flocks throughout production. While there is no clear indication of what titer level would make a bird susceptible to reovirus challenge in the field, it appears that most birds in Flock A/A were negatively impacted by the three-day IT challenge with malabsorption strain 2408 as 17 out of the 20 had a 20-day body weight below the lightest bird in the Flock B/A IT-challenged group (data not shown).

A conservative estimate might be that all chicks with titer groups 0 and 1 would be in danger of an early infection. Using this standard, 45% of Flock A/A would be considered suspect. It is interesting, though, that Flock B/A weights were unaffected by IT challenge even though 15% of the birds were in titer group 1 (data not shown). Thus, lateral transmission of 2408 seemed to play a role in Flock A/A but not in Flock B/A. The 23-30% weight depression in Flock A/A is more severe than one would expect to see in the field, and no doubt was due to the large dose of reovirus used in this study. The high, early dose may also explain why there was such severe weight

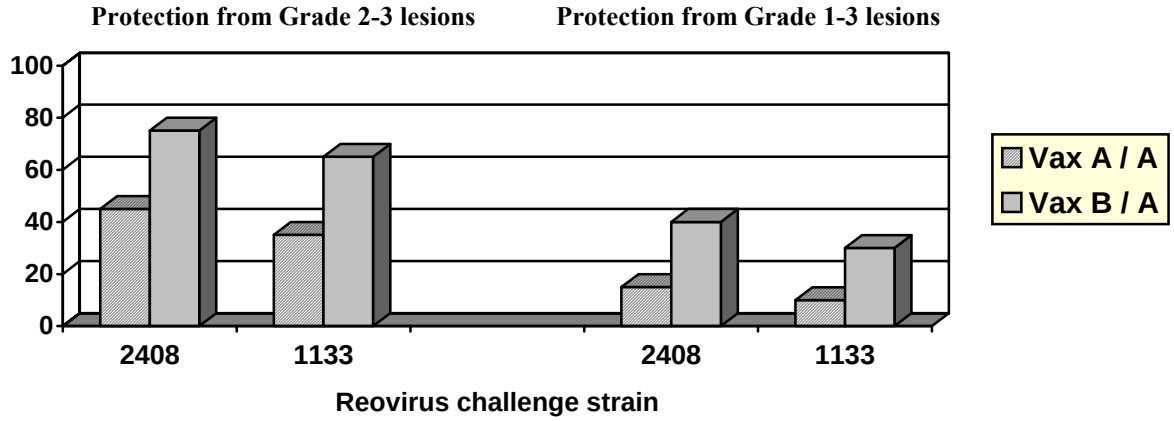
depression in Flock A/A despite live reovirus vaccination. Unfortunately, there were no control groups that did not receive live reovirus vaccine, so the actual effect of live vaccine could not be assessed in the study.

This study also demonstrates that the foot pad challenge method can also assay maternal immune status. There was a significant difference in average lesion scores between the sham inoculated controls and the challenged birds ($p<0.05$). Saline injection did not cause any swelling, while FP challenged Flock B/A averaged mild scores (0.85-1.05) and Flock A/A averaged mild-to-moderate scores (1.50-1.55). Likewise, protection rates from lesions were dramatically different (Figure 1). Allowing for mild swelling, about twice as many Flock B/A birds were considered protected. Using a zero tolerance for FP swelling, that ratio rose to three times as many Flock B/A birds being protected.

Necropsy did not reveal anything remarkable about the birds other than foot pad swelling and, in the IT challenged groups, some weight suppression and bursal atrophy. There was no gross evidence of enteritis or other internal lesions that have been reported in the literature. However, reovirus-induced enteritis and malabsorption is typically transient when not complicated by other enteric agents. Thus, it should have run its course by the evaluation period at 20 days of age. Unfortunately, no observations were made for feed passage or loose droppings in the first few days post IT challenge. Regarding gross bursal atrophy, 21% of Flock A/A IT challenged progeny had significant atrophy compared to only 10% of Flock B/A progeny.

To summarize, in this study lower reovirus maternal antibody levels translated into significantly lower protection levels using two different controlled reovirus challenge routes. How well the two progeny challenge methods employed in this study approximate relative protection levels in the field is not yet clear. However, this study suggests that broiler flocks placed in an environment containing high levels of pathogenic reovirus may be especially susceptible if they received low to marginal levels of reovirus maternal antibodies.

Figure 1. Protection against varying levels of FP swelling.



THE PATHOGENESIS OF TURKEY ORIGIN REOVIRUSES IN SPECIFIC PATHOGEN FREE POULTS

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ABSTRACT

Recently a group of avian reoviruses collected from commercial turkey flocks experiencing poult enteritis complex (PEC) have been described that are genetically distinct from previously characterized avian reoviruses (1, 3). The sequences of the S3 gene segments of numerous chicken origin avian reoviruses (CRV's) have been reported and overall have 84% identity, whereas the S3 gene from these turkey origin reoviruses (TRV's) shares only 64% identity with the S3 gene of CRV's (1, 3). Within the TRV's there is between 94.7 and 100% nucleotide identity (3). Presently, the S1 gene sequence has not been determined for any of the TRV's, but the fact that it can not be amplified with RT-PCR conditions and numerous primers for the CRV S1 gene suggests that the sequence is substantially different. Currently, the incidence and distribution of the TRV type viruses is unknown.

The functional importance of this genetic difference, which represents only 2 of the 11 gene segments is unclear. The S1 gene encodes the σC (or σ3) outer capsid protein, which is believed to be the cell attachment protein and is a major antigenic determinant (2). The function of the σB (or σ2) protein encoded by the S3 gene has not been confirmed in avian reoviruses, but it contains dsRNA binding domains and is believed to be the equivalent of the σ3 gene in mammalian reoviruses which is an outer capsid protein.

In this study the pathogenesis of four TRV isolates has been evaluated in specific pathogen free poults. At three days post-hatch specific pathogen free (SPF) Small White Beltsville poults obtained from Southeast Poultry Research Laboratory flocks were divided into groups and each group was inoculated with approximately $10^{4.0}TCID_{50}$/ bird by the intra-tracheal and intra-oral routes with one of the following four TRV isolates: NC/SEP-R44/03 (4), NC/98 (1), NC/85 and TX 98 (3). A fifth group was inoculated with the 1733 CRV as a pathogenic virus control and a sixth sham inoculated group was inoculated with and equal volume of sterile 50% DMEM/50% F12 media (Mediatech, Herndon VA) by the same route. Each treatment group was housed in a separate Horsfall isolator.

At 2, 5, 7 and 9 days post inoculation (DPI) all poults were weighed and two birds from each group were euthanized and necropsied. Ceca, jejunum and ileum were collected from each bird for virus detection by real-time RT-PCR (4). Bursa, thymus, spleen, liver, ceca, jejunum, ileum, liver and heart were collected and fixed in 10% neutral buffered formalin, paraffin embedded and stained with hemotoxylin and eosin by standard methods for microscopic evaluation. Birds dying during the experiment were necropsied and gross lesions recorded.

Statistically significant ($p < 0.05$) (determined by the one way RM ANOVA using the Student-Newman-Keul test (SigmaStat 3.0, Systat Software, Richmond, CA)) decreases in treatment group mean body weights as compared to the sham inoculates were observed in all reovirus inoculated groups except NC/85 at 5 DPI. Body weights in the NC/SEP-R44/03 and 1733 groups were significantly lower 7 DPI and body-weights in all reovirus inoculated groups were significantly lower at 9 DPI.

Clinical signs in TRV inoculated SPF poults were generally mild or absent, although diarrhea was noted in the NC/98 and TX/98 treatment groups on day 7 PI. No gross lesions were observed.

Poults inoculated with reovirus isolate NC/SEP-R44/03 had severe bursal atrophy at 2, 5 7, and 9 DPI. At 2 DPI there was severe lymphocytolysis in the bursa. At 5, 7, and 9 DPI there was diffuse lymphoid depletion in the bursal follicles. Mild crypt hyperplasia with heterophil infiltration was observed in the duodenum and jejunum at 2 and 5 DPI. Lymphocytic infiltrates were present in the liver at 5, 7, and 9 DPI, in the pancreas at 7 DPI, and in the heart at 9 DPI. No microscopic lesions were observed in the tissues of sham inoculated poults.

Virus was detected by RRT-PCR in intestinal tissues at 2 DPI in TX/98, NC/SEP-R44/03 and NC/98 inoculated groups, 5 dpi in NC/98 inoculated poults and at 7 DPI in 1733 inoculated poults.

Overall, disease presentation was mild, where the greatest clinical effect was on body weights. The long term effects of infection could not be determined with this experimental design. Severe bursal atrophy in some treatment groups suggests that infection with these viruses at an early age may result in

immunosuppression. Interestingly, the disease presentation of 1733 in poults was much less severe than what is observed in chickens.

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HEMAGGLUTININ SEROTYPING OF *AVIBACTERIUM* (*HAEMOPHILUS*) *PARAGALLINARUM* FROM ECUADOR

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ABSTRACT

The reclassified bacterium *Avibacterium* (*Haemophilus*) *paragallinarum* is the etiological agent of infectious coryza, an important respiratory disease affecting chickens of all ages (1). Currently, nine hemagglutinin serovars distributed into three serogroups are recognized as follows: A-1, A-2, A-3, A-4; B-1; and C-1, C-2, C-3, and C-4 (2). These strains are sourced from several countries and some of them are geographically restricted: A-3 in Brazil, C-1 in Japan, C-3 in South Africa and Zimbabwe, and A-4 and C-4 in Australia. Also, within a geographic region, a smaller number of serovars seems to dominate, for example, serovars A-1, C-2, and C-4 in Australia and serovars A-1, A-2, B-1, and C-2 in Mexico (3).

In the present research work, a total of forty-five isolates of *A. paragallinarum* from Ecuador were hemagglutinin serotyped as previously reported (3). Serovars A-3, B-1, and C-1 were identified. Recognition of the serovar A-3 from this country could to confirm a geographic relationship. However, the identification of serovar C-1 is an interesting finding while it has been regarded as restricted to Japan. It is unknown the antigenic and epizootiologic relationships between C-1 serovars from these countries. Differences on the cross-protection and virulence between all the nine recognized serovars of this bacterium have been recently reported by Soriano *et al*. (4). Prevention of infectious coryza by worldwide used vaccines could be improved by the knowledge of the hemagglutinin serovar distribution.

These results highlight the importance of the hemagglutinin serotyping of *A. paragallinarum* isolates from all poultry areas where this bacterium is present.

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THE EVALUATION OF AN INACTIVATED OIL BASED INFECTIOUS CORYZA VACCINE IN BROILER BREEDERS

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ABSTRACT

A commercial, killed oil vaccine produced from combined local Page serogroup A and C3 *Haemophilus paragallinarum* field isolates was evaluated for its ability to protect broiler breeders against infection induced drop in egg production following challenge. The vaccine was administered to 100 Cobb broiler breeder pullets as a single 1 mL intramuscular injection at 17 weeks of age. At 20 weeks of age the pullets were split into two groups of 50 and transferred to the laying pens. The first group of birds was challenged with a NAD-dependent Page serogroup-C field isolate at 32 weeks of age and the second group was challenged with a NAD-independent Page serogroup-C field isolate at 37 weeks of age. In each case the non-challenged group acted as a negative control.

Vaccination provided sufficient protection to suppress clinical signs following challenge with both NAD-dependent and NAD independent Page serogroup-C *Haemophilus paragallinarum* field isolates but egg production dropped by 30% to 57% within three weeks of challenge and took six to seven weeks to return to standard.

Following recovery from the initial challenge, cross-challenge of the two groups with the reciprocal, NAD-dependent/NAD-independent challenge strain went unnoticed with no clinical signs or drop in egg production.

While vaccination did not prevent either strain of Page serogroup C *Haemophilus paragallinarum* field isolates from causing a drop in egg production, the challenge itself induced complete protection against reciprocal challenge.

INTRODUCTION

Infectious Coryza (*Haemophilus paragallinarum*), is endemic to the densely populated poultry production areas of South Africa. Broiler breeder flocks are routinely vaccinated twice during rear with commercial killed oil *Haemophilus paragallinarum* vaccines and yet egg-production drops of 10-40% are common when birds are infected during lay. The emergence of the NAD-independent strain of *Haemophilus paragallinarum* serotype A and C in the late eighties, early nineties appeared to increase the severity of this disease (4).

The aim of this trial was to establish whether an autogenous bivalent *Haemophilus paragallinarum* serotype A and C strain vaccine was capable of protecting broilers breeders from infection induced drop in egg production.

MATERIALS AND METHODS

One hundred Cobb, broiler breeder pullets derived from the same parent flock were vaccinated at 17 weeks via the intramuscular route, with 1 mL of a killed oil infectious coryza vaccine containing Page serotypes A and C3. The birds were transferred to a trial pen facility at 20 weeks and randomly separated into two equal groups of 50. Each group of birds designated group I and group II, were housed in separate rooms and managed according to the Cobb breed guide. The hens came into production at 25 weeks and reached peak production at 32 weeks of age.

Each bird was challenged by intranasal administration of approximately $1x10^6$ viable organisms of field strain *Haemophilus paragallinarum* isolates retrieved from clinically sick broilers and typed using monoclonal antibodies. These isolates were store at -70^{o}C and amplified in embryonated eggs prior to use.

Group I was challenged at 32 weeks with an NAD-independent, Page serotype C3 *Haemophilus paragallinarum* field isolate and Group II was used as a non-challenged control. At 37 weeks Group II birds were challenged with an NAD-dependent Page serotype C3 *Haemophilus paragallinarum* field isolate and the recovered Group I birds were used as the non-challenged controls.

Following recovery from the first part of the trial the two groups were used for a cross-challenge study. At 43 weeks the birds in Group I were challenged with the NAD-dependent, Page serotype C3 *Haemophilus paragallinarum* field isolate and Group II birds were cross-challenged at 44 weeks with the NAD-

independent, Page serotype C3 *Haemophilus paragallinarum* field isolate.

Eggs were collected and production recorded daily.

RESULTS

Two percent of the birds in both group I and II developed signs of mild upper respiratory infection (depression, conjunctivitis, a serous nasal discharge, and a respiratory snick) within four to seven days of challenge and feed finishing times were slightly delayed.

Within a week of challenge the egg production began to drop drastically and by week two, post challenge Group I had dropped by 30% and Group II by 57%. Egg production recovery began four weeks post challenge and returned to standard between the sixth (Group 1) and seventh (Group II) week post challenge. Cross challenge at 43 and 44 weeks went unnoticed.

DISCUSSION

This study is unique in that the vaccine was evaluated on its ability to prevent egg-production drop following challenge. Egg production studies are time consuming, tedious and expensive so vaccines are usually evaluated on their ability to prevent clinical signs of disease (1). Despite protecting the birds from clinical signs of disease the 57% drop in production seen in the Group II birds after challenge is in excess of that previously reported for non-vaccinated flocks (2).

Since commercial broiler breeders were used instead of SPF birds, rearing and vaccination program and disease exposure could have influenced the vaccine response. It is however common practice to administer several vaccines simultaneously in the field so the results should reliably reflect the protective effect of coryza vaccination under field conditions.

The killed oil vaccine administered as a single 1 mL intramuscular injection at 17 weeks of age provided sufficient protection to suppress the clinical signs of challenge with homologous NAD-independent serotype C field isolates but gave little or no protection against egg production drops. Field strain challenge provides protection against both clinical disease and egg production drops. The cross protection induced by exposure to NAD-dependent and NAD-independent strains indicates that these strains are serologically similar.

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CHALLENGE EFFECT OF *GALLIBACTERIUM ANATIS* ON LAYING HENS

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ABSTRACT

There are several reports were *Pasteurella haemolytica*-like organisms have been involve in salpingitis and peritonitis problems in laying hens. We have been studied *P. haemolytica*-like organisms since 1998 and we found a direct relation with their isolation and their effect on egg production of infected flocks in the field. Using a phenotypic and genotypic analysis we confirmed isolated organisms are *Gallibacterium anatis*. Groups of commercial and SPF birds were challenge with 6 different isolates of *Gallibacterium anatis*. Lesions in the reproductive organs, characteristics of the eggs, egg production and percentage of reisolation were recorded. After challenge, bacteria were reisolated from infected birds; almost 100% of affected birds showed lesions in the reproductive organs, and the egg production decreased

until 65%. The challenged birds had the same clinical signs as the birds infected in the field.

INTRODUCTION

There are several antecedents where *Actinobacillus salpingitidis/P. haemolityca*-like complex have been involved in reproductive and respiratory problems in chickens of different regions of the world (1, 2, 3, 4 and 5). Recently Christensen *et al*. (6) proved that the above bacteria belong to a new genus identified as G*allibacterium.* In our study we have monitored laying hen flocks in Mexico with problems in egg production and associated with the presence of *Gallibacterium* (7)*.* The objective of this study was to evaluate the egg production in laying hens after a challenge with different isolates of *Gallibacterium anatis*.

MATERIALS AND METHODS

Source and isolation of the bacteria. Six isolates were used in the study. Three were obtained from the same flock at three ages: 20/0 weeks (OVA isolate), 26/5 weeks (PULTA isolate), and 30/0 weeks (TQR isolate). This flock was affected and showed a decrease in egg production and depression. The other three isolates (07990, Avicor and 4895) were selected from our bacteria collection and were obtained from affected flocks with similar clinical signs. (Table 1)

Bacterial phenotypic identification. The phenotypic analysis of the isolates used in the study was made according to the methodology described by Christensen *et al*. (6); and their identification was confirmed in the Department of Veterinary Pathobiology from The Royal Veterinary and Agricultural University of Denmark by hybridization with the *Gallibacterium*-specific fluorescent probe Gan850, using the procedure described by Bojesen *et al*. (8).

Experimental chickens. Seventy-seven SPF chickens, Babcock strain, (19 weeks of age) and 70 commercial chickens, Hy-line strain, (29 weeks of age) were used. Groups of 11 and 10 birds respectively were housed in separately egg and egg production was recorded before and after the challenge.

Challenge test. The isolates were swabbed onto ovine blood agar plates (BBL) and incubated overnight at 37ºC. The cells were harvested with a loop and a cell suspension was made into sterile tryptose broth (DIFCO). The cell suspension was adjusted to optical density of 2.0 at 540 nm using a spectrophotometer. Every bird was inoculated with 0.2 mL of suspension by intravenous route. The challenge dose for bird was obtained using a plate count standard methodology. Clinical signs, egg production, and mortality in each group were scored daily for 72 hours after infection. At the end of 72 hours the surviving birds were sacrificed and gross lesions in the reproductive organs were registered and scored. Organ samples (liver, ovary, and heart) were also taken for bacteria reisolation.

Bacteria reisolation. Samples of heart, liver, ovary, and egg yolk were taken under sterile conditions and put into 20 mL of sterile tryptose broth incubated overnight at 37ºC. Samples of every culture tube were taken and cultured over an ovine blood agar plate (BBL) and streaked for identification of characteristic *Gallibacterium* ß-hemolyitc colonies. The suspicious colonies were identified as *Gallibacterium* with catalase, oxidase, urea, and indole tests.

RESULTS

According to the phenotypic test, the six isolates used in the study were identified as *Gallibacterium anatis* biovar 1, 3, 4 and 17 (Table 1). After challenge, the birds showed depression and prostration, opaque-greenish diarrhea, and cyanotic and displaced combs. In all the challenged groups the egg production was affected, being more aggressive in the commercial birds groups (Table 1). After 12 hours of challenge some of the eggs had no shell, or it was soft and thin with the six isolates used; 24 hours post-challenge some eggs had deformed shell. Both commercial and SPF birds showed peritonitis, hemorrhages in the gonads, ovarian atrophy and regression, deformed and ruptured follicles, and non-functional oviduct.

In all groups the challenge bacteria were reisolated from heart, liver, and ovary; but their reisolation from egg yolk was negative for SPF and commercial birds as shown in Table 1.

DISCUSSION

Hansen (1) published the first evidence that *P. haemolytica*-like microorganisms had relationship for reproductive organs in chickens. Recently in Mexico, *Gallibacterium anatis* isolates have been involved in field outbreaks associated with considerable economical losses due to a drop in egg production (7). The clinical signs and gross lesions recorded in SPF bird were the same as they observed in field conditions (7). The results showed that *Gallibacterium anatis* infections can produce egg production disorders in laying hens and reproductive organs are affected after a challenge with these bacteria. Further studies to characterize the virulence factors playing a key role for *Gallibacterium* virulence must be conducted.

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ACKNOWLEDGEMENT

The authors wish to tank Dr. Anders Miki Bojesen and The Royal Veterinary and Agricultural University for kindly help us in to confirm the identification of the isolates used in this study.

Table 1. Results three days after challenge.

Bacteria titer CFU/dose (0.2 ml)	Biovar [a]	Birds with lesions in reproductive organs		Egg Production (%)		Re-isolation	
		SPF	Commercial	SPF 40 %[b]	Commercial 85%[b]	SPF	Commercial
07990 $1.1X10^7$	3	9/10 (90%)	9/10 (90%)	18 (-22)	40 (-45)	2/10 (20%)	4/10 (40%)
Avicor $3.2X10^7$	4	8/11 (72%)	10/10 (100%)	15 (-25)	20 (-65)	1/11 (9%)	2/10 (20%)
4895 $1.6X10^8$	4	8/11 (72%)	7/10 (70%)	9 (-31)	27 (-58)	3/11 (27%)	4/10 (40%)
OVA $1.6X10^8$	3	9/11 (82%)	8/10 (80 %)	15 (-25)	33 (-52)	2/11 (18%)	6/10 (60%)
PULTA $1.6X10^8$	17	7/11 (63.6%)	10/10 (100%)	18 (-22)	23 (-62)	4/11 (36%)	4/10(40%)
TQR $1.1X10^7$	1	8/11 (72%)	7/10 (70%)	27 (-13)	30 (-55)	3/11 (27%)	1/10 (10%)
Control group N/A	N/A	0/11	0/10	40	90 (+5)	0/11	0/10

a = According with Christensen *et al*. methodology (2003), b = Egg production before challenge

ERYSIPELAS IN LAYERS: INVESTIGATION ON AGE SUSCEPTIBILITY AND PATHOGENICITY

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SUMMARY

Erysipelas is a bacterial disease caused by *Erysipelothrix rhusiopathiae*. The bacterium was identified more than 100 years ago and recognized as the etiologic agent of swine erysipelas (1). Since then, it has been identified as the causative agent in several different species of mammals, birds, and other animals. In avian species the disease is most common in turkeys (12), although erysipelas has been reported sporadically in several other species of poultry including chickens, ducks, geese, pheasants, and quail. Several significant outbreaks have been reported from chickens (2,6,8,9,10,11,14). *E. rhusiopathiae* is fairly resistant to various environmental and chemical factors (4), and is able to survive for long period of time in the environment (3).

Erysipelas was reported in laying hens in Germany many years ago but was not seen again in layers until 1998, when an outbreak occurred on a farm with 43,000 free range laying hens. The 34-week-old hens developed increased mortality accompanied by a drop in egg production. The birds showed depression, ruffled feathers, somnolence, and diarrhea. Gross lesions consisted of enteritis, salpingitis, and peritonitis with generalized congestion of internal organs, and hemorrhage on pericardial fat, abdominal fat and heart. The livers and spleens were enlarged with multiple large irregular zones of necrosis. Bacteriological examinations of internal organs resulted in isolation of *E. rhusiopathiae* (serotype 1). In addition, *Escherichia coli* and *Pasteurella multocida* were isolated from ovaries and peritoneum. Treatment consisting of penicillin in the drinking water was for 5 days. Overall mortality rate at the end of the production cycle had reached 50 %. Another outbreak occurred on a new restarted flock on the same farm one year later (1999), with a similar incidence of high mortality occurring at 34-weeks-of age. The birds had been purchased from another farm as 17-week-old pullets, and had been vaccinated against *E. rhusiopathiae* at 17-weeks using an inactivated vaccine containing serotypes 1 and 2. Further investigation of the farm revealed that the birds were infested with *Dermanyssus gallinae*. The earlier flock had been infested with mites as well.

Three experiments were carried out with SPF chickens to determine the effects of the age of the bird and the route of infection on the pathogenicity of the field isolate of *E. rhusiopathiae*. Birds were infected at 17 weeks, 27 weeks, and 37 weeks of age by either the intramuscular (IM) or oral route of infection, with 2 X 10^{10} CFU/ml *E. rhusiopathiae* field isolate, and observed for 14 days. The results of the experiment showed that the highest mortality rates occurred in the older birds, with 100% mortality observed in the 37-week-old birds inoculated by the IM route, 60% mortality was reported in the younger 27-week-old birds, while no mortality occurred in the 17-week-old age group. In the orally infected 27-week-old birds, 40% mortality was detected, while no mortality was observed the older birds using the same route. The results of the experiment support the contention that mortality due to *E. rhusiopathiae* is age related in layer chickens. On the other hand, Shibatani, *et al.*, (13), were able to demonstrate a 43% mortality in 3-week-old SPF chickens using the IM route of inoculation, indicating that young birds are susceptible to infection as well. The erysipelas outbreaks that have been reported from chickens under field conditions have been observed during the laying cycle (2,7,11). A possible explanation for why the disease is seen in mature laying chickens rather than in young pullets may be found in a report by Chirio, *et al.*, (5). According to the authors, *D. gallinae* may serve as a vector for *E. rhusiopathiae*, and may act as a reservoir host, maintaining the infection between replacement flocks. The red mite infestation is more likely in the layer hens than the pullets.

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MYCOPLASMA SYNOVIAE IN TURKEY PARENT FLOCK AND THEIR OFFSPRING AFTER NATURAL INFECTION

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Mycoplasma synoviae (*M. synoviae*) was first recognized as an acute to chronic infection of chickens and turkeys that produced an exudative tenosynovitis and bursitis; it now occurs most frequently as a subclinical infection of the upper respiratory tract. *M. synoviae* is an egg-transmitted disease, but the rate is low (probably <5%), and some hatches of progeny of infected flocks may be free from *M. synoviae*. Lateral transmission is similar to that of *Mycoplasma gallisepticum*, but the rate of spread is generally more rapid. *M. synoviae* isolates vary widely in pathogenicity.

In a turkey breeder farm during a routine control *M. synoviae* DNA could be detected in the tracheal swabs collected from the hens at the fifth week of production. Further investigations of hatching eggs revealed positive results. The breeder flock was immediately slaughtered.

For further investigations 13 hens were taken and reared separately in experimental facilities for eight weeks. At several intervals tracheal swab and blood samples were collected for bacteriological, PCR, and serological investigation. Then after the birds were humanely killed, the internal organs (trachea, air sacs, ovary, oviduct, and joints) were examined for *M. synoviae*. In addition, hatching eggs were taken from infected flock and hatched under experimental facilities. The hatched poults were reared eight weeks and examined at several intervals as mentioned above.

Isolation of *M. synoviae* was done according to the method described by Kleven, (3) in mycoplasma broth and agar –bouillon and mycoplasma–agar with supplementation of substitutes made by company Oxoid. The PCR was carried out using the method described by Marois *et al*., (1). For antibody detection, commercially available ELISA test kit was used (IDEXX, USA). The ELISA procedure and the interpretation of the results were performed according to the instructions of the manufacturers.

The results of the investigation of tracheal swabs and the serum samples from breeder hens over eight weeks are shown in Table 1. *M. synoviae* could not be

isolated from all investigated samples during the entire investigation period. However, *M. synoviae* DNA could be detected in all examined pooled tracheal swabs. In addition, antibodies at different level were found. At necropsy, *M. synoviae* DNA could be detected from tracheal swabs and ovary. Examination of air sacs, oviduct, and joints revealed negative results (Table 1).

Out of 112 hatching eggs used, 25 eggs were infertile and/or dead in shell eggs. The hatched poults were reared under experimental facilities and investigated over eight weeks. Out of the 87 hatched poults, 25 died at the first day of age. The obtained results on mycoplasma investigations are shown in Table 2. *M. synoviae* could not be isolated from all investigated samples of yolk sac and tracheal swabs during entire investigation period at 1, 4, 6, and 8th weeks of age. However, *M. synoviae* DNA could be detected out of examined yolk sac pool sample (pool out of 5-10 swabs) collected at the first week of age. Detection of *M. synoviae* DNA out of tracheal swabs was negative. In addition, maternal antibodies could be detected at the first week of age. At necropsy *M. synoviae* DNA could be detected from tracheal swabs. Examination of air sacs and joints revealed negative results.

In conclusion, we can say the *M. synoviae* infection was diagnosed by PCR test and ELISA test in a turkey breeder flock without clinical signs; however, the isolation of the organism was negative. The PCR procedures are comparable in sensitivity to isolation (2). The DNA of *M. synoviae* could be detected in the trachea and ovary from the hens. The vertical transmission is demonstrated, since *M. synoviae* DNA could be detected in the yolk sac of hatched chicks, and also maternal antibodies were detected in the sera of poults.

M. synoviae is an egg transmitted disease, and the only effective method of control is the production of turkeys form MS-free parent flocks (4). Besides eradication policy, effective biosecurity measures to prevent introduction of the infection, the routinely control of parent stocks in short intervals for *M. synoviae* infection is essential to guarantee a very early diagnose of *M. synoviae* infection. PCR is an effective method for early recognition. Since no antimicrobials regardless of dosage or length of treatment can eliminate the infection, medication of breeder flocks is of little value in preventing egg transmission.

Table 1. Results of the investigation of tracheal swabs and serum samples of the 13 breeder hens.

Weeks after stocking	Bacteriological examination	DNA –Detection PCR*	Antibody detection ELISA
0	0/13**	2/2	1/13
1	0/13	2/2	1/13
2	0/13	2/2	5/13
3	0/13	2/2	6/13
4	0/13	2/2	6/13
6	0/13	2/2	4/13
8	0/13	2/2	6/13

*Pool of 6 to 7 tracheal swabs **- No. of positive / No. of tested samples

Table 2. Results of the investigation of yolk sacs, tracheal swabs and serum samples of hatched poults.

Age in weeks	Bacteriological examination		DNA –Detection -PCR*		Antibody detection ELISA
	yolk sac	trachea	yolk sac	trachea	
1	0/20**	0/10	2/2	0/3	3/5
4	n.d.	0/10	n.d.	0/4	0/35
6	n.d.	0/10	n.d.	0/3	0/30
8	n.d.	0/10	n.d.	0/3	0/32

n.d.: not done * pool out of 5-10 swabs **- No. of positive / No. of tested samples

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CAMPYLOBACTER JEJUNI INOCULATION IN IMMUNOSUPPRESSED AND NORMAL BROILER CHICKS

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ABSTRACT

Two hundred one-day-old broiler chicks were obtained from a local hatchery. The chicks were subdivided into 10 groups of 20 chicks and housed in Horsfall units. Commercial broiler feed and water was provided ad libitum. Five groups of chicks were inoculated with IBDV variant E strain at one day of age. Three isolates of *Campylobacter jejuni* of poultry origin and one of human origin were propagated.

Four groups of chicks were inoculated individually with one of the *Campylobacter* spp. containing 0.5 mL of 1×10^2 CFU of *Campylobacter* by crop gavage at nine days of age.

Four groups of chicks that were inoculated with IBDV variant E strain at one day of age were inoculated similarly with one of the *Campylobacter* spp. One group was kept as an uninoculated control and another one group was kept as an IBDV variant E strain control. At 14, 21, and 28 days of age four chicks were collected at random from each treatment group. These chicks were euthanized, necropsied, and the intestinal tissues were cultured for *Campylobacter* enumeration and histopathology. All chicks were weighed at 7, 14, 21, 28 days of age and statistical analyses performed. The study was terminated at day 28.

Reduced body weights were observed at different weighing intervals in the IBDV variant E strain and *Campylobacter* inoculated groups but not in the only *Campylobacter* inoculated groups. Results of *Campylobacter* enumeration from the ceca were two to four logs higher as compared to the upper and mid intestine samples. *Campylobacter* was not isolated from the intestines of day old broilers or the uninoculated controls at different intervals.

CONSUMER EXPOSURE TO *CAMPYLOBACTER* SPP. FROM PROCESSED POULTRY

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ABSTRACT

Ten of the largest United States poultry integrators cooperatively determined the frequency and levels of *Campylobacter* spp. contamination. We wished to determine the consumer exposure to *Campylobacter* spp. associated with U.S. broiler carcasses over a 13 month period. Among each poultry complex, rinses from 25 randomly selected, fully-processed carcasses were taken monthly from individual flocks. Thirteen processing plants around the country cooperated in this assessment. Direct plating methods from the rinses were conducted to provide an estimate of contamination. Among 4,200 samples, approximately 74% of the carcasses had no detectable levels of *Campylobacter* spp. Approximately 3.6% of the commercially processed broilers yielded more than 10^5 cells of *Campylobacter* spp. per carcass.

INTRODUCTION

Campylobacter spp. is reported as one of the most frequent causes of bacterial disease in the United States. CDC estimates that approximately 1.6 million cases occur each year. CDC further suggests that poultry borne transmission accounts for close to 50% of the human cases. Consequently, the poultry industry wishes to control the frequency and levels of the organism associated with the processed product.

In response to this health concern, the industry wished to determine the levels presently occurring on poultry carcasses across the country. With such information, we hope to formulate a strategy designed to reduce and control the transmission of the organism from the raw product.

MATERIALS AND METHODS

Survey study. Thirteen processing plants distributed in poultry producing centers across the United States were enrolled in the study. These plants represented 10 of the nation's largest integrators. Once a month, for 13 consecutive months, one flock per processing plant was selected for sampling. Methods previously described (Stern and Robach, 2003) were used. Briefly, 25 broiler carcasses were randomly selected and aseptically taken from the chiller tanks. Carcasses were individually washed in 400 mL of buffer and the rinse samples were sent on ice overnight to the microbiology laboratory conducting the analysis. Microbiological methods entailed directly plating of dilutions from the rinse materials onto Campy-Cefex medium, and incubating for 36 to 48 hours at 42°C under reduced atmospheres. Colony forming units with typical translucent appearance were placed under phase-contrast microscopy and observed for corkscrew-like appearance and movement. These suspect colonies were subjected to a highly specific latex agglutination assay to confirm the presence of the organism. Colony forming units per carcass were determined and recorded.

Campy Check samples. Laboratory personnel were trained for the protocol involved in enumerating *Campylobacter* spp. from broiler rinses. Prior to the survey study, to determine the reproducibility of the method after one month of practice, encoded sample rinses were sent on ice overnight to the participating laboratories. The coded rinses were sampled to determine the levels of the organism contained in the inoculated samples as indicated above. The data were reported to a central laboratory, the code was broken and, the variation was determined. The entire procedure was repeated after the survey study had been completed to determine whether greater consistency among the laboratories had been gained.

RESULTS AND DISCUSSION

Laboratory variation in the levels of *Campylobacter* spp. was observed among the inoculated samples (Figure 1). The participating laboratories were able to distinguish between the various levels in the check-samples.

Overall, ~74% of the processed carcasses did not yield *Campylobacter* by the method used (Figure 2). The central question is what accounted for that variation. Certain complexes never reported *Campylobacter* contaminated carcasses. Within the means reported for each flock, individual carcasses ranged as much as $\pm$ 2 logs. What explains such variation within a flock? Nationally, 3.6% of the carcasses did yield $\geq 10^5$ cfu *Campylobacter*.

The United States poultry industry has reduced levels of *Campylobacter* spp. on processed broilers by more than 10-fold over the past 10 years. Our study is providing data for a science based regulatory position. When compared with other European countries by using similar quantitative methods, the United States has lower levels of the organism per carcass. We still need to learn what level of reduction is required to assure the public health. Further reductions in the sporadically high levels found on individual carcasses may be of substantial importance.

Figure 1. *Campylobacter* "check" samples-September 2004.

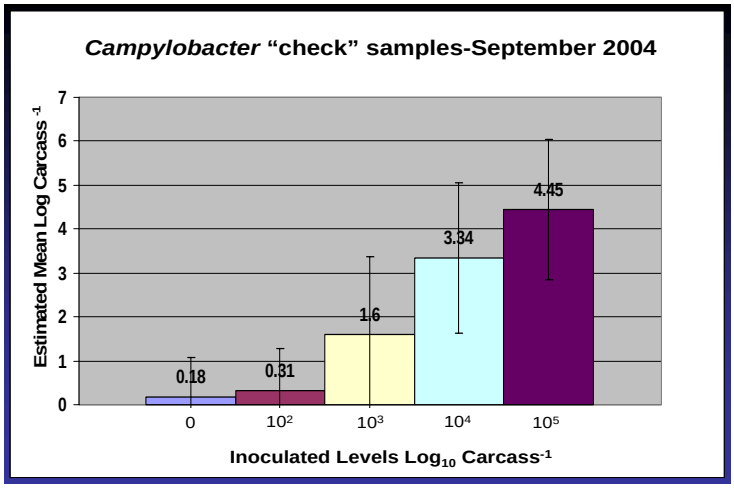


Figure 2. Overall estimates of *Campylobacter* levels for commercial carcasses processed from September 2003 to September 2004 (n = 4,200).

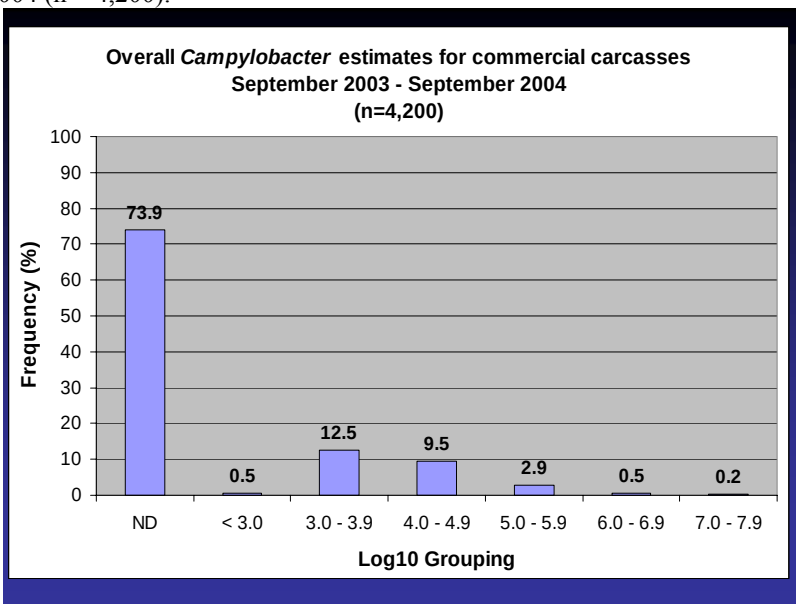
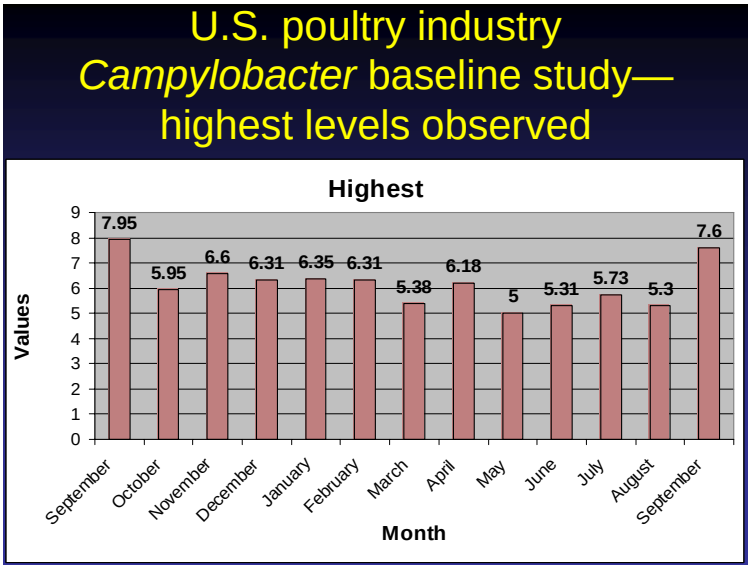


Figure 3. Highest levels of *Campylobacter* spp. observed during each month of the carcass rinse sampling.



THE ROLE OF GLYCOSYLATION IN THE COLONIZATION OF POULTRY BY *C. JEJUNI*

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Campylobacter jejuni is a significant food- and waterborne pathogen both in North America and abroad and a considerable cause of child morbidity in underdeveloped countries. The primary source of *Campylobacter* infection in humans is through the consumption of contaminated poultry products although two recent waterborne outbreaks in Canada and the USA demonstrate that water is also an important source for infection.

We have shown that *C. jejuni* has an *N*-linked protein glycosylation pathway that results in the modification of up to 30 proteins. The Pgl enzymes (for protein glycosylation) required for the biosynthesis of the heptasaccharide are highly conserved. To determine if the complete oligosaccharide must be synthesized before the glycan can be transferred to protein, we have analyzed *pgl* mutants for the presence of the glycan using high resolution magic angle spinning (HR-MAS) NMR. To confirm NMR findings, the glycoprotein Peb3 was examined by mass spectrometry from select mutants. These data indicate that the glycan is transferred as a block with relaxed specificity for the branching glucose.

In *C. jejuni*, disruption of the general *N*-linked glycosylation pathway has been shown to affect host cell interactions, probably through the inactivation of multiple proteins. In order to investigate the therapeutic potential of this pathway, we examined the effect of *pgl* mutations on *C. jejuni* colonization in the chicken commensal host. As expected, *pglB* and *pglH* mutants showed complete loss of colonization while the *pglG* mutant showed similar levels of colonization compared to wildtype.

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

COMPARISION OF LEVELS OF *SALMONELLA ENTERICA* SEROVAR ENTERITIDIS CONTAMINATION OF FLIES OBTAINED FROM ROOMS CONTAINING MOLTED OR NON-MOLTED INFECTED HENS

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ABSTRACT

The commercial table egg business is a major multi-billion dollar industry in the U.S. In order to maximize the effective laying capability of their laying hens, producers must implement a number of management tools. Vaccination, biosecurity, medicated feed, increased stocking densities, to name a few, are used to improve the health of the birds and optimize the useable space within a house. However, these tools can have little impact on an important determinant of productivity, age. As a laying flock ages, its ability to produce eggs diminishes such that it reaches a point where it is no longer economically feasible to keep the flock in lay. Producers have the option of sending their birds to slaughter and bringing on a new flock. This choice suffers from problems such as the cost to raise the birds while receiving no income until they come into lay and the serious situation of disposal of the previous flocks. Because of the growth of the broiler industry and its expansion into commercial areas previously reserved for spent hens, the market for retired layers is minimal. Whereas in earlier times producers might recoup a small sum of money for their old layers from soup manufacturers etc, the current situation dictates that producers must absorb the cost of hen elimination and, on many occasions, are forced to transport birds long distances for the disposal. As a consequence, methods are

continually sought to extend the effective laying life of a flock.

Induced molting remains an important management tool for the layer industry as a means to maximize the effective laying life of a flock. Molting programs involve an estimated 75-80% of the commercial flocks in the U.S., and considering that there is an estimated 256 million hens in the U.S., the number of hens molted annually would be 192-204 million. Approximately one-third of the profits of a flock is estimated to come from molted birds.

While there are many procedures to molt hens, the primary method for molt induction is to remove feed until hens drop a certain percentage of body weight. However, previous experimental studies in ourlaboratory showed that this procedure significantly depressed the cell-mediated immune response in the hens and flow cytometric analysis of peripheral blood from molted and non-molted hens demonstrated that the CD4+ T cells, the helper T cell subset, were significantly decreased in molted hens. An intact immune response is crucial for the health of the bird by providing protection against the wide array of microbial pathogens that attack laying flocks. By compromising that immune protection during situations such as induced molt, the flocks may be vulnerable to infection by a number of these organisms.

One organism in particular, *Salmonella enterica* serovar Enteritidis (SE), was studied extensively with regards to infection during molt. Molted hens shed more SE from their feces, had higher levels of SE in internal organs and remained infected for longer periods. Part of the problem with the higher levels of shedding of SE is the accumulation of high numbers of the organism in the house environment which contaminate house surfaces and infect other poultry as well as non-poultry inhabitants. Houseflies are common residents of poultry houses and are considered to be potential carriers of a variety of disease agents.

The current studies were conducted to discover whether flies become contaminated with SE and, if so, whether the level of SE in the environment will be reflected in the level of SE found on the flies. Two rooms containing equal numbers of hens were either molted via feed withdrawal or not molted (remained fully fed). Flies were released into both rooms and the hens were challenged with 9.2×10^6 SE on day four of molt. Levels of SE were determined on the floors and on the flies over time.

By day two post challenge, significantly more flies were SE+ (85%) in the room containing the molted hens than the non-molted (50%) and significantly more SE could be recovered from these flies as well.By day four post challenge, no significant differences in the percentage of SE+ flies were observed in the two rooms (70% vs. 90% in the non-molted vs. molted hens, respectively) but significantly more SE could be recovered from the flies in the room containing the molted hens. On days seven and 15 post challenge, significantly more flies from the room containing molted hens were SE+ (85% and 90%) compared with flies in the non molted hen room (55% and 30%) and had more SE on their bodies. Interestingly, significantly higher SE numbers were recovered from the floors of the room containing molted hens on only day seven while on the other three test days (two, four, and 15 post challenge), no significant differences in the levels of SE on the floors between rooms could be detected. However, it was noted that the fecal material on the floor of the room containing molted hens was much more moist than in the other room which could increase the numbers of SE that would adhere to the flies.

These results indicate that flies residing in a room containing hens molted via feed withdrawal and infected with SE could become contaminated with substantially more SE than flies from a room containing normally fed hens. As feed withdrawal hens were found to be more susceptible to SE infection – approximately 5×10^4 SE were necessary to infect 50% of fed hens vs. less than 10 SE in hens undergoing molt via feed withdrawal – these flies could serve as an important means of spreading SE through a house. It has been shown that SE readily transmits horizontally to other molted hens either through bird-to-bird contact or via the airborne route. Flies now may also be another mechanism of transmission and this is currently under investigation.

USE OF REAL-TIME PCR TO DETECT THE PRESENCE OF *SALMONELLA* IN ENVIRONMENTAL CULTURES

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ABSTRACT

A real-time PCR assay was used to determine the presence of *Salmonella* in environmental cultures of poultry houses. The assay used the LightCycler® (Roche Applied Science, Indianapolis, IN) Hybridization Probe technology to detect a conserved sequence of the *Salmonella* genus. Cultures, either pre-enrichment or enrichment, were randomly selected for testing. The sample DNA was isolated using 1 mL of the culture broth and the High Pure PCR Template Preparation kit (Roche Applied Science). This kit used a spin column to capture the DNA from the lysed samples. The DNA was subjected to a series of washes to remove PCR inhibitory substances. An elution buffer recovered the DNA for use in the real-time PCR. The real-time PCR consisted of a master mix containing the primers, hybridization probes and buffer, an internal control and an enzyme solution. All of these components were mixed to produce the *Salmonella* PCR reaction mix. The final reaction consisted of 15 µL of reaction mix and 5 µL of sample DNA.

A total of 572 samples were processed and tested for the presence of *Salmonella* DNA. Within this group, 55 samples were both PCR and culture positive. A total of 452 samples were negative when tested with both PCR and culture. Sixty-one samples were PCR positive and culture negative. A subset of these samples were tested with an alternative set of primers and probes specific for the *Salmonella* invA gene sequence (Malorny, *et al*. Applied Environ Micro 2003, 69(1):290-296). The samples tested positive for the presence of this target sequence.

The real-time assay for *Salmonella* was able to consistently detect *Salmonella* DNA in cultures, which were positive for the presence of *Salmonella*. The assay also was able to consistently detect *Salmonella* culture negative samples. The ability to rapidly determine the positive cultures could provide laboratories with a method to quickly screen initial cultures for *Salmonella*. Laboratories could then focus their efforts on determining the identity of the *Salmonella* in the positive cultures.

LABORATORY COSTS OF DETECTING *SALMONELLA ENTERITIDIS* FROM ENVIRONMENTAL DRAG SWABS IN THE CALIFORNIA EGG QUALITY ASSURANCE PROGRAM

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ABSTRACT

Salmonella enteritidis (SE) has been identified as a food safety issue since the mid 1980s (8). The incidence of SE isolations from humans peaked in 1995 and has decreased 46% from 1996 to 1999 in "FoodNet sites" (5). Much of this decrease has been attributed to egg quality assurance programs (5). The California Egg Quality Assurance Program (CEQAP) was established through cooperative input from industry, government and academia. Training and compliance to six core components form the foundation of the program. One component of the program is "environmental monitoring and sampling" which provides a determination of the SE status of a premise. Manure drag swab samples are primarily collected at the end of the laying hen production cycle. Test results allow the producer to know if SE has contaminated the layer environment. Positive results dictate re-evaluation or modification in various components of the program. Cleaning and disinfection procedures, vector control and overall biosecurity plan are all examined prior to incoming pullets.

The cost of monitoring the layer environment is significant and must be taken into consideration by egg quality programs. Sampling types, methods, and cost vary from region to region. Many EQAP, including California's, utilize manure drag swabs to monitor SE in the layer environment (1,3,4,7). In California, pooling drag swabs within a premise has been shown to have no statistical difference over testing individual samples for the presence of SE (2) but does provide for significant cost savings. Samples are collected by trained producer representative or private or state veterinarians. Samples submitted to the California Animal Health and Food Safety (CAHFS) laboratory system usually consists of four to six individual bags each containing a pool of four manure drag swabs. The four manure drag swabs are obtained from randomly selected manure rows or, depending on the facilities, equivalent environmental samples. Typical time required to collect 16 manure rows and pool the swabs into four samples takes between 30 to 60 minutes depending on the layer house equipment (personal communication, Dr. M Bland).

Laboratory costs for SE testing are complex to compute as the outcome of the cost is dependent on the method of *Salmonella* isolation and identification and the prevalence of SE in the layer environment. Culturing procedures for *Salmonella* across the United States are highly variable. A national survey conducted by Waltman and Mallinson found variation in media utilization incubation times and temperature and the number of suspect colonies tested (9). The CAHFS laboratory system's procedure for the detection of SE from environmental samples closely parallels the procedure recommended by the National Poultry Improvement Plan (6). Briefly, the samples are pre-enriched, selectively plated, suspect colonies screened biochemically, serogrouped and serotyped if found to be Group D *Salmonella*. If direct testing failed to detect any Group D *Salmonella*, a delayed enrichment is performed. The number of suspect colonies screened (picks) in either the initial enrichment plating or after DSE plating is three to five colonies. Material costs (Table 1) and labor costs are dependent on testing results.

Utilizing CAHFS 2004 environmental drag swab data, three results are considered. "No *Salmonella* sp. isolated" involves sample setup, primary and DSE plating, suspect colonies screened, and compatible isolates serotyped with poly-O antisera. "*Salmonella* sp. isolated but not Group D" involves additional serotyping with Group D antisera and genus identification. "Group D *Salmonella* sp. isolated" involves the additional costs of confirming the isolate is SE. No *Salmonella* sp. isolated after testing three suspect colonies (poly O antisera negative) on both initial and DSE plating occurred on 47% of 1099 samples. This result utilized materials costing $18.30 and took 32 minutes to complete. The median salary range of a Laboratory Assistant III and a Staff Research Associate II including benefits at UC Davis is $22.19 per hour ($0.37/min). This results in $30.14 per sample if no *Salmonella* sp. is isolated. The *Salmonella* sp. isolated but not Group D result occurred on 51% of the 2004 CEQAP samples, had a material cost of $23.82, had a labor cost $17.76 for a total cost of $41.58. The additional costs of this particular result were due to testing with antisera for Group D and the necessary biochemical confirmation of a *Salmonella* sp. The Group D *Salmonella* sp. isolated result occurred on 2% of the 1099 samples. Test cost and time are similar to the previous result but require serotyping costs ($13.65) and shipping cost as an infectious substance ($25.00) resulting in a total cost of $80.62. An independent cost analysis for SE testing of environmental drag swabs performed at the San Bernardino Branch of CAHFS (Kinde) was similar, although the labor costs were higher. Total laboratory cost for testing CEQAP samples during 2004 amounted to $40,556. The Turlock Branch of CAHFS has been involved in parallel testing of a SE specific PCR test that costs $9.66 per sample including technician time. Additional benefits of the SE PCR test include reducing turn-around-time from 7-14 working days to three working days. This promising test could substantially reduce testing cost for laboratories and the reduced turn-around-time would be of benefit to producers.

Although egg quality assurance programs have been extremely successful in decreasing the incidence of SE associated with egg consumption, there are costs to these programs of which laboratory costs are only a portion. Laboratories involved in egg quality programs and SE testing need to consider both material costs and technical personnel costs. Both costs are related to a number of factors; sample number, sample type, media utilized, and exact procedure followed. Overall function of laboratory operations and job functions are also impacted and must also be considered.

ACKNOWLEDGEMENTS

Sally Channing-Santiago, Cathryn R Bauer and Jennifer Kato of the CAHFS – Turlock Branch bacteriology section.

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Table 1. Material costs for sample setup, selective plating, suspect colony screen and identification.

Sample setup & plating		DSE plating		"Pick" Workup	
Whirl Pack Bag	$0.15	Whirl pack bag	$0.15	TSI slant	$0.45
Tetrathionate broth (225mL)	$3.88	Gloves (pair)	$0.16	LIA slant	$0.51
Iodine for tetrathionate(20mL)	$0.24	16x125 test tube	$0.27	Poly O antisera	$0.49
1% Brilliant green(10mL)	$0.34	Test tube lid	$0.06	D_1 antisera	$0.53
Swabs	$0.04	Tetrathionate broth	$0.16		
XLT-4 agar plate	$1.02	Iodine for tetrathionate	$0.01		
Brilliant green w/nov.	$1.02	1% Brilliant green	$0.01	*Salmonella* ID	
Gloves (pair)	$0.16	Swabs	$0.04	Urea	$0.54
		XLT-4 agar plate	$1.02	S Citrate	$0.39
		Brilliant green w/nov.	$1.02	SIMS	$0.39
				ONPG	$0.53
				ODC	$0.49
				BHI	$0.39

RISK FACTORS ASSOCIATED WITH HEPATITIS SPLENOMEGALY SYNDROME IN LAYING HENS IN ONTARIO, CANADA

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ABSTRACT

High mortality in a commercial layer flock in Ontario, Canada prompted an investigation that included weekly post mortem examinations of birds dying during the eight week mortality period, followed by a retrospective epidemiological investigation of mortality patterns of 22 flocks from the same premise over the previous six years. Forty-four percent of the 285 birds necropsied had lesions compatible with hepatitis/splenomegaly syndrome (HSS), 11% had lesions of fatty liver hemorrhagic syndrome (FLHS), and 23% had lesions consistent with a combination of HSS and FLHS. The remaining 22% of the birds that died during that period had lesions that did not relate to liver disease. Hepatitis E virus RNA was identified by RT-PCR in bile from affected birds. No other pathogen was identified in birds with HSS or those with a combination of HSS and FLHS. The flock was fed an 11% flax diet for production of omega-3 enriched eggs. The retrospective study demonstrated that over the past six years all 11 flocks fed flax diets showed a mortality pattern similar to that of the outbreak flock and this pattern was not observed in 11 flocks fed regular flax-free layer rations. Flax diets and Hepatitis E virus may be risk factors in the initiation of HSS in laying hens.

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

VITAMIN B2 (RIBOFLAVIN) DEFICIENCY IN CHICKENS

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ABSTRACT

Riboflavin (Vitamin B2) is a water soluble vitamin and is a cofactor for various enzymes in many systems of the body. Some of the cofactors are important in the oxidation-reduction reactions involved in cell respiration. Riboflavin deficiency is most commonly reported in chickens but it has also been reported in turkeys and pigeons. Deficiency of riboflavin in chickens can cause peripheral neuropathy resulting in a condition called "curled-toe paralysis". Other signs due to riboflavin deficiency include weakness, slow growth, loss of weight, leg paralysis without curled-toes and walking on hocks with the aid of their wings. Riboflavin deficiency in the hen can result in decreased egg production, increased embryonic mortality and poor hatchability.

A search of CAHFS - Fresno data base for riboflavin deficiency in chickens between 1990 and 2004 revealed seven cases. Five were in broiler chickens, one was in layer pullets and one could not be identified. The age of the affected birds ranged from nine days old to 27 days old. Clinical signs in the chickens included paralysis, walking on hocks and occasionally walking on their wings. The incidence of clinical signs in various flocks ranged from 20 % in one flock to 40 % in another. Other signs included splayed legs, abnormal droppings, uneven size of birds and increased mortality in one flock. Gross lesions included curled-toes in four out of seven submissions. The sciatic nerves were pale yellow, edematous and mild to moderately enlarged with a loss of cross striations in two submissions. Long bones were soft in birds from two submissions. Microscopic examination of the peripheral nerves revealed axonal degeneration, interstitial edema and proliferation of mononuclear cells and infiltration of lymphocytes. Analysis of several feed samples for riboflavin revealed levels ranging from 1.1 mg/kg to 2.4 mg/kg of feed. Normal requirement of riboflavin for broiler chickens is considered to range from 4.6 mg/kg to 5.0 mg/kg of

feed. Prompt supplementation of birds with multiple vitamins resulted in dramatic improvement in clinical signs of most birds.

A search of the entire CAHFS data base for riboflavin deficiency in chickens revealed nine additional cases in addition to the seven cases reported here. Riboflavin deficiency in chickens may be more common than realized before.

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THE EFFECT OF SODIUM TOXICITY ON VIABILITY AND FERTILITY OF BROILER BREEDER MALES

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SUMMARY

Unintended sodium intoxication was identified as the cause of high mortality in 10 day old broiler breeder males. Ascites, mortality and testicular edema were the hallmarks of the intoxication. Microscopic examination of multiple organs revealed lesions consistent with sodium toxicity. Feed analysis confirmed the clinical and microscopic observations. Surviving broiler breeder males were individually identified and maintained until 30 weeks of age, along with unaffected males from the same supplier. Sperm motility, fertility and hatchability of eggs from inseminated hens were evaluated and compared with the fertility and hatchability of breeder flocks housed in the same poultry company of origin.

INTRODUCTION

Sodium intoxication may occur with various salts of sodium, notably sodium chloride and sodium bicarbonate. Levels of Na+ in the feed and water are additive. Young chickens are much more sensitive to Na+ toxicity than adults, possibly due to the relative immaturity of their kidneys. A variety of toxic levels have been reported. A level of 8900 ppm has been reported as toxic. Sodium becomes toxic at levels much higher than 0.5% of the diet, albeit a level of 0.35% will result in increased water intake and watery feces due to increased renal output. Excess sodium in the diet resulting in increased water intake may lead to water intoxication. Clinically affected chickens express increased mortality, diarrhea, distended abdomen, and may appear dyspneic, depressed and often display nervous signs. Heart failure and ascites are some of the immediate problems observed with sodium toxicity. Acute diarrhea, dehydration, kidney damage, weight loss and death are seen in cases of intoxication with high levels of sodium. Kidney damage is thought to occur as a result of ischemia derived from increased red blood cell rigidity. At lower levels there is also watery diarrhea, but body weight gain is unaffected due to water retention. Right ventricular hypertrophy and dilation, valvular insufficiency, edema, and ascites develop within days of high sodium intake. Microscopic pathology has been characterized and includes pulmonary edema, hydropericardium, myocardial hemorrhage, cystic dilation of seminiferous tubules, nephrosis, and non-specific enteritis that is often accompanied by eosinophilic infiltrates. The objective of this study was to determine if sodium toxicity has a detectable impact on male fertility, as related to sperm motility and hatchability of eggs from hens inseminated with sperm from males that had been accidentally intoxicated with sodium at an early age.

Clinical history. Three thousand (3000) broiler breeder males hatched in January of 2004 expressed high mortality during the first 10 days of age. Clinical examination of the chickens in the affected flock

revealed severe depression, lethargy, watery diarrhea, dyspnea, nervous signs and abdominal distention. Broiler breeder pullets placed in the same farm and in a separate pen in the same house were unaffected and had received a different feed. Mortality had reached 19.4% by 10 days of age. Upon necropsy, the affected chickens displayed a large amount of abdominal ascites fluid, hydropericardium, pulmonary and renal edema, hepatomegaly, and severely enlarged testicles. Tissue samples were obtained for virus isolation to rule out adenoviral hepatitis and to confirm a suspected toxicosis. Feed samples were analyzed for calcium and sodium content. The feed formulation was reviewed and the actual ingredient inclusion levels were examined in the feed mill records.

Feed analysis. The calculated sodium inclusion level in the feed was 0.97%, as opposed to a standard level of 0.16%. The calcium level in the feed was 0.9%, in comparison to 1.00% recommended for chickens of the affected age. Despite the fact that the calcium level was approximately as desired, concomitant calcium toxicity was initially suspected, since the inclusion level of limestone was approximately 3.45 times the required level. The inclusion levels of all other nutrients were within normal limits.

Histopathology. Microscopic examination of the liver, pancreas, intestine, and spleen failed to reveal lesions consistent with adenoviral inclusion body hepatitis or other infectious conditions. Severe nephrosis and glomerular sclerosis with granulocytic infiltration were observed in the kidneys. The seminiferous tubules of the testicles were cystic and severely distended. Pulmonary edema was present in some of the chickens sampled.

Immediate intervention. Upon a clinical diagnosis, the suspect feed was withdrawn at 11 days of age and immediately replaced with a fresh batch. Mortality was promptly attenuated after removing the suspect feed. However, the mortality pattern in the affected flock was not fully normalized until five weeks of age. By then, cumulative mortality had reached 30.2%.

Evaluation of sodium toxicity effects on reproductive performance. Due to the extremely high mortality it was necessary to replenish the flock with an additional 1300 males at approximately two weeks of age. At this time, 100 males from the original affected group were identified using wing bands. At approximately 20 weeks of age, all males (affected and non-affected) were co-mingled and made into a single flock of males. At 24 and 30 weeks of age, 60 males from the affected group were sampled to assess sperm production, sperm motility and testicular tissue integrity. At 30 weeks of age, 60 males from the affected group and 50 control males from the non-affected group were sampled to assess sperm production, sperm motility and microscopic testicular tissue integrity. Hens were inseminated with sperm obtained from the affected and unaffected control males in order to assess hatchability.

RESULTS

Although it was theorized that the intoxicated males would exhibit impairment of their reproductive ability, no significant effects were observed in sperm production or sperm motility in comparison with males from the unaffected group. Likewise, eggs from hens inseminated with sperm from males stemming from the affected group exhibited hatchability that was similar to eggs from hens inseminated with sperm from unaffected males. Histologically, residual edema was seen in a few seminiferous tubules in the testicles of affected males at 24 weeks of age, but not at 30 weeks of age. It was concluded that the reproductive fitness of males surviving sodium toxicosis and reaching adulthood is not significantly affected.

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CAUSES OF MORTALITY IN TWO COMMERCIAL TURKEY STRAINS RAISED UNDER FIELD-LIKE CONDITIONS

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ABSTRACT

Causes of mortality were determined for two commercial turkey strains (A and B). Both were obtained from a single turkey integrator located in North Carolina and were raised together under field-like conditions. A total of 2003 day-old hens divided equally between each strain were placed together in a single conventional curtain-sided poultry house at the Teaching Animal Unit of the College of Veterinary Medicine. Strains were identified by different trimming patterns of toe 1 (left for strain A; right for strain B). Toes 2, 3, and 4 were trimmed for all birds. Birds were fed commercial feed provided by the integrator and given municipal chlorinated water ad libitum throughout the growing period. Drinkers were cleaned and disinfected and litter was raked daily. Brooding, heating, ventilation, and lighting were done according to the integrator's guidelines. The average weight at 77 days was 5.99 kg for strain A and 6.56 kg for strain B. Birds were processed at 79 days of age.

Total mortality was 193 birds (9.6%). One-hundred-ninety dead or cull birds were necropsied (155 [7.7%] died; 35 [1.7%] culled). Three birds that died were not available for necropsy. Mortality for strain B was 3.3% higher than for strain A (11.3% vs. 8.0%). In the first two weeks, mortality for strain B was considerably higher than for strain A, due primarily to omphalitis/peritonitis/septicemia (O/P/S). The rise in spontaneous cardiomyopathy (SP) in strain A was observed by late week 3 and remained consistent through the whole period. Notwithstanding, differences between mortalities stayed similar until processing. Weekly mortality for the different strains is presented in Figure 1. Necropsy results are presented in Table 1.

In summary, there were substantial differences in total mortality as well as causes of mortality between the two strains. O/P/S in strain B was more than three times greater than in strain A (74 [7.4%] vs. 24 [2.4%]). In contrast, strain A had an incidence of SP that was more than twice as high as strain B (26 [2.6%] vs. 9 [0.9%]). Mortality attributed to musculoskeletal conditions was not substantially different between the two strains (6 [0.6%] vs. 4 [0.4%]). Starve-out mortality was slightly less than twice in strain A than in strain B (7 [0.7%] vs. 4 [0.4%]).

The possibility that the differences were related more to the specific breeder flocks and not strain of turkey cannot be determined, as only one breeder flock producing each strain was examined in this study. Examination of additional flocks of each strain produced by different breeder flocks would be needed to make this determination. However, it is apparent that overall mortality and causes of mortality can be attributed to strain and/or breeder flock factors and that this should be considered when excess or unusual mortality in turkey flocks is investigated.

Figure 1. Weekly Mortality of Turkey strains A and B.

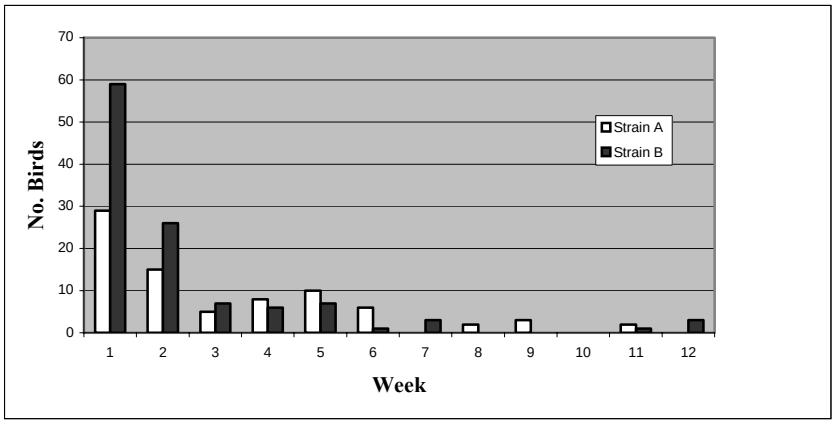

Table 1. Comparison of causes of mortality between turkey strains A and B.

Cause of Mortality	Strain A n =998			Strain B n = 1003		
	Died (%)	Culled (%)	Total (%)	Died (%)	Culled (%)	Total (%)
Omphalitis/Peritonitis/Septicemia	20 (2.0)	4 (0.4)	24 (2.4)	67 (6.7)	7 (0.7)	74 (7.4)
Spontaneous Cardiomyopathy	22 (2.2)	4 (0.4)	26 (2.6)	8 (0.8)	1 (0.1)	9 (0.9)
Musculoskeletal Diseases	0 (0.0)	4 (0.4)	4 (0.4)	0 (0.0)	6 (0.6)	6 (0.6)
Starve-Out	7 (0.7)	0 (0.0)	7 (0.7)	3 (0.3)	1 (0.1)	4 (0.4)
Other	14 (1.4)	5 (0.5)	19* (1.9)	17 (1.7)	3 (0.3)	20** (2.0)
Total	63 (6.3)	17 (1.7)	80 (8.0)	95 (9.5)	18 (1.8)	113 (11.3)

*Ruptured Yolk Sac – 2,Trauma – 2, Umbilical Hernia – 1, Perirenal Hemorrhage Syndrome – 1, Histomoniasis – 1, Not determined – 6, Culled (Hemopericardium) – 1, Culled (Persecution) – 2, Culled (Runting – Stunting) – 1, Culled (Scoliosis) – 1, Culled (Histomoniasis) – 1.

**Ruptured Yolk Sac – 3, Trauma – 3, Intestinal torsion – 1, Peritoneal Hernia – 1, Perirenal Hemorrhage Syndrome – 2, Not available for necropsy – 3, Not determined – 4, Culled (Trauma) – 1, Culled (No lesions) – 1, Culled (Runting – Stunting) – 1.

EFFECT OF ANTIBIOTIC IN TURKEY SEMEN EXTENDERS ON FERTILITY, HATCHABILITY AND EMBRYONIC MORTALITY

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SUMMARY

The study on artificial insemination of Beltsville Small White turkeys was carried out to find out the effect of semen diluents namely Lake's diluent, Brown's diluent and addition of gentamicin in diluents on various semen characteristics, fertility, hatchability and embryonic mortality. Lake's diluent with gentamicin at 5 µg per ml showed a significant increase in sperm motility and live sperms with significant reduction in abnormal sperms compared to other treatments.

Accordingly significant improvement in fertility and hatchability was observed in semen extended with Lake's diluent with gentamicin. The fertility parameters like total fertility, total hatchability, and fertile hatchability were in favor of semen extended with diluents containing gentamicin at 5 µg per mL irrespective of the diluent used.

Artificial insemination is a powerful tool to get optimum fertility in domesticated avian species, especially in turkeys owing to their heavy body weight that hampers natural breeding. Turkey semen is more likely to be contaminated as it issues over the papillae on the wall of the cloaca during collection. Cloaca is the most probable place of contamination of the semen, where it comes in contact with urine, feces, and transparent fluid from lymph folds in the cloaca either singly or in combination resulting in the death of a proportion of spermatozoa. With these in view, an attempt has been made to study fertility and hatchability in turkeys as influenced by antibiotic in turkey semen extenders.

MATERIALS AND METHODS

Experimental design. Five mature toms and 30 hens from the Beltsville Small White variety, age 40 weeks and belonging to the same hatch, formed the study material. The semen collected from four selected toms were pooled and divided into four equal parts. One part of the pooled semen was diluted with Lake's diluent, second part was diluted with Brown's diluent, third part with Lake's diluent with gentamicin at 5 µg per mL, and the fourth part with Brown's diluent with gentamicin at 5 µg per mL. The diluents were added to the semen at the dilution ratio of 1:3. Each group of diluted semen was inseminated into six selected hens once in a week at 4:00 p.m. after oviposition. A control group was maintained by allowing one selected tom in the pen along with six selected hens for natural mating

Collection and incubation of eggs. The hatching eggs were collected, one week after the first

insemination and continued for one week after the last insemination. The eggs were collected twice a day labeled properly indicating the treatment number, bird number and the date of lay. The eggs collected were fumigated as per North (1984) and stored at a temperature of 15.0 - 18.0^{o}C and 75-80 percent relative humidity. The eggs collected over a period of one week were set for hatching. On the 24th day the eggs were candled and live germs were placed in the pedigree boxes dam wise after removing infertiles and dead germs and then transferred to the hatcher. On the 28th day the hatch was taken out and the number of dead-in-shell and good and weak chicks hatched out were recorded. Altogether, twelve hatches were taken for this study. The number of infertiles, dead germs, dead-in-shells, and chicks per each treatment in each hatch were recorded.

Microbiological study. The diluted semen of the four treatments and raw semen were subjected to bacteriological study at the Central University Laboratory. The study was aimed at assessing the effect of addition of gentamicin (5 µg per mL) in the diluted semen on the bacterial load after collection. Five samples in each treatment were inoculated in nutrient agar and MacConkey agar. Each sample was inoculated at three different dilution rates according to the procedure prescribed by Barker and Breach (1). The plates were incubated at 37^{o}C for 24 hours. After incubation the counts were made using a colony counter. The number of bacteria in the diluted and undiluted semen samples is calculated by multiplying the dilution factor.

RESULTS

Dilution of semen in Lake's diluent with antibiotic has resulted in significant ($P<0.01$) increase in fertility rate compared to control and semen diluted in Brown's diluent without antibiotic. Semen diluted in Lake's diluent, Lake's diluent with antibiotic, and Brown's diluent with antibiotic recorded comparable fertility.

Semen diluted in Lake's diluent with antibiotic recorded significant ($P<0.01$) increase in total hatchability compared to Lake's diluent, Brown's diluent, and control group. Addition of antibiotic in Lake's diluent has resulted in significant ($P<0.01$) increase in hatchability. The control group recorded significantly ($P<0.01$) lower total hatchability compared to artificially inseminated groups using semen diluted in different diluents.

Lake's diluent with antibiotic recorded lower dead-in-shell than the other treatments. However, dead-in-shell was significantly ($P<0.01$) reduced by the addition of antibiotic in Lake's diluent compared to non-antibiotic treatment

The highest bacterial count in terms of $\log_{10}$ values was recorded in raw semen irrespective of the media compared to semen extended in Lake's diluent and Brown's diluent. No bacterial growth was noticed in semen samples diluted with both diluents containing 5 µg of gentamicin by cultural methods.

DISCUSSION

Addition of antibiotic has significantly improved the fertility in Lake's diluent which was comparable with that of Brown's diluent with antibiotic. Similarly, Trienhoven *et al.* (9), Millar and Garwood (3), Millar *et al.* (3a) obtained improved fertility in Tyrode solution containing antibiotic. Addition of gentamicin at 5 µg level has significantly improved the fertility irrespective of the diluents owing to significant improvement in fertilizing ability in the absence of contaminants.

Sexton *et al.* (7,8) has concluded that among forty antibacterials studied, gentamicin was one among the few found to prevent bacterial contamination without affecting the viability of spermatozoa. Further, Omprakash *et al.* (6) obtained significant improvement in fertility with semen diluted in Lake's diluent containing 5 µg of gentamicin. Nimai singh *et al.* (4) also observed that 96% of gram negative bacteria mainly contaminating the semen were sensitive to gentamicin.

The findings of this study concur with the observations of Cooper and Rowell (2) who stated that survival of an embryo to hatch depends on the quality of fertilizing spermatozoa.

Addition of antibiotic has reduced the bacterial load in semen and resulted in better fertility and hatchability in Lake's diluent with antibiotic. Wilcox and Shorb (11), Trienhoven *et al.* (9,10) and Sexton *et al*. (7,8) also arrived at similar conclusions.

Omprakash (5) also observed significant lower embryonic mortality in chicken semen diluted with Lake's diluent and Beltsville Poultry Semen Extender containing gentamicin at 5 µg per mL.

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Table 1. Fertility and embryonic mortality of turkey eggs as influenced by different diluents and antibiotic.

	Control	Lakes diluent	Browns diluent	Lakes diluent with Antibiotic	Browns diluent with Antibiotic
Percent total fertility	77.27^{a} ±5.50	90.16^{bc}±1.22	85.00^{ab}±1	95.45^{c}±1.19	89.84^{bc}±2.40
Percent total hatchability	49.74^{a}±6.50	75.52^{b}±1.98	68.13^{b}±2.5	87.32^{c}± 2.03	78.08^{bc}± 3.9
Percent fertile hatchability	59.98^{a}±6.66	83.00^{bc}±2.42	80.46^{b}±2.72	91.99^{c}±2.24	86.63^{bc} ±3.02
Percent dead germ	0.83±0.83	1.93±1.08	4.05±1.47	2.16±0.96	3.05±1.69
Percent dead-in-shell	26.70^{a}±2.35	12.70^{b}±2.05	12.82^{b}±2.10	5.97^{c}±1.84	8.71^{bc}±2.26

Note: Means bearing different superscripts differ significantly ($P<0.01$).

USE OF GIS FOR EVERYDAY INDUSTRY POULTRY HEALTH MANAGEMENT

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SUMMARY

In Georgia, an industry poultry Geographic Information System (GIS) was implemented in 2003. The company owned data is managed at a central location and used exclusively for Poultry Health Management issues. Although it is critical to be GIS-ready in the event of a Poultry Health Emergency (such as HPAI or END), the goal of this paper is to describe the advantages of using a GIS system on a regular basis, and to describe a few non-emergency uses of a Poultry GIS system.

Day-to-day use of GIS in poultry health. We found that because of the intrinsic dynamics of the poultry industry, farm data changes at a rate that makes it merely impossible to use a GIS that is not updated at least every six months. Maps produced with data that is more than six months old are simply incorrect and unusable. Using GIS day to day helps find incorrect data between updates, and helps remind the operator about the importance of updating. Waiting for an Emergency Poultry disease to use GIS may mean

having to relocate premises and make corrections to many data entries. Using GIS often also allows for all companies involved to become not only familiar with the potential uses of the system, but also comfortable with it.

Non-emergency uses of GIS. We have used GIS for the purposes of Emergency Preparedness, Biosecurity, Epidemiology, Industry Communications and Non-Emergency Disease Management.

Poultry disease preparedness exercises have been set up in a tabletop format where a potential "index case" is decided upon. The system can be used as it would in an emergency situation. This helps getting familiar with the system and also helps getting some much needed "practice" for producing the reports needed during an outbreak.

There are situations where a company may need to locate a highly biosecure breeder farm within an area of the state. We have used GIS to help determine the distances of the potential sites to other poultry. Biosecurity projects also included the analysis of land cover around large breeder complexes and the change in the land cover over time. Change in landcover (especially because of deforestation) could lead to increased exposure of the farm due to the disappearance of natural physical boundaries (trees). Remotely sensed images (CIR imagery) were used for this purpose.

We have used GIS in an attempt to better understand the epidemiology of infectious diseases such as vaccinal Laryngotracheitis. Proximity of broiler farms to adult (vaccinated) flocks and to pasture where litter may be spread were factors taken into consideration in this analysis.

We have improved our understanding of poultry density within the state of Georgia and how density relates to disease dynamics. The highly accurate and constantly updated system also gives us the capability to view changes from year to year as it relates to changes in our industry. With diseases such as vaccinal laryngotracheitis and mycoplasmosis, we use GIS to locate flocks and notify industry and to determine the best live haul routes to the processing plant.

INFECTIOUS BRONCHITIS: AN OVERVIEW OF EUROPEAN EPIDEMIOLOGICAL SITUATION

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SUMMARY

A survey on epidemiology of infectious bronchitis (IB) in Europe from the first appearance of the virus in the 1950s is reported. Many new serotypes or variants have been isolated in about 40 years with different spread within a country or among different countries. The recent rapid spreading in Western-Europe of the strains reported also as "It-02" is very interesting. At least some of these strains showed antigenic similarities to AZ-27/98 serotype, initially isolated in 1997 in Italy.

INTRODUCTION

Avian infectious bronchitis is an acute highly contagious disease of the respiratory and/or urogenital tract of chickens caused by a Coronavirus, first identified in 1936 in the USA (Massachusetts strain) and reported in 1948 in Europe, where the disease spread rapidly since the 1950s.

IB continues to be a major cause of economic loss to the poultry industry worldwide due, above all, to antigenic variability of the virus and to secondary bacterial complications. Since 1956 (8), new serotypes of IB virus (IBV) have been reported in various continents (1,6,15,19, etc.). To date, over 50 different serotypes have been detected in the world, and these are probably a small proportion of IBV existing or forming.

Molecular studies have shown that a new virus serotype or variant can emerge as a result of only few changes in the amino acid composition in the S1 part of virus spike protein and of particular virus replication mode (discontinuous transcription), with the majority of the genome remaining unchanged (3). The sharing of so many antigens in different virus isolates might suggest that currently available vaccines should be more or less able to provide complete protection against challenge with viruses belonging to different serotypes; nevertheless, the use of two different vaccinal serotypes seems to induce a broader protection (4,5,16). A permanent monitoring of circulating virus strains in an area would be advisable in order to adapt

vaccination schemes to the field situation. Sometimes a co-circulation of vaccinal and wild type virus has also been demonstrated.

The objective of this paper is to give an overview of the situation of IB in Europe, particularly in recent years, and to compare with the ones of North America and other continents.

EPIDEMIOLOGY AND CONTROL ASPECTS OF INFECTIOUS BRONCHITIS

The first isolation of a new serotype of IBV in Europe goes back to 1965 in Italy with the nephropathogenic 1731 PV strain (12). Besides Massachusetts serotype, many other serotypes or variants of virus spread more or less widely in the last 40 years in different countries. Some of them persisted in an area for a variably short or long period, whereas most have a transient or sporadic appearance (1,6,15,19). The most persistent serotypes or variants have been the following strains:

a) AZ-23/74, mostly nephropathogenic, was reported in Italy in 1974 and spread with serious damage through the country for at least 25 years. Then it seemed to be apparently missing (14,17).
b) D274 and D1466, with respiratory tropism, but not too pathogenic (Dutch variants), was first reported in 1983 in the Netherlands (5). It then spread in other European countries. However, many isolates were highly embryo-adapted and therefore suspicious of vaccinal origin.
c) CR 88121, reported in 1986 in France and 793B or 4/91, reported in 1991 in United Kingdom, mostly respiratory; afterwards this serotype appeared in other European countries, where it is apparently still spread (4,7,9). Also for this serotype it has been suspected what said for Dutch variants regarding the much embryo adapted isolates.
d) AZ-27/98 or Fa-6881/97, respiratory and /or nephropathogenic, was first reported in 1997-98 in Italy (18,19). The results of a wide investigation allow, most likely, to regard these strains as the progenitors or the first resorted isolations of so called "It-02" strains, spread and manifested in many countries of Western- Europe in 2001-04 (13,21).

The RT-PCR tests, reported in a previous paper (19), showed that Fa-6881/97 strain would appear to be related to 793 B strain, unlike the results of serological tests; whereas AZ-27/98 strain, antigenically identical to Fa-6881/97, would appear not related to the most common European and North American serotypes so far reported.

More complete investigations are obviously required and they are actually ahead in some European laboratories as molecular characterization and phylogenetic analysis, by sequencing part of the genome, the subunit S1 (genotype), of numerous European isolates of IBV, including the so called "It-02"strains. Even though some differences have been observed in the sequence of the nucleotides, and consequently of the amino acids by RT-PCR and sequencing, it does not exclude that at least part of the isolates in question could belong to the same serotype Fa-6881/97 or AZ-27/98; and consequently, induce a cross- immune reaction *in vitro* and *in vivo*. The virus can probably undergo some genetic change, even if maintaining the same or similar immunological characteristics (cross-reaction in virus neutralization test). In fact, in order to establish a difference between two strains, only an assessment of a partially different genotype is insufficient. Also needed, and probably of greater importance, is an assessment of the immunological characteristics that are most important from a disease control standpoint (i.e., immunization). Instead, the genotype could have major importance in virological and epidemiological research of the disease and phylogenetic analysis of virus.

Regarding the coupling in the schemes of vaccination of live attenuated Massachussets strain (mostly H120) used worldwide with other more or less attenuated but possibly stable strains, these last have been the AZ-23/74 strain in Italy (16), D 274+ D 1446 strains, first used in The Netherlands, then also in other European countries (5), 4/91, first used in UK, more recently applied in many European countries and maybe in other areas (4), B 1648 strain in Belgium (11); that is alike to what occurs for Arkansas DPI strain, used in many states of USA. The AZ-27/98 strain is still in a phase of attenuation and control of stability, innocuity and efficacy (20).The results so far obtained with different strains would have been tempting, but sometimes controversial – particularly about the use of such variants in countries different from the original ones, sometimes too thoughtlessly, as before reported. Modified live vaccines have been proved to revert to a more or less marked virulence, also because they often contain heterogeneous sub-populations of virus (likely a small pathogenic viral sub-population residue). As regards the combination of live (priming) and inactivated vaccines, in oil emulsion (mono-, bi-or polyvalent) as booster in layers and breeders, that was demonstrated to be very efficacious, particularly if inactivated vaccines contain a good selected protectotype as second virus and because these last are killed.

Another very interesting aspect of IBV, as far as it is possible to know, is the epidemiology, i.e. the presence and the spreading of the different IBV

serotypes in different Continents. About 20 emergent serotypes in North-America, did not spread to other Continents; the only exceptions are the Massachusetts strains, probably also due to its wide use as vaccinal strain. Similarly the European (over 25, of which at least 12 in Italy), Australian and Asiatic serotypes (about 16) apparently did not spread elsewhere. Few it is known about IBV in Central-and South-America and Africa (each with two supposed variants). However, the research on the matter is rather poor and it would be worth to conduct more studies. Investigations, carried out in Italy on many isolates since the '60s, evidenced for the majority of them the belonging to Massachusetts serotype; on the contrary, most of IBV strains were not neutralized by any antisera from American and Australian serotypes (15). Also in Europe, with exception of 793 B (or 4/91 or CR 88121) serotype (2,4), the Dutch variants (5) and AZ-27/98 (likely "It-02") serotypes ,which had some spread in the Continent (13,18), many variants or serotypes were located, for a more or less variable time, only in the country or area of origin.

Sometimes the spreading of a virus among countries or areas in more or less close proximity could be due at least partly to its improper introduction in such areas by birds, or often by forced attenuated vaccines sometimes and unfortunately based only on the ground of serological instead of clear virological tests. In fact, in Italy many isolates obtained from affected chickens induced mortality and specific lesions in embryos since the first passage (Zanella, personal observation). On the contrary, the wild virus would normally require three to five blind passages before adapting to the embryo and induce clear pathognomonic lesions. Therefore, it is necessary to pay particular attention to such evidences before assuming as naturally appearing in an area or country of new but sometimes "manipulated" strains.

In conclusion, it would be necessary to verify by a continuous and in-depth epidemiological surveillance, wide application of improved virological and serological methods, exchanges of data and materials among different laboratories, and accurate bibliographic consultation (!) how much the new variants or serotypes of IBV are diffused in the same and in different areas or countries and to determine the risk factors and the modes of viral spreading.

ACKNOWLEDGEMENTS

The authors are very grateful to Dr. P. Massi, G. Tosi, A. Moreno Martin, C. Fallacara, G. Cattoli, C. Terragino and K. Worthington who kindly supplied the IBV It-02 strain and the respective antiserum and above all for the useful exchange of opinions and experiences.

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TRENDS AND UNIQUE FINDINGS FROM ELEVEN YEARS OF TYPING INFECTIOUS BRONCHITIS VIRUS

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INTRODUCTION

Infectious bronchitis virus (IBV) is a group III coronavirus that causes a highly contagious upper-respiratory tract disease in chickens. Different serotypes of the virus have been identified using virus neutralization testing in embryonating eggs, and modified live vaccines have been developed to control the disease. But different serotypes do not cross protect. Therefore, the serotype of the virus causing the disease must first be determined so that the birds can be properly vaccinated.

In 1993 a new molecular typing method for infectious bronchitis virus (IBV) was introduced, which revolutionized the way IBV is diagnosed and controlled. That method uses the reverse transcriptase-polymerase chain reaction (RT-PCR) and restriction fragment length polymorphisms (RFLPs) to identify regions in the spike gene that correlate with serotype (1,2). It is extremely rapid and has lead to the identification of a tremendous number of virus isolates, which wasn't possible with the traditional virus-neutralization test in embryonating eggs.

Examining virus isolation data over time is important for understanding evolutionary trends in IBV and in coronaviruses in general. At the Poultry Diagnostic and Research Laboratory (PDRC, University of Georgia, Athens, GA) we receive clinical samples for IBV typing from all over the world. Herein, we summarize the number, origin and type of IBV isolates identified at PDRC between 1994 and 2004.

MATERIALS AND METHODS

Viral RNA extraction and RT-PCR/RFLP was conducted as previously described (1). Prior to 1997, primer set S1OLIGO3' and S1OLIGO5' were used as described (2), whereas after 1997 the sequence of the 5' primer was redesigned and designated NEWS1OLIGO5' (1). In 2000, the 3' primer was redesigned and designated Degenerate3' (3).

Isolates of IBV were identified from cases submitted to the PDRC diagnostic laboratory over an 11-year period. Theses isolates are for the most part from the Southeastern region of the United States (AL, AR, GA, KY, MS, NC, SC, TN, VA). Isolates were also obtained from CA, CT, DE, FL, IA, IL, KS, MD, MI, MN, MO, NJ, NY, NE, OH, OK, PA, and TX, and foreign isolates were obtained from Canada, Chile, Mexico, Peru, Israel, and Saudi Arabia.

RESULTS AND DISCUSSION

In general each year shows a biannual distribution pattern of virus isolations (Figure 1) with the most isolations occurring during the summer months (May, June and July), followed by the winter months (December, January, and February). It is interesting that most IBV isolates are made during the summer when many companies cut back on bronchitis vaccination.

The fewest number of isolates identified on an annual basis was 20 in 2003, and the most was 318 in 1997. By far the most common type of IBV identified was Ark-DPI, which ranged from 23% of total

isolations in 1997 to 65% of total isolations in 2003. With the exception of only a few years, the next most common isolates were Mass and Conn. The most common vaccine types used in the U.S. are Ark, Conn, and Mass. Thus, it appears that vaccine type viruses are the most frequently isolated IBV types except in years where a new variant virus has emerged. However, it must be noted that a distinction between a vaccine and field virus of the same serotype cannot be made with the typing techniques used in this study. The predominance of Ark-DPI virus isolations each year is interesting. Perhaps the Ark-DPI types have a selective advantage over other types of IBV in the upper-respiratory tract of chickens, or persist for longer periods in the birds, or are more easily isolated. Whatever the mechanism, persistence of Ark type viruses is truly unique among IBV types and warrants further study. It also appears that Ark type viruses are evolving since new Ark-like types continue to emerge. It is logical to assume that persistence of Ark in chickens provides an opportunity for that virus to undergo antigenic drift.

An unusually high number of isolates were typed in 1997 due to an outbreak of a new variant virus designated Georgia Variant (GAV). In fact 1997 was the only year where a virus type (GAV, 25% of isolations) other than Ark-DPI (23% of isolations) was the predominate type identified. An unusually high number of isolations also occurred in 1999, which coincided with the identification of a new variant virus during the winter of 1998 designated Georgia 98 (GA98). That virus is similar to the DE072 type virus and both continue to be isolated.

Examining the origin of IBV types shows that some viruses tend to be geographically restricted to a given area. The CAV isolate has only been isolated in California. Likewise the 97-8147 isolate has only been isolated in Mexico. No foreign virus types have been detected in the U.S. Finally, it is interesting to note that some highly characterized serotypes of IBV were not isolated during the 11-year period. Those types include FL, Gray, Holt, Iowa, and JMK.

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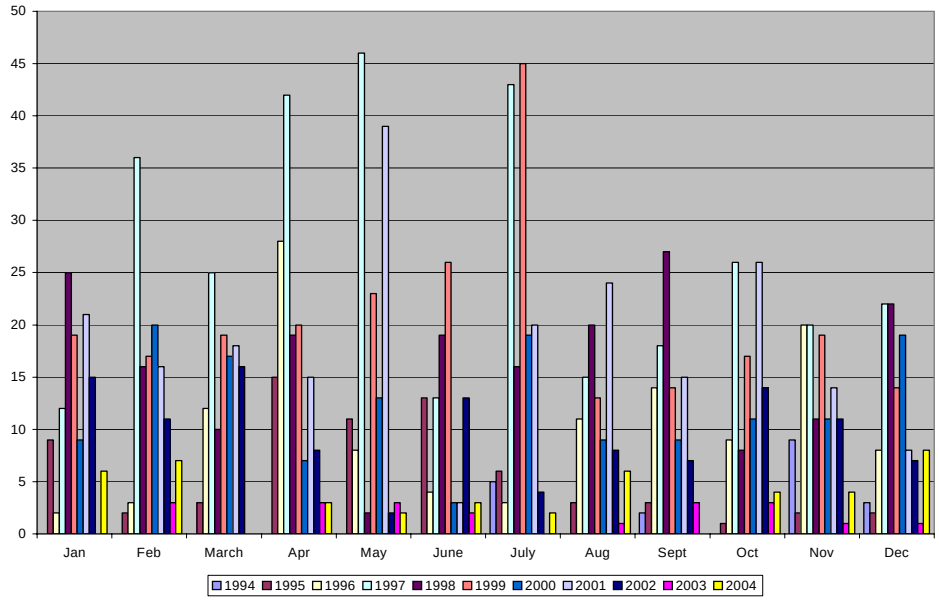
Figure 1. Total IBV isolations at PDRC by month over an eleven-year period.



A SURVEY OF INFECTIOUS BRONCHITIS VIRUS ISOLATES IN THE SOUTHEAST U.S. FROM 2001-2004

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ABSTRACT

Infectious bronchitis virus (IBV) continues to be an important respiratory pathogen of poultry. Routine serological testing using ELISA and HI is useful for monitoring the serological status of a flock. However, virus isolation and serotyping is considered the gold standard for identifying field variants and new serotypes. In our laboratory, we utilize virus isolation in specific pathogen free (SPF) embryos and hemagglutination (HA) activity of neuraminidase-treated allantoic fluid with chicken red blood cells (CRBC) for initial IBV detection. HA positive samples are confirmed by RT-PCR. We have observed a high correlation (>95%) between HA and RT-PCR. Occasionally, some neuraminidase-treated IBV samples do not hemagglutinate CRBCs. In this case, mortality patterns along with lesions consistent with IBV are used to identify samples for RT-PCR testing. The RT-PCR/RFLP method is used to serotype all IBV isolates.

A survey of IBVs isolated at the Poultry Diagnostic and Research Center primarily from broilers during 2001-2004 reveals Ark DPI as the predominant serotype in the southeastern U.S. representing approximately 62% of IBV isolations annually. Other Ark-like viruses were isolated in 9% of IBV cases annually during 2001-2003, but declined to 3% during 2004. Ark 99 was rarely isolated during this time. During the past four years De O72 was isolated in <3% of cases and a steady decrease in Ga 98 isolations were observed (9% in 2001 to 0 in 2004). Mass followed by Conn isolates represented less than 10% of IBV serotypes in 2001-2003. In 2004, Mass isolates increased to 15%. Typable IBV variant viruses, or viruses having RFLP patterns unlike reference strains, were isolated in less than 5% of cases during the past four years. Virus isolation and RT-PCR/RFLP continues to be the superior method of detecting and serotyping IBV isolates.

INFECTIOUS BRONCHITIS VIRUSES IN CALIFORNIA: OBSERVATIONS MADE ON SEQUENCING DATA

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ABSTRACT

Between 2001 and 2004, 368 isolations of infectious bronchitis virus (IBV) were made from submissions of sick chickens to the CAHFS laboratory system for necropsy. Further testing by screening with monoclonal antibodies suggested that some of the IBVs could be of vaccine origin. Other IBVs were characterized by direct automated cycle sequencing of the hypervariable region of the S1 gene. This has resulted in a number of IBVs that appear to be specific to California as judged by searches in NCBI GenBank. These include Cal 99 *AY514485*; CA/603/01 *AF425648*; CA/389/01 *AY038997*; and two recent introductions which are not in GenBank.

In broilers, the clinical signs most commonly observed in chickens over 35 days of age have been associated with airsacculitis and condemnations. In layer chickens the clinical signs have been more variable.

The case histories and the relationships of some of these isolates to IBVs isolated in the 1980s will be presented. The IBV history in California flocks over the last four years has been variable as it has overlapped with the 2002-2003 Exotic Newcastle disease outbreak in Southern California, and sporadic cases of low path avian influenza (H6N2) in 2001-2003; thus, some cases of IBV may have been missed or masked by other diseases.

MONITORING THE COURSE OF INFECTIOUS BRONCHITIS VIRUS INFECTION IN CHICKENS USING REAL-TIME RT-PCR

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INTRODUCTION

Infectious bronchitis (IB) is a disease of the upper respiratory tract in chickens. It is of economic significance to the poultry industry due to the high morbidity and production losses associated with the disease. The etiologic agent of IB is infectious bronchitis virus (IBV), a positive-sense RNA virus that belongs to the *Coronaviridae* family.

To control the disease, it is imperative to quickly and accurately detect the presence of the virus within an infected poultry flock so that subsequent flocks can be properly vaccinated. Recently, a specific and highly sensitive TaqMan® RRT-PCR assay for the detection of IBV RNA from biological samples was reported (1). Although the assay is quantitative, the correlation between copy number of viral genomes detected by RRT-PCR and infectious bronchitis disease progression has not been determined.

The purpose of this study was to monitor the course of IBV infection in chickens using the IBV Taqman RRT-PCR assay. The reason for the study was to determine the copy number of viral genomes that is biologically significant when using RRT-PCR as a diagnostic tool. Herein, we report the IBV Taqman RRT-PCR data and its correlation with clinical signs, histopathology scores, and ELISA titers.

MATERIALS AND METHODS

One hundred and twenty-eight specific pathogen free (SPF) chickens were divided into four groups of 32 chickens. Each group was housed in a separate Horsfall isolation unit in four separate filtered air, positive pressure houses. The day before inoculation, blood and tracheal swab samples were taken from two birds representing each group. The next day, each group was inoculated intranasally with 50 microliters (μL) of the Arkansas DPI strain of IBV with the following titers: Group 1 – no inoculum, Group 2 - 1.6 X 10^1 EID_{50}/mL, Group 3 - 1.6 X 10^3 EID_{50}/mL, and Group 4 - 1.6 X 10^5 EID_{50}/mL.

Five birds from each group were checked for clinical signs, necropsied and sampled on 1, 5, 10, 14, 21, and 28 days post inoculation (d.p.i.). Tracheal swab samples were taken at each time point, while tracheal tissue and blood samples were taken at every time point except at 1 d.p.i.

Tracheal swab samples were placed in 200 μL of 1X PBS and stored at –70 C until processed for RNA extraction. RNA extraction was performed using a High Pure RNA Isolation Kit (Roche). The RNA was used as template in a IBV Taqman RRT-PCR as previously described (2004, AVMA). The number of viral RNA copies was determined by comparison with a standard curve generated from 10-fold serial dilutions of a known concentration of runoff RNA molecules synthesized from a DNA template containing the 5' end of an IBV genome. Tracheal tissue samples were fixed in neutral buffered formalin and processed for histopathology analysis and scored for typical IBV induced lesions. Serum was collected from each blood sample and tested for IBV specific antibodies (IDEXX IBV ELISA).

RESULTS AND DISCUSSION

The IBV Taqman RRT-PCR, histopathology scores, and ELISA results for each group at each time point are summarized in Table 1. Note that the birds used in this study were SPF for IBV as confirmed by negative results for IBV Taqman RRT-PCR and ELISA analysis performed on samples taken prior to virus inoculation. From our data, no clear correlation between copy number of viral RNA genomes and clinical signs, histopathology scores, or ELISA can be found. There are several possible reasons for this lack of correlation. One possibility is that clinical signs, histopathology, and serum antibody levels may lag behind viral replication, making it difficult to correlate them with viral RNA copy number. Furthermore, specific to our study, the histopathology results probably did not correlate due to increased scores for group 1 caused by mechanical damage to the tracheal tissue while obtaining tracheal swab samples, as evidenced by the diffuse and multifocal ulceration observed for group 1 without the presence of lymphocytic infiltrates or loss of respiratory cilia characteristic of IBV infection. The lack of correlation with serum antibody levels may be that 1) the IDEXX IBV ELISA measures IgG, whereas the initial exposure

of these chickens to IBV would have produced an IgM response and 2) the viral antigen used in the IDEXX IBV ELISA is from a different serotype than the virus used in our experiment.

Although the lack of correlation in the study was disappointing, there were some interesting findings. One of the main advantages of Taqman RRT-PCR is its sensitivity. In previous work, it was determined that the IBV Taqman RRT-PCR assay was sensitive down to five copies of target RNA (1). The sensitivity of the IBV Taqman RRT-PCR allowed the detection of viral RNA out to 28 d.p.i. This represents an improvement compared to a reported assay that only detected viral RNA from tracheal swab samples out to 14 d.p.i. (3). Although advantageous in most respects, the high sensitivity of the IBV Taqman RRT-PCR assay can lead to false positives. With regard to our IBV Taqman RRT-PCR system (including RNA extraction and RRT-PCR), a false positive rate of 7% was observed. Three experimental samples from group 1 (one each from day 1, 5, and 21) and one extraction control were weakly positive when they were expected to be negative. It seems likely that these positives were due to cross-contamination during the RNA extraction process, emphasizing the importance of taking every precaution to limit contamination at every step during the process.

At 1 d.p.i., the IBV Taqman RRT-PCR results clearly showed that each group received different doses. A 28-fold difference of viral RNA was measured between groups 2 and 3, while groups 3 and 4 differed by 64-fold. Based on the copy number of viral genomes, the virus load plateaued regardless of dose at 5 d.p.i. To our knowledge, this is the first report of the plateau effect for different doses of IBV, but agrees with a past report that detected the highest amount of IBV viral RNA in the trachea at day four as compared to days two and six after experimental infection (2). For group 2, there was a 20,000-fold increase in viral RNA between 1 and 5 d.p.i., as compared to a 77.9-fold increase for group 3 and a 1.4-fold increase in viral RNA for group 4. At this time, it is not clear what causes the fold increase differences for each group. It may be that there is a more vigorous virus replication when a low dose is given or it may be due to saturation of target cells when a higher dose of virus is given, allowing only for a minimal increase in overall viral RNA.

It was also interesting to note that there was an inverse relationship between the virus dose given and the copy number of viral genomes present in the trachea at 28 d.p.i. Group 2 received the lowest dose of virus, but had the highest copy number of viral genomes (1,313 copies) at 28 d.p.i. Group 3 received the second highest dose of virus, and had the second highest copy number of viral genomes (38 copies) at 28 d.p.i. Group 4 received the highest dose of virus, but had the lowest copy number of viral genomes (0 copies) at 28 d.p.i. This inverse relationship warrants consideration with respect to IBV vaccination because it may have an affect on the overall quality of the immune response.

Clearly, further work needs to be conducted that compares virus isolation data and copy number of viral genomes. Most likely, this will be the best way to establish a biologically significant cutoff value for data obtained from the IBV Taqman RRT-PCR assay. Based on our data, we did not find any clear correlation between copy number of viral genomes and clinical signs, histopathology, or ELISA results. Therefore, at this time we are unable to establish a biologically significant cutoff value for the copy number of viral genomes as determined by IBV Taqman RRT-PCR.

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Table 1. IBV RRT-PCR, histopath, and ELISA results for Groups 1-4 at 1, 5, 10, 14, 21, and 28 d.p.i.

	Clinical signs[a]	Copy number[b]	Histopathology score[c]	ELISA[d]
Day 1				
Group 1	0/5	15	ND	ND
Group 2	0/5	2,667	ND	ND
Group 3	0/5	74,880	ND	ND
Group 4	0/5	4,852,810	ND	ND
Day 5				
Group 1	0/5	1,624	3.0	0/5
Group 2	1/5	11,786,970	2.8	0/5
Group 3	1/5	5,832,593	2.3	0/5
Group 4	3/5	5,709,111	2.4	0/5
Day 10				
Group 1	0/5	0	1.4	0/5
Group 2	2/5	831,283	2.6	0/5
Group 3	5/5	545,163	3.0	0/5
Group 4	2/5	20,112	2.2	1/5
Day 14				
Group 1	0/5	0	1.0	0/5
Group 2	3/5	11,296	3.4	1/5
Group 3	2/5	4,866	3.4	0/5
Group 4	0/5	1,299	2.6	0/5
Day 21				
Group 1	0/5	4	2.1	0/5
Group 2	0/5	141	2.6	0/5
Group 3	0/5	3,016	2.6	0/5
Group 4	0/5	155	2.6	0/5
Day 28				
Group 1	0/5	0	2.4	0/5
Group 2	0/5	1,313	2.4	1/5
Group 3	0/5	38	3.2	0/5
Group 4	0/5	0	1.8	3/5

[a]Number positive/Total
[b]Average number of viral RNA molecules from 5 tracheal swabs as determined by IBV Taqman RRT-PCR
[c]Average tracheal lesion score from 5 tracheal tissue samples using a scale of 1-4, with a score of 1 being normal and a score of 4 being severe
[d]Number positive/Total using IDEXX IBV ELISA

IMMUNOGENICITY AND SAFETY OF AN ATTENUATED GEORGIA TYPE INFECTIOUS BRONCHITIS VACCINE

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SUMMARY

Schering-Plough Animal Health Corporation (SPAHC) developed an attenuated Georgia type infectious bronchitis (IB) vaccine (AVIMUNE™ IB98). The result of an immunogenicity study showed that the percentage of IBV culture-negative birds in the three vaccinated groups was 91.7%, 92.0%, or 96.0% after challenged with homologous serotype of infectious bronchitis virus (IBV). The Georgia type IB vaccine protected greater than 90% of birds at a minimum protection dose of $10^{2.1}$ EID_{50}, indicating that the vaccine will provide excellent protection for chickens against IB caused by Georgia type IBV. The safety of the vaccine was tested under experimental and field conditions. A ten-fold overdose safety study showed that the vaccine virus was the mildest among the viruses tested, including two existing commercial IB vaccine strains. The vaccine virus did not revert to virulence after five passages in specific pathogen-free (SPF) chickens. The safety of the vaccine was also rigorously assessed under commercial conditions using 68,864 broiler chickens at three geographic locations. The results of the field safety study supported the conclusion that the Georgia type IB vaccine appeared to be safe when administered at a full dose to day-old chicks via cabinet spray.

INTRODUCTION

Avian infectious bronchitis (IB) is a significant respiratory disease in chickens and remains a primary concern in commercial poultry operations. The causative agent of IB is infectious bronchitis virus (IBV), a member of the family *Coronaviridae*. Many serotypes of IBV have been found around the world. Georgia type (also referred to as GA98 serotype IBV) is a newly emerged serotype, which has caused IB outbreaks in the southeastern United States in recent years (2). It is well known that no cross protection can be provided against this new serotype by heterologous serotypes IB vaccines, such as Massachusetts (Mass), Connecticut (Conn), or Arkansas (Ark) types of vaccines. One exception is the Delaware Variant type IB vaccine (Shor-Bron™-D) which has been shown to provide partial protection against the Georgia type IB.

MATERIALS AND METHODS

Chickens and chicken eggs. Specific pathogen-free (SPF) chickens and eggs were used for immunogenicity and all experimental safety studies, including overdose safety (10X) and reversion to virulence studies. The chickens and eggs were provided by Hy-Vac or Charles River Laboratories. Commercial broiler chickens were used for the field safety study.

Virus. A field isolate of Georgia type IBV was purified and attenuated by SPAHC.

Immunogenicity study. To test immunogenicity of the vaccine, three groups of 25 birds were vaccinated via cabinet spray (CS) with three dose levels of Georgia type IB vaccine virus, respectively. Two groups of 15 chicks served as unvaccinated unchallenged or unvaccinated challenged controls. All the vaccinates and one group of unvaccinated birds were challenged by eye drop (ED) with a homologous serotype (heterologous strain) IBV at a dose of $10^{3.0}$ EID_{50} at 21 days post-vaccination. Tracheal swab samples were collected at five days post-challenge. Virus re-isolation from the swab samples was conducted in embryonated SPF chicken eggs as required by government regulations (1).

Overdose safety. To test safety of the Georgia type IB vaccine under an overdose situation, three groups of 25 day-old SPF chicks were inoculated by ED with one of the following viruses: Mass type IB vaccine strain, Delaware Variant type IB vaccine strain, or a candidate Georgia type IB vaccine strain. The dose used for the inoculation was ten-fold higher than the expected typical release dose for the Georgia type IB vaccine. Each bird was scored for rales every day between Days 4 and 8, and on Days 10, 12, 14, 16, 18, and 21 post-inoculation. Rale scoring was conducted blindly as follows: No rales (score=0), Mild

(score=1), Moderate (score=2), and Loud (score=3). In addition, all respiratory IB signs, which include gasping, coughing, sneezing, nasal discharge, wet eyes, and sinus swelling, were observed for 21 days post-vaccination. Total Virulence Score (TVS) for each virus was calculated based on the severity of rales, the ratio of birds showing high rale scores, and the total number of days and birds in each group that showed IB respiratory signs. The scores were based on internal standards developed by SPAHC.

Back passage study. To provide assurance that the vaccine virus will not revert to virulence after being administered in chickens, a back passage study was conducted. A group of 10 chickens at one day of age were inoculated with the vaccine virus by ED route at a dose of $10^{4.3}$ EID_{50}. The birds were observed daily for clinical sign of IB. Tracheal swab samples were collected for virus isolation at five days post-inoculation in 9 to 11 day-old embryonated eggs. Positive samples were pooled after each passage and used as inoculum for the next passage. These procedures were repeated four more times for a total of five successive passages. Rales were scored daily as follows: No rales (score=0), Mild (score=1), Moderate (score=2), and Loud (score=3). To further assure the safety of the vaccine virus after successive passages, the final passage material was inoculated into 10 birds and clinical signs were compared to a group of 10 birds inoculated with the original master seed virus (MSV). Observation period were over a 21 day.

Field safety study. To test safety of the vaccine in day-old chicks under commercial conditions, a field safety study was conducted according to Good Clinical Practice (GCP) and other relevant guidelines (3, 4). Briefly, Georgia type IB vaccine pre-licensing serials (PLS) were administered to one-day-old commercial broiler chicks using a spray cabinet at the hatcheries of three participating farms in three geographically disparate farms. The experimental vaccine was administered in addition to the standard IB vaccine regimen applied to the flocks. The total number of vaccinated chicks were 68,864 (farm No. 1, n=23,900; farm No. 2, n=21,800; farm No. 3, n=23,164). Control flocks not vaccinated with Georgia type IB were included at the same sites. The total number of control chicks were 68,865 (farm No. 1, n=23,900; farm No. 2, n=21,800; farm No. 3, n=23,165). The flocks were monitored for potential vaccine reactions post-vaccination. The overall mortality (including culled birds) was recorded from the day of placement until processing. Each treatment group (house) was processed separately after grow-out. Overall livability was calculated as follows: total number of live birds processed/total number of enrolled birds. The condemnation data was obtained from the USDA Condemnation Certificates following slaughter.

RESULTS AND DISCUSSION

Immunogenicity study. In a previous study, the immunogenicity of Shor-Bron™D was examined. Birds vaccinated with Shor-Bron™D at a full dose using cabinet spray were 80% protected against the challenge from a Georgia type IBV. The immunogenicity of the Georgia type IB vaccine was examined in this study. No virus was recovered from negative control birds (100% negative). The virus was recovered from all positive control birds after the challenge. The percentage of culture-negative birds in the three vaccinated groups following challenge was 91.7%, 92.0%, and 96.0%, respectively. The Georgia type IB vaccine protected greater than 90% of challenged birds with a minimum protection dose of $10^{2.1}$ EID_{50}. These results indicate that, at the release dose, the Georgia type IB vaccine will provide excellent protection for chickens against challenge from a homologous type IBV.

Overdose safety. No severe respiratory signs of IB were observed in any group except for one bird in the Mass vaccinated group that showed sinus swelling on Days 9 and 10 post-inoculation. The TVS for the Mass, Delaware Variant, and Georgia types IBV vaccinated groups were 25, 9, and 6, respectively. These results showed that the Georgia type IB vaccine candidate virus was the mildest among the viruses tested in this study. The results indicated that the vaccine virus was well attenuated and safe even when administered at a ten-fold dose.

Back passage study. The average rale scores from the first to fifth passage were 0.30, 0.24, 0.16, 0.14, and 0.16, respectively. No other IB clinical signs were observed. Average rale scores for the chickens inoculated with final passage material or the MSV were 0.15 and 0.23 over the 21 days of observation, respectively. These results indicated that the vaccine virus did not revert to virulence after five back passages in chickens.

Field safety study. The safety of the Georgia type vaccine was rigorously assessed using large numbers of broiler chickens at three geographic locations. No severe respiratory/systematic clinical signs or adverse vaccine reactions were observed in any of the vaccinated flocks during the study. At farm No. 1, the overall livability from Day 0 until processing was 0.4% higher in the Georgia type IB vaccinated flock than that in the control flock. The total condemnation rate following slaughter for the vaccinated flock was 0.13% higher than that in the controls. At farms No. 2 and No. 3, the overall livabilities were 0.8% or 1.5% lower in the vaccinated flocks than those in control flocks. The total condemnation rates for the vaccinated flocks were 0.25% and 0.01% higher than those in control flocks,

respectively. The results showed that both overall livability and condemnation rates for the Georgia type IB vaccinated flocks were comparable to the controls, and within a range considered normal for the type of management conditions. Use of the Georgia type IB vaccine as an additional IB vaccine (second or third) applied to the vaccinated flocks without causing unusual clinical signs, mortality, or condemnations further supports the safety of this vaccine for field use. In conclusion, the Georgia type IB vaccine, AVIMUNE™ IB98, is safe for one day-old chicks when administered at a full dose using a spray cabinet under commercial conditions.

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HIGH INCIDENCE OF FALSE LAYERS IN (RE) PRODUCTION HENS SUPPOSEDLY ATTRIBUTED TO A JUVENILE INFECTIOUS BRONCHITIS VIRUS INFECTION

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FIELD CASES

Very severe egg production problems were recorded in 10 commercial chicken flocks, which belonged to different breeds, i.e. six broiler parent flocks, two layer parent flocks, and two layer flocks. In total, seven different commercial lines were involved.

The production problems were characterized by lay stagnation before peak production, with maximum egg production percentages ranging from 30 to 55% in apparently healthy flocks.

At clinical inspection of the affected flocks the chickens seemed in good health with good plumage, large, bright red, glossy comb and wattles, and bleached eye ring and beak. Moreover, the birds were alert, lively and showed normal activity. In some instances some birds appeared to be standing more upright while showing a pendulous abdomen at palpation. The characteristics of the pubic bones (flexible and wide apart) and the aspect of the vent (large, moist and bleached) of these and other birds, which at post-mortem showed oviduct abnormalities, matched the external characteristics of good layers.

An average of 20 birds per affected flock were submitted for post-mortem and further analysis.

HISTORY

Six cases of nephropathogenic infectious bronchitis virus (NIBV) infections were diagnosed in broilers, layer rearing pullets, and broiler rearing pullets between December 2003 and March 2004 in the southern part of the Netherlands and in a central area of the country called the Veluwe. Affected birds showed respiratory symptoms (conjunctivitis, nasal exudate, rales, and sneezing) and increased mortality ranging from several to 10%. Moreover, the litter in the houses where these flocks were allocated was wet. From trachea, lung, and kidney tissues an unidentified infectious bronchitis virus variant was isolated, which was different from the nephropathogenic D8880 (also known as the B1648 serotype) strain (see virology) that is usually associated with the rare outbreaks of NIBV in Western Europe.

An unexpected follow up was the occurrence of high incidence of false layers in a cluster of three broiler parent lots, which were hatched and reared together, and suffered from a nephropathogenic infectious bronchitis infection at the rearing farm at two weeks of age. According to the integrator these flocks had been vaccinated against infectious bronchitis virus at the hatchery. Later additional cases emerged primarily in the area where the rearing farm of

the first three cases was located, and subsequently in other areas of the country.

MACROSCOPY

The oviducts of birds from these flocks belonged to any of the following three categories:

1. Normal oviducts.
2. Hypoglandular oviducts with near to normal morphology.
3. Oviducts with evident abnormal morphology, which were found in 100% of three submissions and in 10 to 65% of chickens of remaining submissions.

Hypoglandular oviducts with near to normal morphology were characterized by focal circular and longitudinal sections lacking glandular tissue, while the length in general was similar to that of normal healthy counterparts. The hypoglandular tissue was located mostly in the caudal part of the magnum. Moreover, there was no liquid accumulation or lumen obstruction in these birds. Sometimes egg concrements were found in the isthmus.

Oviducts with evident abnormal morphology: cystic and non-cystic.

Non-cystic oviducts with evident abnormal morphology represented a minority. These were characterized by various grades of apparent absence of glandular tissue ranging from small hypoplastic sections to nearly complete absent oviducts.

Cystic oviducts. In a number of cases large to very large cystic dilatations were seen in the magnum, which was separated from the isthmus by an occluding constriction. Fluid accumulation was also found in the isthmus, but to a much lesser extent than the magnum or in some cases even absent. The oviduct walls of magnum cysts were thin and transparent, while the cysts were filled with colorless fluid, sometimes with flaky material. The walls of the affected isthmus had partial development of glandular tissue and were not transparent. In most cases the whole oviduct appeared as an enormous cyst containing a volume of colorless fluid exceeding one liter.

Ovaries with preovulatory ova in various stages of development were found in most birds with hypoglandular oviducts or with oviducts with evident abnormal morphology submitted for post-mortem. However, there were no signs of ovulation in many of these chickens although a few showed absorption of yolk material in the serosa covering the oviduct and the body cavity at histology.

MICROSCOPY

Varying degrees of hypoplasia and/or atrophy of the glandular tissue of the magnum were found at microscopy, i.e. dilatation of the lumen and slight decrease of the thickness of glandular tissue. Often severe decrease to complete absence of glandular tissue still covered with ciliated superficial epithelium was found. In other instances a thin wall consisting of only a thin muscular layer and stroma with non-ciliated (cubic to flat) superficial epithelium was present. In most cases glandular cells of the magnum were filled with secretum. In a few cases part of the glandular cells seemed devoid of secretum.

Infiltrations with mononuclear cells and plasma cells were found in the lamina propria and the muscular wall. Also, varying quantities of lymph follicles were observed. In some cases yolk material was found in the magnum.

Microscopic abnormalities of the isthmus were similar to those described for the magnum. Occasionally, in both the magnum and the isthmus structures resembling dilatated lymph vessels were found.

In the uterus similar abnormalities to those found in the magnum and isthmus were seen. However, in many cases where cystic dilatation of the oviduct was present, the muscular wall was thick and glandular tissue was not severely atrophied and had retained a productive aspect (glandular cells with active aspect containing secretum; based on the size of the cells and the aspect of cytoplasm). Infiltrations of the lamina propria and the muscular wall were as mentioned above, infiltrations of the muscular wall being more prominent in the uterus.

Active absorption of yolk material was visible in some tissue sections of the serosa covering the oviduct and body cavities. Yolk material was never found within cysts and the uterus.

In a number of cases the right oviduct appeared more prominent than would have been the case in normal birds.

In the kidneys of all birds varying degrees of lymphocytic and plasmacellular infiltrations were found. They were located in the tubular loops of the reptilian type nephron which surround the central veins, and in the stroma of the medullar cone. The perilobular collecting ducts were free of these infiltrative changes as opposed to what is commonly found in infections with nephropathogenic D8880.

VIROLOGY

Cecal tonsils, trachea, lung, and kidney tissue from the six initially affected flocks with nephropathogenic infectious bronchitis virus were used for virus isolation. Virus isolation and identification by monoclonal antibodies (MoAbs) was performed as described (5) and virus neutralization as described by Davelaar and others (4). The isolates showed positive

reactions with a MoAb for the Massachusetts serotype and no reactions with MoAbs representing the D274, D1466 and D8880 serotypes. However, the neutralization tests indicated that the isolate (D388) was not a representative of the Massachusetts serotype and confirmed that is was not a strain of the NIBV D8880 serotype either. The D388 strain was inoculated in SPF broilers and commercial birds and showed to be nephropathogenic and could be detected well in the oviducts of the infected birds.

Further typing of the D388 strain is in progress.

DISCUSSION

In the present cases severe and permanent lesions of the oviduct consistent with abnormalities described in laying hens following infectious bronchitis virus exposure at day-old were found (1, 3). In some cases the submitted hens showed hypoglandular oviducts with near to normal morphology, but most frequently oviducts with evident abnormal morphology and cyst formation were found.

Infections with infectious bronchitis virus may occur soon after hatching and induce clinical symptoms and increased mortality. Later such flocks may suffer from severe egg production stagnation (maximum egg production of 50% or less) due to permanent damage of the reproductive system. Although in mature chickens, infectious bronchitis virus may affect egg production, the external and internal egg quality and induce respiratory symptoms, the extensive and permanent damage of the oviduct as seen in birds infected with the virus at an early age, is not encountered.

Specific maternal antibodies will protect the oviduct from permanent damage due to infectious bronchitis virus infection at very young age (2). Since all commercial (re)production flocks in the Netherlands are extensively vaccinated against infectious bronchitis virus, the emergence of flocks with high incidence of false layers suggests that either the parent flocks were not vaccinated (well) against infectious bronchitis or a "new" serotype was introduced to a susceptible population. Based on the flock's history, the virology and sequence work the latter seems most likely to be the case.

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DETECTION OF INFECTIOUS LARYNGOTRACHEITIS VIRUS BY PCR SPECIMENS STORED ON FTA® FILTER PAPER

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ABSTRACT

Speed and accuracy of detection is extremely important in suspected cases of infectious laryngotracheitis (ILT). Traditionally, histopathology looking for syncitia cell formation with intranuclear inclusion bodies or direct fluorescent antibody tests (DFAT) have been used. These two tests have the disadvantage of requiring about one day for tissue processing once the samples are received in the lab and a pathologist to read the slide.

We have previously shown that the Whatman FTA® filter papers can be used to successfully collect, inactivate, and transport poultry pathogens from the field to the diagnostic laboratory where they can then be rapidly processed for detection by PCR. We have shown that the FTA paper will inactivate the ILTV/Trachvax on contact and it is still detectable after 70 days storage at room temperature. In an attempt to determine the best method for sampling ILT infected chickens to the FTA paper compared to histology, virus isolation and DFAT, 125 four-week-old broiler chickens were infected with $10^{4.2}$ $TCID_{50}$/bird CEO vaccine (4x normal dose). All samples were collected seven days after challenge.

It was found that 100% of the eyelid impressions onto FTA were PCR positive, 60% of trachea impressions, 60% of trachea scrapings, and 8% of trachea swabs were PCR positive from FTA. Twenty-five (25%) percent of the samples were virus isolation positive and 0% were positive by histology and DFAT.

Under the conditions of this experiment, it can be concluded that the best sample for PCR detection using the FTA paper is eyelid impressions followed by either proximal trachea impressions or tracheal scraping. One reason the histology and DFAT were unsuccessful may be due to the method of challenge which results in minimal clinical signs in the birds and/or the timing of sample collection for these two tests.

VITAMIN A DEFICIENCY IN TURKEY POULTS

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A group of five- to six-week-old commercial turkey poults experiencing mortalities of 71-85% was diagnosed with hypovitaminosis A based on histopathology and toxicological analysis of serum, liver, and feed. Aside from increased mortalities, other signs included depression, inappetence, and wobbly gait. There were interesting gross lesions observed such as the presence of dry pale yellow exudate in the lumen of the bursa of Fabricius, increased mucus exudate in the sinuses, and prominence of papillae on the mucosa of the oral cavity. The main histopathological finding was squamous metaplasia of epithelial cells in various organs including the conjunctiva, crop, esophagus, sinuses, oral mucosa, bronchi of the lungs (occasionally), proventriculus, and the bursa of Fabricius. Vitamin A levels in the liver considered normal for poultry is 60-300 ppm. In the liver samples tested, vitamin A concentration was extremely low (0.1 ppm, 1/7 poults) to undetectable (6/7 poults). Analysis of the feed revealed undetectable amounts of vitamin A (detection limit of 0.5 ppm). Serum levels were also below the accepted concentration of 0.3-1.7 ppm.

Management changes such as the removal of old feed, cleaning of feed bins and troughs, and the addition of a vitamin pack in the ration was immediately implemented after a diagnosis was made. Some birds recovered after this change in the diet but unfortunately, the majority of them remained poor doers. Vitamin A analyses of liver and serum a week after changes in the diet showed a slight increase in concentrations although they were still below accepted normal levels for poultry in general. Literature is abound with studies and reports on vitamin A deficiency in chickens, but a natural occurrence and a histopathological description of avitaminosis A has not been reported in commercial turkeys.

(A full-length article will be submitted for review and consideration for publication in *Avian Pathology* journal.)

GENETIC AND TOXIN CHARACTERIZATION OF *CLOSTRIDIUM PERFRINGENS* FROM DIFFERENT AVIAN SPECIES

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ABSTRACT

Relationships between *Clostridium perfringens* isolates were analyzed from 24 cases of different avian species submitted between 1997 and 2004. The main criteria for selecting the isolates were diagnosis of necrotic enteritis associated with *C. perfringens* in the bird. The observations are important to assist the understanding of the epidemiology of clostridial enteritis in birds.

Multiplex polymerase chain reaction (PCR) assay was used to detect the genes for the major toxins (alpha, beta, epsilon, and iota), enterotoxin (*cpe*) and toxin beta2 (CPB2) from the *Clostridium perfringens* isolates. All isolates were classified *C. perfringens* type A. In addition, most isolates from all avian groups had the gene for CPB2. Recently, this gene has been found in a few isolates from broilers with *C. perfringens*-associated hepatitis (1). In our study, the gene for the CPB2-toxin was detected in all *C. perfringens* isolates from chickens with acute necrotic enteritis and no liver involvement.

Furthermore, recent studies of the CPB2-toxin strongly suggest an association between this toxin and clostridial gastrointestinal diseases in domestic animals, including necrotic enteritis in piglets and typhlocolitis in horses (2, 3). In order to investigate the possible connection with enteric disease, the expression of the CPB2 toxin was determined by polyacrylamide gel electrophoresis (SDS-PAGE). Only 41% expressed the toxin in vitro. Since the correlation between presence of the gene and expression is low, it suggests that CPB2 toxin may not cause necrotic enteritis alone in birds. However, the ability of this toxin to exacerbate the disease is not known.

Finally, pulse-field gel electrophoresis (PFGE) was used to analyze genetic variations among the isolates. In general, a high genetic diversity was found between isolates. Quail isolates were identical and chicken isolates were possibly related. Most turkey and psittacine isolates were unrelated within and between avian groups.

In conclusion, most isolates had the gene for CPB2 toxin; but less than half expressed CPB2 toxin in vitro. More work is required to determine the role of this toxin in necrotic enteritis in birds. Also, the subtypes of *C. perfringens* from chickens with necrotic enteritis may be more closely related than other subtypes that cause clostridial enteritis in other avian species.

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(The full-length article will be published in *Veterinary Microbiology*.)

ANTIBODY IMMUNE RESPONSE AND PROTECTION CONFERRED IN LAYER HENS BY MUCOSAL VACCINES OF *AVIBACTERIUM* (*HAEMOPHILUS*) *PARAGALLINARUM*

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ABSTRACT

The reclassified bacterium *Avibacterium* (*Haemophilus*) *paragallinarum* is the etiological agent of infectious coryza, an important respiratory disease affecting chickens of all ages (1). The economic impact in layer hens is due to a significant reduction in egg production. The parenteral vaccination against infectious coryza in susceptible chickens is the main strategy for preventing of disease (3)

In the present work, hemagglutination-inhibition (HI) antibody response and protection conferred by an *A. paragallinarum* (strain 221, serovar A-1) vaccine administered by nasal, oral, ocular, and subcutaneous routes were evaluated. Furthermore, induced both serum and tracheal IgG and IgA isotypes were investigated by ELISA. All vaccines contained 109 CFU/mL and chickens were vaccinated in two occasions.

One-hundred percent protection against clinical signs of infectious coryza was conferred by subcutaneous-, ocular-, and nasally-administered vaccines. The highest IgG, IgA, and HI antibody titers were conferred by subcutaneous and nasal vaccines. Nasal vaccine conferred the highest IgA titers in both sera and tracheal washings. A good protection was conferred on chickens by a similar concentrated, *A. paragallinarum* intratracheal administrated vaccine (2).

Obtained results showed that nasal vaccination of susceptible chickens against infectious coryza conferred a solid protection against nasal challenge, likely based on the induction of IgA isotype. Future work is focused on the nasal-subcutaneous combinations for increasing both serum and local antibody titers. This vaccine practice could decrease the handling of layer flocks.

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HEALTH STATUS OF THE HOODED CROW (*CORVUS CORONE CORNIX*) IN NORTHERN ITALY

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SUMMARY

During the last year the health status of 203 hooded crows (*Corvus corone cornix*) was monitored in Northern Italy. Gross and microscopic lesions were found mainly in the gastrointestinal and respiratory tracts (splenomegaly, necrotic hepatitis, and airsacculitis). Bacteriological examination detected *Campylobacter* spp. (13.9%), *Salmonella typhimurium (*9.7%), *Yersinia* spp*.* (5.5%) and *Chlamydophila abortus* and *C. psittaci; Pasteurella multocida* was isolated from six birds with severe prostration. Virological and serological examinations for Newcastle disease virus, avian influenza virus and West Nile disease virus were negative. No blood protozoans were found, but oocysts of *Isospora* spp. in the feces (70%) were frequent. *Dispharynx* spp. in the proventriculus and flagellated protozoans as *Trichomonas* spp. and *Hexamita* spp. were found in the upper digestive tract and adult nematodes as *Diplotriena* spp. in the air sacs. Cestodes were a fairly constant finding in the small intestine.

INTRODUCTION

In the last few years the hooded crow population has been markedly increasing in number so that this species is now widely distributed all over the country. The diffusion of this species is related to its role of scavenger and its ability to living in metropolitan areas. No information exists about the health status of this species in Italy. The purpose of our study was the control of the health status of the hooded crow, which is known as a host for many transmissible diseases of domestic animals and humans.

MATERIAL AND METHODS

The health status of 203 hooded crows from Northern Italy was monitored for one year. Pathological, histological, bacteriological (search of *Salmonella* spp*., Yersinia* spp*., Campylobacter* spp., *Clamydophila* spp.), virological, serological (for avian influenza virus, Newcastle disease virus, West Nile disease virus), and parasitological examinations were performed.

RESULTS AND DISCUSSION

Two-hundred three hooded crows (117 males and 86 females) were examined and 77% of the birds were yearlings.

At external examination the feathers were in good condition; two birds were infected by lice. Ticks and other external parasites were never detected.

Gross and microscopic lesions were found mainly in the gastrointestinal and respiratory tracts and consisted of splenomegaly (5.4%), necrotic hepatitis (4.9%), and airsacculitis (6.4%). No histological lesions were present in the brain.

Campylobacter spp. has been isolated in 13.9 % of the birds; *Yersinia* spp. (5.5%) and *Salmonella typhimurium* (9.7%) were isolated from intestine of healthy birds. *Pasteurella multocida* was isolated from 3% of crows with severe prostration. This infection was probably due to ingestion of domestic geese dead for fowl cholera in the same area. A similar case was reported in Texas in 1981 (1), but the role of the crow as reservoir of *P. multocida* is still uncertain.

C*hlamydophila psittaci* and *C. abortus* were detected in two crows of 10 birds examined.

We found no blood protozoans although oocysts of *Isospora* spp. were frequent in the feces (70%). *Trichomonas* spp. and *Hexamita* spp. were present from 6% of the birds; *Dispharinx* spp. in the proventriculus, *Capillaria* spp. in the small intestine, and *Diplotriaena* spp. in the air sacs were present. *Raillietina* spp. was a fairly constant finding, also in birds in good state of nutrition.

All the birds showed no serological titers for avian influenza virus and Newcastle diseases virus, in contrast with data available in literature (2, 3). In our study no bird positive for West Nile disease virus was found, but in other countries the crow is proven to be a reservoir for this virus (4).

On the basis of our results we can conclude that even in the absence of clinical signs, the hooded crow

can be a source of infectious agents for humans and animal species.

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IMMUNE RESPONSE IN CHICKENS WITH DIFFERENT INFLUENZA VACCINE FORMULAS AND SCHEDULES

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SUMMARY

Although the high pathogenic virus has been absent from commercial poultry in Mexico since 1995, one of the methods used to control the disease is the use of an inactivated vaccine. We wanted to know which formula and schedule provided the best immune response in chickens. We vaccinated 700,000 broilers from a commercial farm. The chickens were bled weekly after vaccination and the immune response was evaluated using HI trial. The result has been processing.

INTRODUCTION

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

MATERIALS AND METHODS

Chickens. 700,000 meat-type chickens (broilers) were used in this experiment; they came from breeders who vaccinated with AIV. The chickens were reared in a commercial farm. All the chickens were bled at seven weeks of age. No chickens showed any respiratory sign before being bled.

Commercial vaccine. It contained two inactivated antigen with mineral oil like adjuvant, one was Newcastle (La Sota) and AIV with the official strain (A/Chicken/Mexico/232/94/) (H5N2).

Experimental vaccine. The vaccine contained antigen of AIV inactivated and aluminum gel like adjuvant.

HI test. This test was performed according with OIE. We used the same strain from the vaccine in this assay.

Vaccination schedule. See Table 1.

RESULTS

See Table 2 for results of Immune response in chickens vaccinated with different AIV schedules.

DISCUSSION

In spite that millions of chickens have been vaccinated with AIV, the low pathogenic AIV is moving in chicken farm, it would be associated with the following aspects:

1. Chickens contact AIV at an early age – sometimes the field virus affects the flock at two weeks of age.
2. The AIV is constantly affecting the poultry areas, so the amount of virus in the environment is too high.
3. The presence of maternal antibodies in the progeny is very important. The majority of the breeders are vaccinated, and so their progeny has humoral antibodies. In the trials that we made we watched the catabolism of the antibodies finish around the fourteenth day of age. It does not matter if the progeny have high or low amounts of antibodies to AIV. The maternal antibodies will block the virus from the vaccine in part, if the vaccine is applied at an early age; the amount of antibodies in these chickens is less compared with chickens with low maternal antibodies at the time of vaccination. The immune response will be appropriate if the vaccine is applied at 10 days of age in chickens with maternal antibodies, we had been proved it.
4. The vaccine schedule is another important aspect. We demonstrated that immune response is significantly affected when we applied two doses of vaccine with a minimum interval between, instead one doses when the maternal immune is almost disappeared, it will probably happen because the antibodies that were produced in the first vaccination will be attached to virus from the second immunization, and so the immune response will be low compare with the chickens that received just one dose of AIV vaccine.

The results found in this study could differ from other researchers because they probably performed their studies in others regions and different farms; moreover, in the immune response, important participatory factors like breeder stock, gender, type of chicken (meat or egg), immunological status of the flock, immune suppression due to diseases, nutritional disorders or mycotoxins, stress level, type of facilities, administration of vaccination (by crew), chickens sampling amount, serum quality (not hemolysis), performance of the HI test (the result would be different between labs), and HA concentration per dose could play a role. Additionally, it is normal that some of the chicks (0 to 14%) will not respond to antigen. This could be named immune system idiosyncrasy.

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ACKNOWLEDGEMENTS

The technical assistance of MVZ. Olga Martinez R. is gratefully acknowledged. The technical information from Gisela Erf. Ph.D. and Robert Webster, Ph.D. is gratefully acknowledged.

Table 1. Vaccination schedule.

Group	Day vaccinated	Type	Dose (mL)
1	10	Oil	0.5
2	10	Oil	0.5
2	18	Oil	0.5
3	10	Oil	0.5
3	18	OH3	0.5
4	10	OH3	0.5
5	10	OH3	1.0
5	18	Oil	0.5
6	10	OH3	0.5
6	18	Oil	0.5
7	Control	Control	Control

Table 2. Immune response in chickens vaccinated with different AIV schedules.

Group	M.G (log^{10})
1	1.86
2	0.84
3	0.78
4	1.84
5	1.54
6	1.34
7	0.00

THE CHALLENGE TRIAL – A GOLD STANDARD TEST TO EVALUATE THE IMMUNE RESPONSE IN LAYERS VACCINATED WITH *AVIBACTERIUM* (*HAEMOPHILUS*) *PARAGALLINARUM*

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SUMMARY

Infectious coryza is an upper respiratory tract disease of chickens with the major impact occurring in multi-age flocks. We investigated whether layers vaccinated and challenged with *Avibacterium* (*Haemophilus*) *paragallinarum*, despite lacking detectable serum antibodies, would resist the challenge. We used a commercial and an experimental bacterin to immunize layers and compared hemagglutination inhibition (HI) antibodies and protection. We found that, despite the absence of detectable HI antibodies, chickens were protected. We suggest that the vaccination-challenge trial is the gold standard for the evaluation of the immune response to infectious coryza vaccines.

INTRODUCTION

Infectious coryza is an upper respiratory tract disease of chickens that can appear in all ages of chickens with the major impact occurring in multi-age flocks. The economic impact of the disease in well-managed flocks tends to be due to an increased culling rate and a reduction in egg production (10%–40%) (1).

The disease can have a much greater impact than the relatively simple scenario described above. As an example, a recent outbreak of the disease in older layer birds in California, which was not associated with any other pathogen, caused a total mortality of 48% and a drop in egg production from 75% to 15.7% over a three-week period (5).

The causative agent of infectious coryza is the bacterium *Avibacterium* (*Haemophilus*) *paragallinarum* (2). This organism has traditionally been regarded as requiring nicotinamide adenine dinucleotide (NAD, V factor), but not hemin (X factor), for growth in vitro (1). Two serotyping schemes exist: 1) the Page scheme which recognizes serovars A, B and C (Page, 1962) and 2) the Kume scheme which recognizes serogroups A, B and C with four serovars each within serogroups A and C (3).

The hemagglutinin antigen of *Av. paragallinarum* plays a key role in serotyping, immunity and pathogenicity (1). For Page serovar A organisms, there is a reported close correlation between HI titer and both protection (9, 11) and clearance of the organism from the nostrils of vaccinated chickens (10). Purified hemagglutinin antigen from a serovar A organism has been shown to induce protective immunity (7). For both serovar A and serovar C the assessment of mutants lacking hemagglutination activity has shown that the hemagglutinin antigen plays a key role in colonization (13, 14).

However, the only fully characterized hemagglutinin gene from *Av. paragallinarum* to date has not varied between the different serovars (6), suggesting that this gene – the *hagA* gene – may not be the serotypic specific hemagglutinin.

In Mexico disease associated with *Av. paragallinarum* is enzootic, occurring mainly in densely populated egg-type chickens zones. Vaccines against *Av. paragallinarum* are widely used and are often evaluated by the challenge test. The aim of this study was to see if we could show a correlation between HI antibodies and protection as defined in a vaccination-challenge trial.

MATERIALS AND METHODS

Bacteria. The *Av. paragallinarum* strain (Hpg6) used in this study was originally isolated from a Mexican chicken and has been shown to belong to Kume serovar A1.

Experimental vaccine. This vaccine was monovalent and included the Mexican Hpg6 serovar A1 strain. The strain was grown overnight in a non-commercial medium that contained peptones, dextrose, thiamine, starch, sodium chloride, 0.0025% NAD, and 3% filter-sterilized heat-inactivated chicken serum.

The vaccine contained 1x $10^{8.6}$ CFU/mL. The bacteria were inactivated with thimerosal. The vaccine used a mineral oil like adjuvant.

Commercial vaccine. This product contained serovars A, B, and C of *Av. paragallinarum* and used a mineral oil like adjuvant.

Experimental groups. All chickens used in this study were healthy layer chickens obtained from a commercial farm known to be *Mycoplasma gallisepticum* and *M. synoviae* free. Group 1, which consisted of 70 chickens, was vaccinated at four weeks of age with the above experimental vaccine. Group 2, which consisted of 30 chickens, was vaccinated at four weeks of age with the above commercial vaccine. All the chickens were vaccinated intramuscularly with 0.5 mL of suitable vaccine. Group 3, which consisted of 15 chickens, were unvaccinated controls.

All chickens showed no hemagglutination-inhibition antibodies to *Av. paragallinarum* before being vaccinated. The chickens were placed on a commercial chicken farm. No chickens showed any respiratory sign before the challenge trial.

HI test. Hemagglutinating antigen with neither enzymatic nor chemical treatment was used and was prepared as previously described (3, 4). The HI test was essentially performed as described by Blackall *et al.* (3, 4) except that the chicken sera were tested at dilutions from 1/5 to 1/160. All chicken sera were absorbed prior to HI testing by diluting 1/5 with 10% fresh chicken RBCs. The serum/RBC mix was held at room temperature for three hours and then 4^{o}C overnight. The suspension was then centrifuged and the supernatant retained and regarded as a 1/5 serum dilution.

Challenge methods. When the birds were 15 weeks of age, the three groups were challenged by the intrasinus route with 0.1 mL of an overnight broth culture of *Av. paragallinarum* strain Hpg6 containing 1x10^{6} colony-forming units per milliliter. Clinical signs of infectious coryza were recorded from the first to the seven days after inoculation. The presence and degree of any nasal discharge and facial swelling in the challenged chickens were recorded. Seven days after inoculation, the chickens were euthanized, and the presence of the challenge organism was investigated by swabbing both infraorbital sinuses with a sterile cotton swab subsequently streaked onto a blood agar plate that was cross-streaked with a nurse colony of *Staphylococcus epidermidis*. A protected chicken was defined as one showing no clinical signs, no gross lesions at post-mortem, and failed to yield any challenge organism on culture of the sinuses.

RESULTS

The results of both the HI testing and the vaccination/challenge trial are shown in Table 1.

DISCUSSION

Infectious coryza is generally under control in Mexico, with the majority of commercial vaccines containing Page serovars A, B and C of *Av. paragallinarum*. The common vaccination schedule in Mexico involves a minimum of two doses, all given before the start of egg production. Often, the serological response to a number of the routine vaccines used in layers is checked, e.g. Newcastle and IBV.

We felt it important to check if an HI assay would be appropriate to evaluate the immune response of coryza-vaccinated chickens. Despite failing to detect any HI antibodies, 70% of the chickens vaccinated with the experimental vaccine were protected while 90% of the chickens given the commercial vaccine were protected. We believe that the difference in protection levels between the two vaccines could be due to the amount of antigen that the commercial vaccine contains. As the commercial vaccine contains three strains (to cover all three Page serovars), it is possible that the higher overall antigenic load – combined with some cross-serovar protective antigens – may have given a better level of protection. In contrast, the experimental vaccine contained just the local Kume serovar A1 strain.

In comparing this work with prior studies on HI antibodies in chickens, it is important to note that a range of different HI tests have been used in the literature. Hence, comparisons with our work must be done with some care as it is possible that different HI tests may have different sensitivities. At this stage, there is still no universally accepted HI methodology for detection of antibodies to *Av. Paragallinarum*.

The protective antigens of *H. paragallinarum* have not been definitively identified. While many studies emphasize the role of the HA antigens, it has also been suggested that the capsule of *Av. paragallinarum* contains protective antigens (8). Using both a Page serovar A and C strain, a crude polysaccharide extract was shown to provide serovar-specific protection (8).

It is possible that antibodies other than those targeting HA antigens can also provide protection to vaccinated chickens. Certainly, our work has shown that vaccines that fail to elicit a HI antibody response in the HI methodology we have used can still be effective vaccines.

With this finding, we conclude that challenge test is the gold standard test to check the immune response in chickens that have been vaccinated with a single dose of an infectious coryza vaccine.

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Table 1. HI antibody titer and protection of chickens vaccinated with *Av. paragallinarum* and challenged.

	A1	
Vaccine	GM* (log10)	Protection (%)
Experimental	0.00	70
Commercial	0.00	90
Control	0.00	0

*Geometric mean

IN VITRO SUSCEPTIBILITY OF THREE RECENTLY ISOLATED ARTHROPATHIC *MYCOPLASMA SYNOVIAE* STRAINS FOR ENROFLOXACIN, DIFLOXACIN, DOXYCYCLIN, TYLOSIN AND TILMICOSIN ANALYZED WITH THE MICRO-BROTH DILUTION TEST

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INTRODUCTION

In the Netherlands clinical relevant isolates were not detected for decades until recently when arthropathic and amyloid-inducing strains were observed (3). Similarly, in Australia arthropathic *M. synoviae* isolates were not described until 1990 (5), suggesting that the strains present before in both countries did not have the potential to induce infectious synovitis.

At the same time as outbreaks of arthropathic and amyloid-inducing *M. synoviae* strains were found in brown layers, an increase in arthritis cases were reported in turkeys. A recent field survey (7) showed that in some of these cases *M. synoviae* could be isolated from affected joints. Similar joint lesions were induced experimentally in young turkey poults with *M. synoviae* isolates obtained from spontaneous cases (4).

Economic losses caused by arthropathic *M. synoviae* strains can be reduced by means of antibiotic treatment at the beginning of infection. Knowledge of the antibiotic sensitivity profiles of the mycoplasma strain involved can be of great value in order to maximize the efficacy of the treatment. Here we report on the *in vitro* susceptibility of three recently obtained arthropathic *Mycoplasma synoviae* isolates for enrofloxacin, difloxacin, doxycyclin, tylosin and tilmicosin.

MATERIALS AND METHODS

Mycoplasma strains. Two strains were isolated from arthritic joints of commercial meat turkeys in 2001 and 2002, respectively. Another strain originated from arthritic joints of commercial layers (year 2000). The *M. synoviae* ATCC 25204 (WVU1853) dating from 1996 was included as a control strain. The susceptibility was tested quantitatively with the micro-broth dilution test according to Hannan (1,2).

Identification and concentration of *M. synoviae* cultures. Frozen *M. synoviae* stock was thawed and 10 decimal dilutions were made in ME broth. Tubes were

incubated for 14 days at 37°C. Concentration was calculated as color changing units (CCU) per mL. As inoculum a concentration of 10^{3-5} CCU/mL was obtained.

Influence of antibiotic solvents on pH and mycoplasma growth. The influence of antibiotic solvents on the pH and mycoplasma growth was assessed. Corrections were not necessary.

Preparation of microtiter plates. Per antibiotic five microtiter plates were filled with 50 µL ME broth per cup in column 2 through 12. Cup A1, C1, E1 and G1 were filled with 100 µL antibiotic solution (64 µg/mL). Cup B1, D1, F1 and H1 received 100 µL antibiotic solvent (rows B, D, F and H are for growth control). Dilutions were made by mixing 50 µL of the first column with the second, then from the second to third and so forth. Plates were sealed and stored until use at -80°C.

Assessment of the quality of microtiter plates. The efficacy of antibiotic solutions in ME broth was assessed by testing them against *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213. The MIC values obtained for both microorganisms were compared to known NCCLS values for both species of bacteria.

Starting the micro-broth dilution test. For each strain two rows (antibiotic and growth control series) were inoculated with 50 µL cultured broth inoculum and incubated at 37°C for two weeks. Final antibiotic concentrations were 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.06, 0.03 and 0.015 µg/mL per column.

Assessment of the MIC. MIC = lowest antibiotic concentration without change of color (red to yellow). Plates were read at day 1, 2, 3, 4, 7, 8, 9 and 14 of incubation (at 37°C). MIC values of day 7 and 14 are reported.

Breakpoints. For doxycyclin, enrofloxacin and tylosin the breakpoints proposed by Hannan *et al*., (1) were used (µg/mL):

Antibiotic	Sensitive	Intermediate	Resistant
Oxytetracycline	≤4	8	≥16
Enrofloxacin	≤0.5	1	≥2
Tylosin	≤8	-	≥16

For difloxacin and tilmicosin the NCCLS breakpoints were used:

Antibiotic	Sensitive	Intermediate	Resistant
Difloxacin	≤0.5	1-2	≥4
Tilmicosin	≤8	16	≥32

RESULTS

The MIC results obtained for *M. synoviae* have been summarized in the Table 1.

DISCUSSION

The inocula used had a concentration ranging from 10^3 to 10^5 CCU/mL, which meets the concentration proposed by Hannan.

The MIC results obtained for the *E. coli* and *S. aureus* ATCC strains were within the range of the corresponding NCCLS data (data not shown), suggesting that the quality of the plates was satisfactory for all five antibiotics.

After thawing the plates no change in broth color due to possible pH changes was observed. The change in color observed in the control rows was therefore attributed to mycoplasma growth.

The MIC obtained after an incubation period of seven days was reported because the MIC data of the ATCC strain corresponding to that data show the best agreement with MIC values published before (2).

All strains are sensitive for enrofloxacin, doxycyclin, tylosin and tilmicosin, except the chicken strain, which is resistant to enrofloxacin. One turkey strain is sensitive for difloxacin, while the remaining strains are intermediate to it.

As specific breakpoints for difloxacin are not known (NCCLS data were used) and bearing in mind that it is as enrofloxacin a quinolone, we speculate that the ATCC strain and the turkey strain from 2001 will probably be sensitive for difloxacin as their MIC does not change with time. The chicken strain is probably resistant for this compound.

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Table 1. MIC results obtained for *M. synoviae*.

Strain/isolate	Antibiotic	MIC at day 7/14 (µg/mL)	Profile
ATCC	Enrofloxacin	0.5/1	S/I
Chicken 2000	Enrofloxacin	4/16	R/R
Turkey 2001	Enrofloxacin	0.5/1	S/I
Turkey 2002	Enrofloxacin	0.25/0.25	S/S
ATCC	Difloxacin	1/1	I/I
Chicken 2000	Difloxacin	2/4	I/R
Turkey 2001	Difloxacin	1/1	I/I
Turkey 2002	Difloxacin	0.5/0.5	S/S
ATCC	Doxycyclin	0.125/0.25	S/S
Chicken 2000	Doxycyclin	0.125/0.25	S/S
Turkey 2001	Doxycyclin	0.06/0.125	S/S
Turkey 2002	Doxycyclin	≤0.015/0.0125	S/S
ATCC	Tylosin	≤0.015/≤0.015	S/S
Chicken 2000	Tylosin	≤0.015/≤0.015	S/S
Turkey 2001	Tylosin	≤0.015/≤0.015	S/S
Turkey 2002	Tylosin	≤0.015/≤0.015	S/S
ATCC	Tilmicosin	0.5/0.5	S/S
Chicken 2000	Tilmicosin	0.25/0.25	S/S
Turkey 2001	Tilmicosin	0.25/0.25	S/S
Turkey 2002	Tilmicosin	0.125/0.06	S/S

EXPERIMENTAL INDUCTION OF *MYCOPLASMA SYNOVIAE* ARTHRITIS IN TURKEYS WITH FIELD ISOLATES ORIGINATING FROM TURKEYS AND CHICKENS

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INTRODUCTION

Mycoplasma synoviae has been identified as the causal agent of infectious synovitis (4), however it has also been associated with respiratory disease, especially airsacculitis.

Mycoplasma isolates from airsac lesions tend to induce airsacculitis, while those originating from joint lesions are prone to cause synovitis and arthritis (1). How the difference in tropism is dictated, is currently unknown. In some countries like the United States of America respiratory and synovitis strains alternated each other dominating in the field for periods as long as 10 to 20 years. In the Netherlands clinically relevant isolates were not detected for decades until recently when arthropathic and amyloid-inducing strains were observed (2). Similarly, in Australia arthropathic *M. synoviae* isolates were not described until 1990 (3), suggesting that the strains present before in both countries did not have the potential to induce infectious synovitis.

At the same time as outbreaks of arthropathic and amyloid-inducing *M. synoviae* strains were found in brown layers, an increase in arthritis cases were reported in turkeys. A recent field survey (5) showed that in some of these cases *M. synoviae* could be isolated from affected joints. The arthropathogenic nature of both a chicken and a turkey *M. synoviae* isolate was evaluated in turkeys.

MATERIALS AND METHODS

Ninety six turkeys, six-weeks of age, were obtained from a commercial farm free of *M. gallisepticum* and *M. synoviae* infections. Control of the mycoplasma free status was performed by means of serology and PCR of trachea swabs.

The birds were divided in three experimental groups:

Negative control group (n = 24)

Infected with *M. synoviae* chicken isolate (36)

Infected with *M. synoviae* turkey isolate (36)

The birds were inoculated intravenously at eight weeks of age. Half of group 2 received a second inoculum four weeks later.

The turkeys were housed in floors pens at a stock density of 1.17 birds/m^2, each group in a separate room. Water and feed (a commercial turkey pellet) were provided *ad libitum* and a lighting scheme of 16 hours light and 8 of dark was applied.

Birds of group 2 were first inoculated with 10^5 cfu *M. synoviae* (chicken/NL/Dev/801979Rob/00). This strain originated from brown layers with joint lesions (2). Half of the birds that were inoculated for the second time received 10^8 cfu of the same strain. The third group was inoculated with 10^8 cfu *M. synoviae* (turkey/NL/Dev/SP1240/01) originating from turkeys with joint pathology (5). Preparation of the inocula and control of the mycoplasma concentration was performed as described (2).

The birds were subjected to post-mortem at nineteen-weeks of age. Blood samples were collected for serology. Routine bacteriology and mycoplasma culture of joint specimens was performed. Finally, affected joints were fixed in formalin for histopathology (hematoxylin & eosin stain).

The occurrence of microscopic joint lesions was assessed semi-quantitatively:

0 = 90-95% of the tissue samples is not altered. In the remaining area scant free lying plasma cells and/or granulocytes can be found. In some cases hypertrophy of the synovial membrane is noted and/or a few hemorrhages, which based on morphology are attributed to mechanical stress/damage.

1 = in a larger area as 0 diffuse infiltrates of plasma cells and granulocytes.

2 = in a large part of the tissue sample confluent areas with plasma cells and granulocytes are found. The synovial membrane is altered.

3 = as 2 including lymph follicles and/or (fibrino) purulent exudate.

4 = the lesions described in 3 are found in the whole tissue section, while (fibrino) purulent exudate is noted in the articular pouches.

Samples for bacteriology and histology of the negative control group were only taken from approx. half of the birds.

The overall effect of treatments on the occurrence of joint lesions was assessed with the Chi-Square test. Follow up Chi-Square tests were performed to assess

which pairs of experimental groups differed significantly. Bonferroni's correction developed by Keppel was used to compensate for the alpha inflation. Yates correction was applied for cells with frequency <5%, in other instances Pearson Chi-Square was used. Significant differences in lesion scores between treatment groups were assessed with Kruskal-Wallis test and Dunn's procedure as *post hoc* test.

RESULTS

Excessive synovial fluid with a clear aspect was found in 10/24 negative control birds. In some cases this fluid had a hemorrhagic appearance (6/24). Turkeys inoculated twice with the chicken strain showed a moderate to severe increase of synovial fluid (17/18). In half of the birds the synovial fluid had a whitish aspect, in one it was hemorrhagic, in four clear, and the remaining birds that died before termination of the experiment it was not assessed. Turkeys inoculated once with the chicken strain showed increased synovial fluid in 16/17 of cases. The synovial fluid had a whitish aspect in three birds, hemorrhagic in five and clear in eight birds. Birds inoculated with the turkey isolate had excessive synovial fluid in 32/36 cases. The aspect of the fluid was whitish in six turkeys, hemorrhagic in 11 and clear in 15.

All birds of group 2 inoculated twice with *M. synoviae* showed synovitis and arthritis (18/18) and had an average lesion score of 3.7. The remaining birds of that group had an average lesion score of 2.5, while 8/17 (47%) turkeys had confirmed synovitis. The rate of synovitis and arthritis in group 3 was 15/36 (42%), with an average lesion score of 3.4. Three control birds showed minor inflammatory changes (lesion score 1) in their joint tissue. Two birds of group 2 inoculated once and eight birds of group 3 with minor inflammatory changes (lesion score 1) similar to those found in the negative control group, were not included as arthritic/synovitis birds.

Antibodies against *M. synoviae* were only detected in the groups exposed to this microorganism. All birds inoculated twice with the chicken strain showed positive in the rapid agglutination test (average $\log_2$ titer 3.7). Birds inoculated once showed antibodies in 44% (8/18) of cases (average $\log_2$ titer 2.5). Turkeys inoculated with the turkey isolate showed 71% (24/34) positives (average $\log_2$ titer 2.1).

The routine bacteriological analysis of joint specimens of all groups gave negative results. The mycoplasma culture of joint specimens from group 1, showed a similar outcome. In contrast, *M. synoviae* was reisolated from 2/36 (5.5%) turkeys inoculated with the turkey isolate and from 11/36 (30.6%) of birds inoculated with the chicken strain. Most reisolates from the latter group (10/36 (28%)) originated form birds that had been inoculated with the second higher dose.

When comparing the rate of arthritis between experimental groups only the group inoculated with the higher dose of the chicken isolate was significantly different from all others (Chi-Square $P \leq 0.01$). The same result was obtained after analyzing the microscopic arthritis score (Kruskal-Wallis test $P = 0.000$ and Dunn's procedure $Z > 2.635$) and the *M. synoviae* isolation rates from joints (Chi-Square $P \leq 0.006$).

DISCUSSION

Both, the *M. synoviae* chicken strain and the turkey isolate induced severe joint disease in turkey poults after intravenous inoculation. Respiratory symptoms were not observed during the experiment, nor were airsacculitis lesions found at post-mortem.

Remarkable was the frequent occurrence of overfilled joints with clear or hemorrhagic synovial fluid in negative control birds at macroscopy. These lesions were confirmed at histology as minor inflammatory changes only in three birds with hemorrhagic synovial fluid. Moreover, in a number of other birds of this and the other experimental groups villus formation and/or a few bleedings were found, which based on their morphology could be related to mechanical stress, microtrauma or microangiopathy. Seemingly, mild macroscopic lesions suggestive of arthritis, which seem aseptic judging by the results of histopathology, do occur in a number of non infected and other ways apparently healthy birds. Therefore birds with similar mild lesions and negative mycoplasma culture from joint tissue in other experimental groups were regarded as non-arthritic from the septic point of view. In contrast, the occurrence of moderate to severe accumulation of whitish synovial fluid correlated well with severe microscopic lesions of synovitis/arthritis.

The joint lesions were mostly symmetric, the hock joints being affected in most cases.

The occurrence of a dose-effect was suggested by the results of group 2. Turkeys inoculated the second time with the higher dose showed a higher rate of synovitis/arthritis. Moreover, the lesions were more severe. Nevertheless, a synergistic effect with the first inoculum that was given four weeks earlier can not be ruled out. This makes it difficult to assess differences in virulence between both isolates. However, if group 3 is compared with the birds that received the low dose of the chicken strain, the latter seems to induce the same degree of synovitis/arthritis as the high dose of the turkey isolate, and must therefore be more virulent.

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A COMPARISON OF PERFORMANCE AND THE ANTICOCCIDIAL EFFICACY OF CLOPIDOL TO VARIOUS COMMERCIAL IN-FEED ANTICOCCIDIALS IN BROILER CHICKENS

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SUMMARY

A floor pen study was conducted with broiler chickens to compare performance and the anticoccidial efficacy of clopidol to various commercial in-feed anticoccidials. The challenge inoculum caused a significant effect on performance and high coccidial lesion scores in the unmedicated infected birds. As observed by the average lesion score of 1.4, the coccidial field isolates were slightly resistant to diclazuril. Performance and coccidiosis control were significantly improved by feeding clopidol, nicarbazine, or diclazuril in the starter feed versus a straight salinomycin program. The clopidol and nicarbazine shuttle programs had very similar results and had equal performance compared to the noninfected birds. No significant difference in performance was observed feeding either salinomycin or monensin in the grower feeds. A clopidol starter feed shuttle program should provide equal anticoccidial protection and similar performance to a nicarbazine shuttle program.

INTRODUCTION

Coyden (clopidol) was approved in the USA as a feed additive at 125 ppm of chickens in 1968. Stock (5) and Reid (4) both in 1967 demonstrated that clopidol was very effective against all species of *Eimeria* of chickens. Mathis and McDougald in 1982 (3) examined coccidial field isolates and found that 50% of 37 isolates were resistant to clopidol. Clopidol was last available in the USA in 1989. Mathis and Cope in 2004 (2) examined coccidial field isolates and found that 100% of all *E. acervulina*, *E. maxima*, and *E. tenella* strains/isolates tested were very sensitive to clopidol 125 ppm. The objective of this study was to evaluate the anticoccidial efficacy and related performance of clopidol in the starter feed followed by either monensin or salinomycin in the grower feed using a coccidiosis challenged broiler floor pen study to simulate commercial conditions. The performance of broilers fed diclazuril or nicarbazine in the starter feeds was also compared.

MATERIALS AND METHODS

The 42 day floor pen study consisted of 72 pens starting with 25 male and 25 female broiler chickens per pen. All pens had approximately four inches (10 cm) of fresh pine shavings with the initial stocking density being 0.77 ft^2 (715 cm^2) per bird. The treatments were replicated in eight blocks, randomized within blocks. The treatments were: unmedicated, noninfected; unmedicated, infected; clopidol 125 ppm starter to monensin 99 ppm grower; infected; clopidol 125 ppm starter to salinomycin 55 ppm grower, infected; Nicarb 125 ppm starter to monensin 99 ppm grower, infected;

Nicarb 125 ppm starter to salinomycin 55 ppm grower, infected; diclazuril 1 ppm starter to monensin 99 ppm grower, infected; diclazuril 1 ppm starter to salinomycin 55 ppm grower, infected; and salinomycin 44 ppm starter to salinomycin 55 ppm grower, infected. Finisher feeds did not contain any anticoccidial. Birds received feed appropriate to the treatment from day 0 to day 42. A change from starter to grower in which all previous feed was removed and weighed occurred on day 21. Grower diet was fed from day 21 to day 35. Finisher diet was fed from day 35 to day 42. Feed consumption was measured for each feed type. All feeds contained BMD 50g/ton. On day 15 all birds, except Treatment 1, were exposed to field strains of *E. acervulina*, *E. maxima*, and *E. tenella*. Bird weights (kg) by pen were recorded at days 0, 15, 21, 35, and 42. On days 21 (six days post challenge) and 42, five pre-selected birds from each pen were examined for the degree of coccidial lesions using the system of Johnson and Reid (1970) (1), wherein 0 is normal and 1, 2, 3, or 4 indicate increasing severity of infection.

RESULTS AND DISCUSSION

A significant weight reduction and increased feed conversion was observed on days 21, 35, and 42; with an average of 2.4 coccidial lesion score in the unmedicated, infected birds. As observed by the average lesion score of 1.4, the coccidial field isolates were slightly resistant to diclazuril. Performance and coccidiosis control were significantly improved by feeding clopidol, nicarbazine, or diclazuril in the starter feed versus a straight salinomycin program. The clopidol and nicarbazine shuttle programs had very similar results and had equal performance compared to the noninfected birds. No significant difference in performance was observed feeding either salinomycin or monensin in the grower feeds. This study shows that clopidol is a highly effective anticoccidial. A clopidol starter feed shuttle program should provide equal anticoccidial protection and similar performance to a nicarbazine shuttle program.

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EFFECTS OF INOCULATION SITE ON INFLUENZA A SUBTYPE H9N2 ON IMMUNE RESPONSE

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SUMMARY

In order to study effects site of inoculation, two groups of broiler chicks were inoculated with influenza vaccine subtype H9N2 at subcutaneous of the neck back and in the breast muscle respectively. The results showed that the antibody titer against AI vaccines induced by the subcutaneous route was better than the intramuscular route of injection, and the difference was statistically significant ($P<0.05$).

INTRODUCTION

Inactivated influenza virus vaccines have been used in a variety of avian species, and their effectiveness in preventing clinical signs and mortality is different. However, protection is virus subtype specific. Various inoculation sites have been used in chickens because to make the inoculation procedure easier and produce better protection against disease. The objective of this study was to evaluate the effects of inoculation site of AI vaccine on immune response of broiler chicks.

EXPERIMENTAL DESIGN

One hundred fifty day-old broiler chicks were divided into three equal groups of 50 each. Group A and B chicks were vaccinated with AI oil-emulsion vaccine by neck subcutaneous and breast muscle routes respectively at eight days of age. Group C chicks were kept as unvaccinated control group. Blood samples were collected randomly from five chicks of each group by wing vein at 7,14,21,28, and 35 days after vaccination. The specific antibody titer was determined by routine hemagglutination inhibition (HI) test.

RESULTS

The results showed that mean geometric titer of groups A and B chicks was increased from two weeks post vaccination (PV) and reached maximum at four weeks PV.

DISCUSSION

Various attempts have been made in recent years to increase maintenance of level of antibody titer by using different site of inoculation. Except for the difference in level of antibody response between the two routes, each site has some advantages and disadvantages (3). For example, injection in breast muscle is quick but trimming of vaccination lesions has reduced the popularity of the procedure (1). Subcutaneous injection at the back of the neck can result in serious neurological and/or musculoskeletal problems if done improperly (2,4). Injection under the skin can be more expensive, since it may necessitate one person to hold the bird and another to administer the vaccine, where as injection in the breast can be done by one person, who also holds the bird (2,4).

Antibody response induced by the subcutaneous route of injection was better than that induced by the intramuscular route of injection, and the difference was statistically significant ($P<0.05$). The results were correlated with study of some workers (3,4). Levy *et al*. showed that chickens immunized subcutaneously in the neck region responded better than those immunized intramuscularly (1, 5).

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Table1. Geometric mean titer (log 2) of HI test to avian influenza vaccine.

	Days after vaccination				
Groups	7	14	21	28	35
A	3.00	3.06	4.14	4.92	3.60
B	3.00	2.50	3.15	4.61	2.62
C	3.00	2.50	1.00	0.50	0.12

DIGIT NECROSIS ASSOCIATED WITH SCALY-LEG MITE INFESTATION IN BACKYARD CHICKENS

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ABSTRACT

Scaly-leg mite infestation has been reported in backyard chicken flocks. *Knemidocoptes mutans* is a species of mite infestation in a backyard flock of chickens which resulted in digit necrosis in some birds.

Histologically, the necrotic toes demonstrated *Knemidocoptes mutans* in the stratum corneum. This case documents the association of scaly-leg mite infestation with that of digit necrosis. Ivermectin treatment prevented new case development and reduced the severity of affected birds.

MOLECULAR CHARACTERIZATION OF FOWL ADENOVIRUSES FROM FTA® –LIVER IMPRESSIONS

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ABSTRACT

Inclusion body hepatitis (IBH) is a viral disease caused by adenovirus group I including all 12 serotypes which are distributed worldwide. Identification of IBH can be done by growing the virus in tissue culture, by serology, and more recently by molecular techniques.

The use of FTA® paper as a medium to transport nucleic acid for virus identification has revolutionized the sampling of avian specimens for molecular analysis (1). FTA filter paper (Whatman) is a proprietary cellulose membrane containing lyophilized reagents that lyses eukaryotic and prokaryotic organisms. We have demonstrated that adenovirus serotype 1 spotted on FTA paper is inactivated as shown by lack of cytopathic effects on chicken liver cells in culture. However detection of the virus was still possible by PCR using primers that hybridize conserved regions of the hexone genes of all 12 adenovirus serotypes. Adenovirus 1 was detected by PCR after eight months of storage on FTA at room temperature indicating that the integrity of DNA was not affected by those conditions. Moreover FTA-liver impressions obtained from chickens challenged with adenoviruses European serotype 1, 4, 8, and 11 proved to be a reliable method of sampling clinical specimens for viruses at least within the first three to five days post inoculation as indicated by positive PCR results in the study. Nucleotide sequencing analysis of the amplified products agreed 100% with the serotype of the challenging virus. This is the first report of adenovirus detection by PCR performed directly on liver impression onto FTA cards.

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COMPARISON OF BURSAL HISTOLOGIC LESIONS CAUSED BY DIFFERENT ISOLATES OF TURKEY REOVIRUS

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ABSTRACT

In this study, the pathogenesis of four turkey origin reovirus (TRV) isolates was evaluated. These TRV isolates were collected from turkey flocks experiencing poult enteritis and are genetically distinct from known avian reoviruses (3,8). Specific pathogen free (SPF) poults were inoculated at three days post-hatch with approximately $10^{4.0}TCID_{50}$/bird by the intra-tracheal and intra-oral routes with one of the following four TRV isolates: NC/SEP-R44/03 (10), NC/98 (3), NC/85, and TX/98 (8). Bursa, thymus, liver, spleen, heart, pancreas, duodenum, jejunum, and ceca from each bird were collected at two, five, seven, and nine days post inoculation (dpi) for histopathologic examination. Viral antigen was detected in tissues by immunohistochemical staining (IHC) using turkey convalescent sera as primary antibody. Apoptosis in tissues was also evaluated by IHC.

The most important lesion associated with TRV infection in poults occurred in the bursa. All TRVs studied induced different degrees of bursal atrophy characterized by moderate to severe lymphoid depletion and increased fibroplasia between the bursal follicles. Immunohistochemical staining revealed viral antigen in macrophages in the bursal interstitium and the follicular areas of the bursa and spleen, and in the bursal epithelial cells. Viral staining was strongly positive at five dpi, and lymphocyte depletion was evident at five, seven, and nine dpi. Increased apoptosis of bursal lymphocytes was observed at five dpi. Isolate NC/SEP-R44/03 induced particularly severe lesions in the bursa, and multiple intrafollicular cysts were present at seven and nine dpi.

The damage present in the bursa would most likely result in immune dysfunction in poults infected with TRV. The immunosuppressive activity of chicken reoviruses in chickens has been addressed by several authors and it has been demonstrated that reovirus causes atrophy of the bursa of Fabricius due to lymphocyte depletion (1,4,5,6,9). As in a previous study of reovirus infection in psittacines (7), viral antigen was observed in bursal epithelial cells and macrophages suggesting that the lymphocyte depleting action of TRV is mediated by chemical soluble factors released by macrophages and not directly related to viral infection of lymphocytes. This indirect mechanism of lymphoid depletion has also been suggested for infectious bursal disease virus (2).

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MORPHOLOGY OF *BLASTOCYSTIS* IN THE INTESTINE OF TURKEYS

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ABSTRACT

Blastocystis is a water-borne, ubiquitous parasite that can be found in the intestinal tract of various animals including mammals (humans, primates, rodents, cattle, pigs, etc.), birds, reptiles, fishes, and amphibians. *Blastocystis* has also been isolated from insects. *B. hominis* in humans is becoming increasingly associated with gastrointestinal disease. Among birds, *Blastocystis* has been described in chickens (*B. galli*), turkeys (*M. gallopava*), geese (*B. anseri*), ducks (*B. anatis*), guinea fowl (*B. meleagris*), pheasants, quail, red-legged partridge, grey partridge, peafowl and ostriches.

Blastocystis is a pleomorphic organism. Isolates from humans and animals can range in size from 3 - 200 µm and appear in various forms including vacuolar, granular and cystic. Other forms such as avacuolar, multivacuolar and cells containing filament-like inclusions occur less frequently. *Blastocystis* ranges from round and oval to ellipsoid and ameboid.

The vacuolar form is the predominant cell type which can vary widely in size but averages, 4 -15 µm in diameter. It contains a characteristic large vacuole and a thin rim of peripheral cytoplasm. The nucleus and organelles including Golgi apparatus and mitochondrion-like structures are located within the cytoplasmic rim. The granular form is identical to the vacuolar form except that granules are observed in the cytoplasm or, more commonly, in the central vacuole. Granules may be myelin-like inclusions, small vesicles, crystalline granules or lipid droplets. The amoeboid form may have one or two pseudopodia. The cyst form is generally smaller in size (2 – 5 µm) than the other forms and is surrounded by a thick multi-layered cyst wall. It lacks the large central vacuole but internal contents include one to four nuclei, multiple vacuoles and glycogen and lipid deposits. A loose fibrillar layer may surround the cysts. Cysts are resistant to lysis by water and can survive for long periods at room temperature.

Blastocystis can be isolated from feces in various media and can be identified in wet smears of feces using bright field or differential interference contrast optics. Air-dried or methanol-fixed fecal smears stained with Giemsa, PAS, Wright's, trichrome, Loeffler's methylene blue or iron hematoxylin are useful in confirming *Blastocystis*. Transmission electron microscopy also can be helpful in identifying the organism. Interestingly, *Blastocystis* has not been described in the intestinal lumen of birds or animals on hematoxylin and eosin (H & E) stained sections of the intestine.

In this study the morphology of *Blastocystis* in turkeys was determined by wet and Wright's, Giemsa, and PAS stained smears of cecal contents from five turkeys. Prospective and retrospective histopathologic studies of ceca from 65 cases of turkeys, two days to 15 weeks of age and stained by H & E, Giemsa, and PAS were also undertaken. Cytology of the wet smears and smears stained by different methods revealed primarily vacuolar forms of *Blastocystis* ranging in size from 20 to 50 µm in diameter, mostly round with a large vacuole surrounded by a thin rim of cytoplasm. Nuclei and vacuoles were evident within the cytoplasm. A few multi vacuolar forms were also observed. Wright's stain was better than Giemsa and PAS. H & E sections of the ceca revealed primarily large round to oval or somewhat irregular faintly staining basophilic organisms measuring 15 to 50 µm in diameter in the lumen of the ceca. Often these organisms had an eccentrically placed round to oval reddish body and a single large vacuole or small multiple vacuoles or vesicles. By Giemsa the organisms stained blue and the material within the vacuole faintly brick red or pink. By PAS the organisms appeared deep pink with no vacuoles or appeared faintly pink with single or multiple vacuoles. The periphery and the thin rim of cytoplasm of the organisms appeared faintly blue while the material within the vacuoles stained pink.

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EFFECT OF GENTA-MEX® (GENTAMICIN SULFATE) ON MAREK'S DISEASE VACCINE

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SUMMARY

Two studies were carried out to determine the effect of Genta-Mex®, a veterinary commercial product containing 100 mg of gentamicin base/mL, when included in Marek's disease vaccine. Results of the *in vitro* experiments shows that Genta-Mex has no effect on diluent pH or vaccine titers after 120 minutes when compared with the control group. Results of the *in vivo* experiment show that there was no interference with the behavior of the vaccine in commercial chickens. It can be concluded that the product can be used mixed with Marek's disease vaccine.

MATERIALS, METHODS, AND RESULTS

As the first experiment, the vaccine was tested *in vitro* under laboratory conditions using a commercial HVT (cell-associated) vaccine and three different batches of Genta-Mex at a dose of 5mL/200 mL. pH and viable cell counts were measured in samples from each aliquot of vaccine. Titration was performed at 0, 30, 60, 90, and 120 minutes following the addition of the antibiotic. A 1:1000 dilution was prepared for each sample and inoculated onto four plates of secondary chicken embryo fibroblasts (1 mL per plate). Plates were incubated for five to six days and the HVT plaques counted.

Results show no differences on pH in any treated and control groups. Only minor differences (less than 1.5%) were observed in treated vaccine titers when compared with the control group at the end of the experiment (at 120 minutes).

As the second experiment, the vaccine was tested *in vivo* under field conditions using a commercial HVT-SB1-Rispens vaccine in a multi-age farm (with 2.1 million birds in production) that reported high mortality due to Marek's disease in the past. One group of 10,000 birds were vaccinated at the hatchery with the combination of 5 mL of Genta-Mex with 200 mL of diluent. A group of 2,000 birds remained unvaccinated as negative control group. A third group of 140,300 birds were vaccinated with the same batch of Marek's disease vaccine with no antibiotic, and represents a positive control group. All birds were raised in one single house and received the same vaccination program.

At 14 weeks of age all birds were move to the production site to one single fully automated house. Mortality was recorded every week until birds reached 25 weeks of age. From 16 to 25 weeks of age, 25 affected birds were necropsied every week and nerve samples were sent to the laboratory for histologic examination.

Results show that during the first 15 weeks of age the accumulated mortality in all groups remained low (from 2.53% to 2.65%). Starting at 16 weeks of age the mortality in the unvaccinated control group increases with birds showing signs of Marek's disease. No important increase in mortality was seen in the vaccinated positive control group or in the vaccinated group with Genta-Mex up to 25 weeks of age. Unvaccinated control group ends at 25 weeks of age

with 12.03% mortality. Vaccinated group with no antibiotic ends with 4.83%, and the vaccinated group with Genta-Mex ends with 4.69%. Histology reports determine the presence of Marek's disease lesions in mortality observed in the unvaccinated negative group.

CONCLUSION

It is concluded that the inclusion of Genta-Mex has no negative effect on Marek's disease vaccine when tested *in vitro* and *in vivo*.

Table 1. Effect of three batches of Genta-Mex® on HVT titers (titer per dose).

	PFU/mL				
	0 minutes	30 minutes	60 minutes	90 minutes	120 minutes
Control	7600 (100%)	7600 (100%)	7600 (100%)	7500 (98.68%)	7500 (98.68%)
Batch 05	7600 (100%)	7600 (100%)	7600 (100%)	7600 (100%)	7550 (99.34%)
Batch 06	7800 (100%)	7800 (100%)	7800 (100%)	7800 (100%)	7750 (99.35%)
Batch 07	7700 (100%)	7700 (100%)	600 (98.70%)	7600 (98.70%)	7500 (97.40%)

FX PLUS® 20 (FOSFOMYCIN) AS A NEW DRUG FOR MAREK'S DISEASE VACCINES

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SUMMARY

Fosfomycin is a broad spectrum antibiotic derived from the phosfonic acid with highly therapeutic index and a fast bactericidal effect on bacteria in growth phase. Two experiments were conducted to determine the effect of FX PLUS® 20 when included with Marek's disease vaccine at a dose of 5 mL in 200 mL of diluent. Results of the *in vitro* experiment show that FX PLUS 20 has no effect on diluent pH or vaccine titers after 120 minutes when compared with the control group. Results of the *in vivo* experiment show that there was no interference with the behavior of the vaccine in commercial chickens. It can be concluded that the product can be used mixed with Marek's disease vaccine.

MATERIALS, METHODS, AND RESULTS

As the first experiment, the vaccine was tested *in vitro* under laboratory conditions using a commercial HVT (cell-associated) vaccine and three different batches of FX PLUS 20 at a dose of 5mL/200mL. The pH and viable cell counts were measure in samples from each aliquot of vaccine. At 0, 30, 60, and 120 minutes following the addition of the antibiotic, samples were taken from each aliquot for titration. A 1:1000 dilution was prepared for each sample and inoculated onto four plates of secondary chicken embryo fibroblasts (1 mL per plate). Plates were incubated for five to six days and the HVT plaques counted. Results show an increase of 0.5 on pH and a slightly decrease of 10% in cell counts between all treated groups and the control group. Only minor differences (less than 1.25%) were observed in treated vaccine titers when compared with the control group from 60 minutes to the end of the experiment at 120 minutes.

As the second experiment, an *in vivo* test was performed under field conditions using a commercial HVT-SB1-Rispens vaccine in a multi-age farm (with 1.5 million birds in production), that reported high mortality due to Marek's disease in the past. One group of 12,400 birds were vaccinated at the hatchery with the combination of 5 mL of FX PLUS 20 with 200 mL of diluent. A second group of 2,000 birds remained unvaccinated as negative control group. A third group of 94,100 birds were vaccinated with the same batch of Marek's disease vaccine with no antibiotic, and represents a positive control group. All birds were raised in one single house and received the same vaccination program.

At 15 weeks of age all birds were move to the production site to one single fully automated house. Mortality was recorded every week until birds reach 25

weeks of age. From 16 to 25 weeks of age, 25 affected birds were necropsied every week and nerve samples were sent to the laboratory for histologic examination.

Results show that during the first 15 weeks of age, the accumulated mortality in all groups was slightly high (from 3.12% to 3.45%). Starting at 16 weeks of age, the mortality in the unvaccinated control group increased with birds showing signs of Marek's disease. No important increase in mortality was seen in the vaccinated positive control group or in the vaccinated group with FX PLUS 20 up to 25 weeks of age. The unvaccinated control group ends at 25 weeks of age with 12.79% mortality. Vaccinated group with no antibiotic ends with 5.96%, and the vaccinated group with FX PLUS 20 ends with 5.68%. Histology reports determine the presence of Marek's disease lesions in mortality observed in the unvaccinated negative group.

CONCLUSION

It is concluded that the inclusion FX PLUS 20 has no negative effect on Marek's disease vaccine when tested *in vitro* and *in vivo*.

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INFECTIOUS BURSAL DISEASE SITUATION IN MEXICO

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SUMMARY

Infectious bursal disease virus (IBDV) continues to cause economic problems in the chicken industry. In some countries of Latin America an acute clinical presentation of infectious bursal disease (IBD) has been reported, but so far in Mexico only subclinical IBD has been seen. In order to have information regarding the situation in Mexico, a survey of clinically normal broiler flocks was carried out using Image Processing Analysis (IPA or Imaging) and RT/PCR-RFLP as diagnostic screening tools.

We carried out a chronological survey to identify the time of challenge in a given complex. With this survey we can see when IBD field challenge is at its peak and this helps determine an appropriate IBD vaccination program.

Imaging is a method for estimating lymphocyte concentrations in the bursa of Fabricius. Bursas were analyzed at 21, 28, and 35 days of age. There were varying levels of bursal lymphoid depletion, and the most common time of challenge was in the fourth week.

Regarding RT/PCR-RFLP, Jackwood's method was use with restriction enzymes BstN1, Mbo1, and Ssp1. BstN1 often detects important changes in the first major hydrophilic region (Peak A) and Mbo1 sometimes detects changes in the second major region (Peak B). Both peaks help define the antigenic characteristics of the IBD virus. In Mexico we could observe the presence of 424 and 370 viruses; no samples reacted with Ssp1, which screens for vvIBD viruses.

Lastly, the variable region of VP-2 was sequenced from two 370 patterns recovered at 21 days of age. Their amino acid sequence in Peak B was novel and will be presented.

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PERIPLASMIC PROTEINS AND SUPERNATANT PROTEINS OF *SALMONELLA ENTERITIDIS* BIOVAR ISSATSCHENKO AS IMMUNOGEN AGAINST *SALMONELLA ENTERITIDIS* IN CHICKENS

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SUMMARY

Poultry production is one if the most susceptible to economic losses by sanitary problems. One of these problems is represented by avian salmonellosis. It is a world-wide disease due to egg consumption and/or *Salmonella*-contaminated chicken meat. Control programs include vaccination to confer immunity and reduce *Salmonella* excretion in feces. There are experimental vaccines with some bacterial components like *S. gallinarum* outer membrane proteins (OMP), or enterotoxins related to OMP. It has been observed reisolation reduction from feces in challenged birds or the ability reduce *S. enteritidis* linking to intestinal mucous, suggesting the possibility of these proteins could be potential immunogens to reduce *Salmonella* colonization in birds.

The objective of this work was to evaluate the use of supernatant proteins (SP) and periplasmic proteins (PP) of *Salmonella enteritidis* biovar Issatschenko as immunogen for the control of *Salmonella enteritidis* infection in broiler chickens.

Five micrograms per mL of SP and PP were inoculated by oral route in one day old chickens. The chickens were challenged 15 days later with 10^6/ml/chicken of *Salmonella enteritidis*. There were no signs, mortality, or bacterial feces elimination during all the time of the study. No seroconversion was seen at 15 days posinoculation with *Salmonella enteritidis* biovar Issatschenko SP neither PP in challenged groups. There was no reisolation of *Salmonella enteritidis* in challenged groups.

These results do not reflect if *Salmonella enteritidis* biovar Issatschenko SP and PP could serve as immunogens for the control of *Salmonella enteritidis* infection in chickens, due to *Salmonella enteritidis* behavior, and also the use of the heterologous rapid plate agglutination test antigen.

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EXPRESSION OF AN AVIAN INFLUENZA NUCLEOPROTEIN GENE IN PRO-AND EUKARYOTIC VECTOR SYSTEMS

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ABSTRACT

The Influenza A virus nucleoprotein (NP) is major conservative structural protein. By this reason NP is very useful for immunological screening of serum materials. Influenza A diagnostic is one of the most important parts of a disease control, prevention and eradication system. It requires scaleable, fast and cheap methods for viral antigen and specific antibodies (Abs) detection. ELISA is the obviously suitable immunological method complying with these requirements (1-3). The potential sources of the antigen for ELISA-plate coating are the virus-containing materials and recombinant NP (rNP). The preparation of viral antigen for ELISA usually requires concentrations and some additional procedures. The recombinant NP is easy to produce and standardize its propagation (4). The advantage of rNP in term of sera testing is eliminating of some false positive results. These non-specific results can occur due to immunological response to some non-viral vaccine components produced using of chick embryo and used for the poultry immunization.

The producing of rNP protein of prokaryotic origin is simpler and cheaper than baculoviral one. On the other hand the requirements to prokaryotic rNP purification, which can be essential to avoid the false positive results (due to interaction between bacterial proteins and Abs to this protein), should neutralize this benefits.

In this paper we present (1) the production of full-length NP proteins of an avian influenza virus (AIV) in *Escherichia coli* (*E. coli*) using pTT9 plasmid and baculoviral Bac-to-Bacsystem using HTc shuttle vector; (2) comparison of these rNPs characteristics for ELISA diagnostic tests. The application of pTT9 plasmid gives easy and cheap way to purify rNP using cellulose-binding domain (CBD) encoded in plasmid. The Bac-to-Bac system allows the application of non-purified rNP.

Six primers corresponding to NP gene were designed based on the consensus sequence of isolates available through NCBI database. Outer primer pair was common and inner primer pairs carried restriction sites for cloning into prokaryotic (BamI-EcoRI) and baculoviral system (SalI-SpeI). RNA was isolated from virus-containing suspension with TRIzol reagent (Invitrogen, USA) according to the manufacturer's specification. The purified RNA of /Swine/Hong Kong/9A-1/98 (H9N2) strain (kindly provided by Prof. Kaverin N.V.(5)) and strain A/Chick/Chekhov/72 (H4N6) were used as a template. The RT/PCR was carried out with H^- reverse transcriptase and High-Fidelity polymerase (Fermentas) using outer primer pair. Then nested PCR was performed to obtain NP genes carrying corresponding restriction sites for cloning in two vector systems. PCR products were gel-purified using DNA extraction kit (Fermentas).

Baculovirus expression was carried out using Bac-to-BacBaculovirus Expression System (Gibco BRL, USA) according to the manufacturer's specification. The NP gene was cloned to pFastBacHTc as a donor plasmid. Recombinant pFastBacHTc plasmid was transformed into *E. coli* DH10Bac for transposition into the bacmid. Recombinant bacmids were used for transfection of Sf-21 cells.

E. coli expression: The NP gene was expressed in pTT9 plasmid system in *E. coli* strain M15 according to manufacturers manual.

Presence of 56 kDa recombinant NP protein was confirmed by Western blotting analysis. Harvested and sonified cells were used for ELISA. The detection of rNPs was based on ELISA with NP-specific monoclonal antibodies (MAb) that were produced in the ARRIVV&M. In case of rNPs produced in prokaryotic system ELISA was designed as double-sandwiched ELISA. The direct ELISA system was used for the rNP produced in Bac-to-Bac system.

Our results showed that both rNPs producing systems give satisfactory results in ELISA. The false positive results were not observed, the titers of rNPs were high, and the reaction was stable and reproductive.

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BREAKS DUE TO *MYCOPLASMA GALLISEPTICUM* AND *SYNOVIAE* IN HEAVY BREEDERS IN ITALY: CLINICAL, SEROLOGICAL, AND PRODUCTION PARAMETERS

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SUMMARY

Production results of mycoplasma free or not free heavy breeders of an integrated poultry company in years 2003-2004 in Italy are reported. Some substantial differences have been demonstrated in various parameters, particularly in egg laying.

INTRODUCTION

Mycoplasma gallisepticum (MG) and *Mycoplasma synoviae* (MS) infection are probably the costliest disease problems, due to high economic losses from condemnation or downgrading of carcasses, reduced feed and egg production efficiency and increased medication costs (1). In heavy breeders such infections are enough well controlled in many part of the world by strict application of biosecurity measures; that is applied also in some poultry plants in Italy. However and because of the high concentration of poultry farms, particularly in northern area of the country, breaks of free-condition frequently occur, sometimes with appearance of symptoms and lesions, sometimes only with seroconversion. The breaks are almost always going with damages of zootechnical parameters, particularly in the progeny performances.

The purpose of this survey is to compare the results of some flocks of the same chicken breed, with or without MG and MS infection, belonging to the same integrated poultry Company in the years 2003-04, with a view for 2004-05.

MATERIALS AND METHODS

Chickens. Fifteen flocks of Ross 508 breeders, originally MG and MS free, have been considered: six of them became positive for one or both infections; the relative progeny have been also controlled.

Serological tests. Sera samples from all flocks were tested at two days, 10 and 20 weeks of age in pullet farms and every month during egg laying for MG and MS with ELISA, using a kit of Idexx and rapid plate agglutination (RPA), using stained antigens of Intervet.

Parameters. Percentages of laying, fertility, and hatchability as well as progeny treatment need and destination have been considered.

Antibiotic treatments. Tylosine tartrate has been used by monthly treatments (five days in water at 0.5g /L) of positive breeder flocks or in the first three days and two days and four weeks of life in broilers.

RESULTS AND CONSIDERATION

Six flocks of breeders of the integrated company became infected with mycoplasma, of which one with both mycoplasmas, two with MG and three with MS. The three flocks of birds infected with MG showed

conjunctivitis, more or less evident rales and airsacculitis, increased mortality, variable decrease in egg production, depending on the age at infection and worsening in egg shell quality. The three flocks of birds infected with MS showed no particular symptoms and only low decrease in egg laying.

The periodic antibiotic treatments of seroconverting flocks allowed, most likely, to reduce the losses and the transmission of infection to the progeny. The treatment was extended to the progeny, also assigning them to a shorter age at slaughter and weight (1.7 kg), consequently with further economical losses.

The productive parameters of the free or not free flocks are cumulatively reported in Table 1 and in Figure 1.

In order to increase the biosecurity and to control the horizontal transmission of infection, the Veterinary Service of the integrated company is from time inducing to apply the following severe measures:

a) more technical and health education of staff and workers, with reduction to essential of the visits from veterinarians and technicians in the farm
b) improvement of buildings and furnishings, also for a better cleaning and disinfection between replacements
c) exclusion of farms located in risk zone
d) strictly separate management and service of infected farms, with tendency to eliminate the flock as soon as possible
e) control of egg- packing material introduction in the farm
f) feed supply and egg cases collection outside of the barrier
g) periodic disinfections of external contaminable areas
h) wise transport of birds from pullet to laying farms (accurate disinfection of trucks and cages, chose of transit way, avoiding as much as possible the proximity to other poultry farms)
i) periodic blood sampling and cloacal swabs for serological and bacteriological tests.

Actually (2004-05 period), all pullet and laying farms (36) located in different places of the country are resulting negative for MG and only three became to now positive for MS. Therefore, a considerable progress is achieving in the control of mycoplasmosis, after application of very strict biosecurity measures, with consequent improvement of performances.

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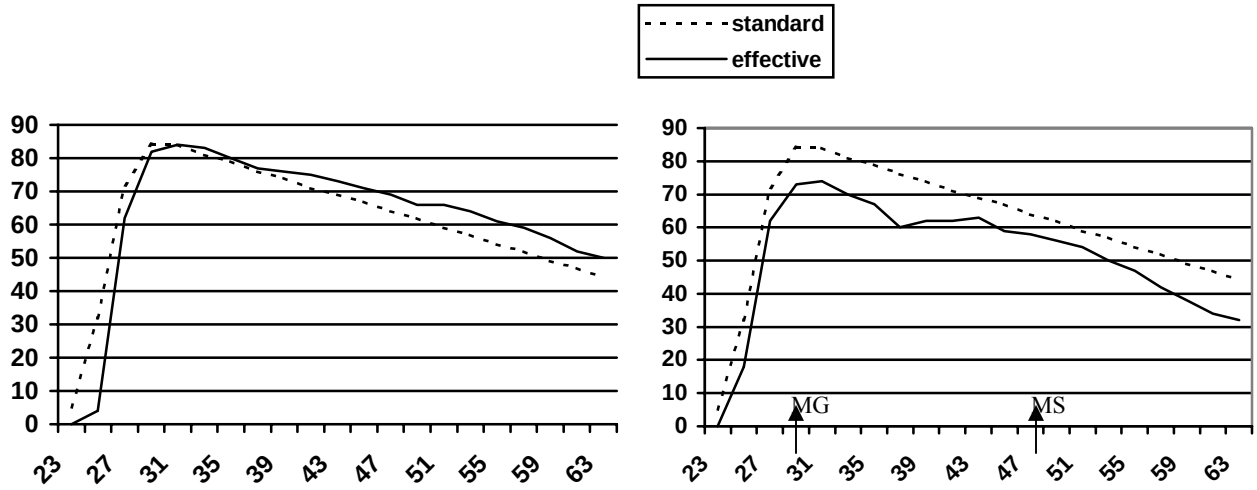
Table 1: Comparative production parameters in breeders free or not free of MG or MS.

Type MG or MS	No. flocks	No. birds	Weeks laying	Mortality (%)	No. eggs production	No. eggs incubation	Fertility (%)	Hatchability (%)
Positive	6	201,000	38.0	13.3 (8.2-25.0)	162.1 (144.4-172.2)	152.8 (139.0-164.3)	87.0 (81.6-89.9)	80.6 (75.9-84.1)
Negative	9	231,000	38.6	10.3 (3.4-18.5)	173.8 (157.7-182.3)	160.6 (143.8-170.0)	87.2 (84.8-89.6)	81.2 (79.2-83.7)

Figure 1: Example of comparative egg-laying between two breeder flocks free or not free from MG.



INHIBITION OF *CLOSTRIDIUM PERFRINGENS* TYPE A ASSOCIATED WITH BROILER NECROTIC ENTERITIS AND THE A-TOXIN PRODUCTION BY HEN EGG WHITE LYSOZYME

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ABSTRACT

Clostridium perfringens type A causes both clinical and subclinical forms of necrotic enteritis in domestic avian species (1). In this study the inhibitory effect of hen egg white lysozyme (2) on the vegetative form of *C. perfringens* and the α-toxin it produced *in vitro* was investigated. A micro-broth dilution assay was used to evaluate the minimal inhibitory concentrations (MIC) of lysozyme against three *C. perfringens* type A strains in 96-well microtiter plates. The MIC of lysozyme against *C. perfringens* was found to be between 150 and 200 μg/mL. Scanning electron micrographs of the cells treated with 100 μg/mL of lysozyme revealed extensive cell wall damage. A quantitative sandwich ELISA for α-toxin produced by *C. perfringens* was developed based on a commercial ELISA kit allowing only qualitative detection. Addition of 200 μg/mL of lysozyme to a standard preparation of the α-toxin resulted in 33% reduction of the toxin level. In another experiment, 50 μg/mL of lysozyme did not inhibit the growth of *C. perfringens* but significantly inhibited the toxin production.

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(A full-length article has been submitted to the journal of *Avian Diseases*.)