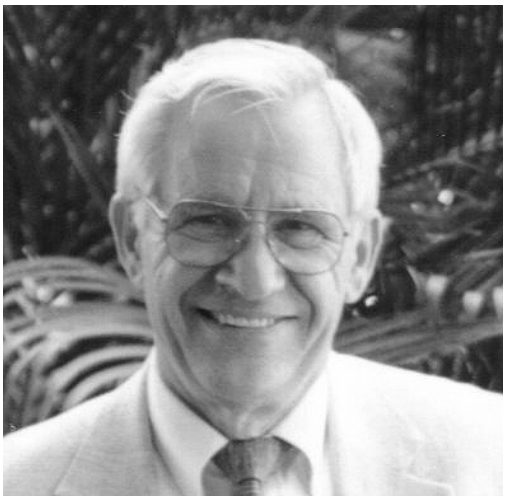
PROCEEDINGS OF THE FIFTY-FIFTH WESTERN POULTRY DISEASE CONFERENCE

March 5-8, 2006 Sacramento, California

55TH WPDC SPECIAL RECOGNITION AWARD

MARION A. HAMMARLUND



The 55th Western Poultry Disease Conference is honored to present the 2006 WPDC Special Recognition award to Dr. Marion Hammarlund.

Marion graduated from Kansas State University in 1953 and began work as a mixed-animal practitioner. In 1955, he moved to Colorado where he began his career in poultry medicine at the veterinary diagnostic laboratory and teaching poultry pathology at Colorado State University. In 1957, Marion was hired by Ralston Purina as a staff veterinarian in St. Louis, MO, and provided technical services and disease management to their poultry clients. In February, 1964, Marion arrived in Southern California to visit the K-M turkey breeding company. It was beautiful sunny weather and a decision was made to relocate from St. Louis if an opportunity developed.

That opportunity came in 1966 when he signed on to work at the Arlington Veterinary Labs in Riverside with Dr. Robert Olsen. This laboratory provided state-licensed vaccines to the poultry industry. Unfortunately, as a consequence of the Exotic Newcastle Disease outbreak in 1970, only USDA-approved vaccines were allowed, forcing Arlington Veterinary Labs out of business. Marion developed new skills and opened his own veterinary clinic for small animals and poultry in Riverside. He sold his practice in 1984, but continues to work as a private veterinary practitioner with special interest in poultry.

Marion met his wife Margaret at Kansas State where they were married in 1950. They have one daughter and one grandson.

SPECIAL ACKNOWLEDGMENTS

The Western Poultry Disease Conference (WPDC) is honored to acknowledge the many contributions and support to the Conference. The financial contributions provide support for outstanding presentations and to help pay for some of the costs of the Conference, thus helping us to maintain a relatively low registration fee for an international conference. More than 40 organizations, companies, and individuals have given substantial financial support. Many companies and organizations, including some that also contribute financially, send speakers at no expense to the Conference. We thank all these people, and acknowledge their support and contribution.

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

Dr. Peter Woolcock would like to thank Rebecca Gonzales for compiling all the titles submitted during October, when I was on vacation. On my return my task of sorting and grouping all the submissions was made much easier. Thanks must also go to Dr. Richard Chin for constantly keeping me on my toes, and thus encouraging me to get everything done within acceptable timeframes. Thanks also to the members of the Title Review Committee, Dr. Carol Cardona, Dr. Bruce Charlton, Dr. Richard Chin, Dr. David Frame, Dr. Stewart Ritchie, Dr. Yan Ghazikhanian, for their helpful comments and contributions when I was compiling the program. It has been an honor to serve as Program Chair and learn more about the behind the scenes activities necessary to organize a conference of this type. WPDC owes a debt to Dr. Richard Chin for the many hours he spends administering and keeping afloat this prestigious organization.

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 Facility, 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. A special thanks goes to Utah State University Cooperative Extension for reproducing the CD-ROM and donating the cost of reproduction 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 original design of the CD label.

55TH WPDC CONTRIBUTORS LIST

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CONTRIBUTIONS CHAIR
Dr. Yan Ghazikhanian

55th WPDC PROCEEDINGS

The Proceedings of the 55th Western Poultry Disease Conference are not refereed, but are presented as a service and a source of information to those attending the conference and to others who wish to gain some insight as to the information presented. Copies of the Proceedings are available in either hardcopy or electronic (CD) formats.

Copies of these Proceedings are available from:
Dr. R. P. Chin
CAHFS-Fresno
University of California, Davis
2789 S. Orange Ave.
Fresno, CA 93725-1919
rpchin@ucdavis.edu

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50th WPDC Anniversary CD-ROM. This CD contains all printed proceedings of the first fifty Western Poultry Disease Conference meetings. Copies can be purchased from the AAAP. Phone: 706-542-5645. Fax: 706-542-0249. E-mail: aaap@uga.edu. Web: http://www.aaap.info/.

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. Hammarlund	G. W. Peterson		
7th PHS				
23rd WPDC – 1974	G. W. Peterson	Craig Riddell		
8th PHS				
24th WPDC – 1975	C. Riddell	Ralph Cooper		
9th PHS				
25th WPDC – 1976	R. Cooper	Gabriel Galvan		
10th PHS				
26th WPDC – 1977	G. Galvan	Don H. Helfer	Hector Bravo	
11th PHS				
27th WPDC – 1978	D. H. Helfer	Art Bickford		
12th PHS				
28th WPDC – 1979	A. Bickford	J. W. Dunsing		
13th PHS				
29th WPDC – 1980	J. W. Dunsing (WPDC)	G. Yan Ghazikhanian	P. P. Levine	
14th PHS				
5th ANECA	Angel Mosqueda T. (ANECA)			
30th WPDC – 1981	G. Y. Ghazikhanian	Mahesh Kumar		
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	Bachoco S.A. de C.V. Productos Toledano S.A.
52nd WPDC – 2003	B. Daft	David H. Willoughby		Roland C. Hartman
53rd WPDC – 2004	D. H. Willoughby	Joan Schrader		G. Yan Ghazikhanian
54th WPDC – 2005	J. Schrader	Stewart J. Ritchie	W.D. Woodward	R. Keith McMillan
55th WPDC – 2006	S. J. Ritchie	Peter R. Woolcock		M. Hammarlund
56th WPDC – 2007	P.R. Woolcock	Bruce Charlton		

Minutes of the 54th WPDC Annual Business Meeting

President Schrader called the meeting to order on Tuesday, 26th April 2005, at 11:06 AM, at the Fairmont Hotel Vancouver, BC, Canada. There were about 35 people in attendance.

APPROVAL OF 53rd WPDC BUSINESS MEETING MINUTES

The minutes from the 53rd WPDC business meeting were reviewed and a motion was carried to approve them as printed in the Proceedings of the 54th WPDC.

ANNOUNCEMENTS

President Schrader acknowledged all the contributors; in particular, those contributing at the Benefactor level, which included the American Association of Avian Pathologists, Canadian Egg Marketing Agency and Merial Select, Inc. She also thanked all the contributors for their generous donations. President Schrader acknowledged the efforts of the current WPDC officers for their work and participation in the organization of this year's meeting. President Schrader asked Dr. Mariano Salem to talk about Dr. Celedonio Garrido Melo who passed away this year. In addition, President Schrader honored Dr. Willis "Woody" Woodward, who also passed away this year, and to whom the 54th WPDC is dedicated, for his tremendous contribution to the poultry industry.

REPORT OF THE SECRETARY-TREASURER

Dr. R.P. Chin presented the Secretary-Treasurer report. There were 206 registrants for the 53rd WPDC held at the Capitol Plaza Holiday Inn, Sacramento, CA, March 7-9, 2004. Contributions for the 53rd WPDC were $25,150, with a total income of $51,384. There were expenses of $52,997 for WPDC for the meeting, resulting in a net loss of $1,613. The current balance in the WPDC account was $45,825. The good news is that the loss was less than anticipated as we had a good number of late registrants (40). The loss was again due to the low number of registrants for WPDC, decrease in contributions and an increase in costs.

Dr. Chin was happy to report that there is an estimated gain of $343 for this year's preliminary budget. This is due to the number of registrants (approximately 270) and the increase in contributions ($36,743) due mostly to $10,000 from Canadian companies as a result of Dr. Ritchie's efforts. Estimated expenses for this year are approximately $80,000. The increased cost is due primarily to additional audiovisual and hotel costs.

Once again, Dr. Chin stated that WPDC needs to look at ways to increase our registration numbers and contributions to break even for future conferences. There were no comments regarding the Secretary-Treasurer's report.

REPORT OF THE PROCEEDINGS EDITOR

Dr. D. Frame presented the Proceedings Editor report. There were 89 papers and a total of 135 pages in this year's proceedings. For this conference, 500 hard copies and 500 electronic copies of the Proceedings were produced. The CD's cost approximately $1.53 each, which is much less than last year's $7.75 each because Dr. Frame created the CD himself. The books cost $5.75 each. As was done last year, the books were produced by Omnipress. As previously mentioned, the CD's were created by Dr. Frame and the duplication was done by CDMan of Vancouver, BC, Canada. The total cost came to $4678.63 for editing and publishing of the proceedings. Dr. Frame investigated copyrighting the proceedings and did not find any advantages to doing so. Dr. Frame also looked into a web-based proceedings, but found concerns with publishing in a web-based proceedings and a refereed journal.

REPORT OF CONTRIBUTIONS CHAIR

Dr. Ken Takeshita reported that there was an $11,000 increase over last year in contributions. $10,000 came in as new contributions from Canada. Dr. Takeshita announced that he will no longer be the contributions chair and felt that another person, a committee or the Secretary-Treasurer can fulfill that role.

REPORT OF LOCAL ARRANGEMENTS CHAIR

Dr. Stewart Ritchie thanked Ms. Shirley Fast and Ms. Catherine McAllan for doing just about all the local arrangements.

REPORT OF TEMPORARY LOCAL ARRANGEMENTS CHAIR

Dr. Joan Schrader reported for Dr. Jim Andreasen that he looked into hotels in Las Vegas, but could not find any suitable hotel for our meeting. Dr. Andreasen will check again, but look at weekday meeting dates rather than WPDC's usual Saturday/Sunday – Tuesday. He will also check into places in Arizona.

OLD BUSINESS

Dr. Chin reported that based on the quotes provided him, next year's conference organizers will again be University of California, Davis's Conference & Event Services.

Dr. Schrader stated that WPDC proceedings will not be web-based because journals may not accept manuscripts already published on a web. Dr. Frame reiterated this, and stated that WPDC prefers to maintain a high quality proceedings with good scientific information (rather than "Results will be discussed.").

There was no action on the motion that we obtain a list of people who use to attend WPDC and find out why they are no longer attending.

Dr. Chin reported that he talked with representatives of the American College of Poultry Veterinarians regarding improvement of their workshop. All agreed that this year's workshop was much improved and probably attracted people to the joint meeting.

Dr. Chin reported that he did additional investigation into what book-sellers do and found that it was not appropriate to charge them a higher rate.

NEW BUSINESS

President Schrader reported that the WPDC Executive Committee nominated Dr. Bruce Charlton for Program Chair-elect of the 56th WPDC in 2007. There were no other nominations and Dr. Charlton was elected unanimously as program chair-elect. Dr. Takeshita resigned as Contributions Chair and suggested Dr. Yan Ghazikhanian as the successor. Though there was a unanimous voted for approval, Dr. Ghazikhanian had reservations, but later agreed to serve as the Contributions Chair. President Schrader nominated the following officers for 2005-2006:

Program Chair: Dr. Peter Woolcock
President: Dr. Stewart Ritchie
Local Arrangement Coordinator: none – responsibilities to be taken over by secretary-treasurer
Contributions Chair: Dr. Ghazikhanian
Proceedings Editor: Dr. David Frame
Secretary-Treasurer: Dr. Rich Chin
Program Chair-elect: Dr. Bruce Charlton

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

There was a discussion on ways to improve attendance to WPDC. Dr. Chin stated that in 2002, there were 195 registrants, in 2003 there were 196 registrants, and 205 in 2004. This year, we currently have 270 registrants, obviously due to the location of the meeting in Vancouver, Canada. Suggestions for improving WPDC included:

1. Dr. Takeshita suggested having a section of talks that provide practical information for veterinarians and possibly producers, similar to the Poultry Health Symposium previously held in conjunction with WPDC.
2. Dr. Rosenwald (through Dr. Bickford) suggested having the meeting at the University of California, Davis.
3. Dr. Mariano Salem suggested including processing and food safety presentations.
4. Dr. Singh Dhillon suggested having the meeting in Seattle, WA.
5. Dr. Fernando Lozano commented that this year's meeting was immediately preceding ANECA (which is April 27 – 30). It would be best not to do this so Mexicans have the chance of attending both meetings. (Sec/Treas Note: We only change the meeting to these dates when it is held in Vancouver, Canada. In addition, ANECA is now being held one week earlier than in previous years.)

6. Dr. Mariano Salem suggested that we encourage national and international pharmaceutical companies to participate.
7. Dr. Ahmad also suggested that we have more presentations on food safety/security.
8. One person suggested coming to Vancouver every other year.
9. After the meeting, one person commented that maybe WPDC should go back to being a smaller regional meeting, thus decreasing overall costs.

Dr. Chin stated that an announcement will be sent via Email to all those registered that the WPDC survey for this year's meeting can be completed online.

President Schrader announced that next year's WPDC will be held in Sacramento, CA March 5-7, 2006, at the Holiday Inn Capitol Plaza. There was a discussion on the days for the meeting, i.e., Sunday through Tuesday or Monday through Wednesday. Dr. Chin commented that WPDC and ACPV are two separate meetings. One person stated that they consider ACPV and WPDC as one meeting and, with the ACPV workshop on Saturday, it was difficult to attend. Dr. Takeshita stated that WPDC should decide on its own as to meeting days and let ACPV decide their own day. A vote was taken and 11 voted for Sunday – Tuesday, whereas seven voted for Monday – Wednesday. Craig Blackmore commented that we should put this question on our survey to get a better representation of everyone who attends.

Dr. Andreasen will continue to investigate meeting in Las Vegas for the 56th WPDC. At the WPDC Executive Committee meeting, Drs. Urquiza and Tamayo of ANECA invited WPDC to join ANECA for another joint meeting. The Executive Committee recommended that the 57th WPDC in 2008, be held in Mexico in conjunction with ANECA. The tentative location is either Puerto Vallarta or Ixtapa.

Dr. Chin commented that Feed Info News Service, an internet news agency, once again asked to publish WPDC papers on their web site. As in previous years, WPDC agreed that this company can talk with individual authors to obtain approval for their papers to be published on the internet.

President Schrader passed the presidency to Dr. Stew Ritchie who thanked those involved in the organization of the meeting. President Ritchie adjourned the meeting at 11:58 AM.

TABLE OF CONTENTS

Note: Both the oral and poster presentations of the 55th 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-FIFTH WESTERN POULTRY DISEASE CONFERENCE

THE USE OF MICROARRAYS IN DIAGNOSTIC MICROBIOLOGY

C. L. Keeler, Jr., M. N. Maughan, and T. W. Bliss

Department of Animal and Food Sciences, University of Delaware, Newark, DE 19716-2150

DNA microarrays (sometimes referred to as DNA chips) have transformed the field of genomics. Whereas previous generations of scientists studied a single gene or a single organism, this technology permits the study of the expression of thousands of genes simultaneously, providing a genome-wide view of complex host pathogen relationships. The result is that scientists now have an additional tool in their arsenal for developing improved methods of disease detection, treatment and prevention.

The concept of immobilizing probe molecules in order to detect targets can be traced back to the 1960s and the development of the immunoassay. DNA microarray technology evolved from the use of the Southern blot, developed in the 1970s. Traditionally, DNA microarrays are composed of treated glass slides (the size of a regular microscope slide) upon which are immobilized hundreds or thousands of gene probes. These gene probes can be derived from PCR products or they can be synthetic oligonucleotides. Proper design of the microarray is critical for obtaining statistically relevant data. In use, the microarray is simultaneously exposed (hybridized) to target samples, traditionally fluorescently labeled cDNA products derived from mRNA. The fluorescent image of the microarray slide which is generated after washing and scanning results in the simultaneous measurement of the expression of thousands of genes, as measured by the amount of mRNA present in the sample at that point in time. Because of the scale of information generated, data analysis and management are critical. Only with the proper management and analysis of this information can the potential of microarray technology be realized.

Diagnostic applications of microarrays initially focused on the detection of inherited human disease traits. However, microarray techniques have also been successfully developed for use in diagnostic microbiology. In a classic paper, Wang *et al.* (9) described a microarray capable of identifying over 140 respiratory viruses. Their technology was validated using clinical specimens from the respiratory tract of human subjects. Wilson *et al.* (10) developed a Multi-Pathogen Identification (MPID) microarray that identified 18 pathogenic prokaryotes, eukaryotes, and viruses. These researchers amplified unique regions of DNA from each microorganism, and then used the microarray to detect the poresence or absence of pathogen-specific DNA sequences with a detection limit as low as 10 femptograms of pathogenic DNA. More recent applications of the microarray allow scientists to readily identify pathogenic microorganisms by performing rapid large-scale sequence analyses. Developing a form of "microbial forensics," Read *et al.* (7) used a microarray to determine the origin of an anthrax infection. More recently, commercial enterprises such as Affymetrix (Santa Clara, CA) have developed pathogen-specific sequencing arrays. By sequencing up to 300 kb of a genome within 48 hours, these arrays can rapidly identify sequence variations between different bacterial or viral strains. "Resequencing" can be used to detect and identify pathogens and improve typing systems.

Our laboratory has developed an avian influenza DNA microarray. Avian influenza (AI) is an economically significant pathogen having great agricultural and public health consequences. Influenza viruses are enveloped, single-stranded RNA viruses belonging to the *Orthomyxoviridae* family and the influenza genome is comprised of eight negative-sense RNA segments. Avian influenza is a type A influenza and is further subtyped by identifying the hemagglutinin (HA) and neuraminidase (NA) surface antigens. Sixteen hemagglutinin subtypes and nine neuraminidase subtypes have been isolated from birds. Reassortment and a high mutation frequency can increase virulence and help AI cross inherent species barriers. Current detection methods are limited to either rapid detection without subtyping, or costly and time-consuming subtyping. We feel that microarray technology is ideally suited for the identification and classification of influenza viruses.

There are several published reports of influenza diagnostic microarrays. One such array identifies strains of equine influenza by using oligonucleotides and differentiates HA1 from HA3 and NA1 from NA2 (8). Other studies have been able to differentiate HA1, HA3, HA5 and NA1 from NA2 of type B human influenza strains (4, 6). Our goal is to identify type A avian influenza, identify and differentiate between hemagglutinin subtypes and neuraminidase subtypes, and finally to determine the geographical origin of viral isolates based on hybridization patterns consistent with phylogenetic analysis.

In theory, probes specific for each of the 16 HA subtypes and 9 NA subtypes can be designed and spotted onto glass slides. Our microarray slides contain PCR products specific for the HA1, HA5,

HA7, and HA9 subtypes, as well as the NA1, NA2, and NA3 subtypes. The slides also contain a pan-influenza probe, based on the matrix (M) gene sequence. Each element on the array is spotted in duplicate in each of four subarrays, yielding eight spots representing each element on the array. The current AI cDNA array contains 20 elements and a total of 160 spots. A panel of 10 AI samples has been used to evaluate the avian influenza cDNA microarray and an example of an array generated in this validation experiment is depicted in Figure 1. Statistical (ANOVA) analysis of spot normalized intensities was used to correctly determine that the strain was a member of the HA5 NA2 subtype and that it belonged to the Mexico HA5 clade.

What is the potential for this system to be used in the field? Currently, the time, cost, and specialized equipment involved relegate the use of this technology to a reference laboratory. However, as the methodology becomes more robust and economical this technique may see wider use. We are already expanding the avian influenza array to include gene probes for Newcastle disease virus (NDV), permitting the same platform to be used to identify and characterize two important avian pathogens. In addition, the development of non-fluorescent based detection technologies should significantly reduce the cost and the need for specialized equipment.

There are other potential applications of microarray technologies in the field of poultry health. Gene-expression profiles of infected animals can provide a "signature" or "diagnostic marker" for infection by specific agents (2). This approach has already been used successfully in a bovine model, where Johne's disease in cattle is associated with the enhanced expression of a number of genes, including IL-5 (3), although it has not been used for poultry. We also envision expanding this approach to flock monitoring. In the future it may be possible to inform a company veterinarian about the immune status of his flocks. It might also be possible to determine if they have been exposed to specific infectious agents or are particularly susceptible to specific pathogens. Microarrays have already been used in production animal models to determine immunological correlates for protection. By characterizing the bovine immune response against experimental challenge with *Mycobacterium bovis,* researchers have correlated the IFN-gamma response and IL-4 mRNA expression with the severity of disease and have thus been provided with a measure for protection (1).

In the field of avian genetics, we are already using microarrays to evaluate the immune response of genetic lines of birds and correlating those results with observations of performance in the field. These experiments can be combined with other powerful genetic tools; SNP analysis, real-time qRT-PCR, and QTL mapping, to enhance breeding programs. By using microarrays for DNA sequence analysis, researchers could readily identify mutations in host "susceptibility genes" that influence a particular genetic stock's risk of acquiring disease.

Microarrays also have great potential in the area of vaccine development. By examining the transcriptional activity of all the genes of a pathogenic microorganism under *in vivo* conditions, rarely expressed but potentially important genes can be identified (5). Increasingly, vaccine manufacturers are including immune enhancing compounds in their formulations. Microarrays can be an attractive tool to use to generate the efficacy data required for licensing and marketing.

The flexibility and high-throughput capability of microarray technology offers tremendous opportunities for infectious disease research. These new tools will allow microbiologists to develop a better understanding of host-pathogen interactions and stimulate the development of novel approaches to infectious disease diagnosis, treatment, and prevention.

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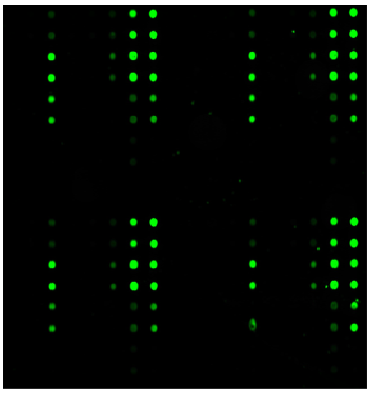
Figure 1. Avian influenza microarray.



THE USE OF ENVIRONMENTAL AIR-SAMPLING IN THE DETECTION OF POULTRY DISEASE AGENTS

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Surveillance for rapid disease detection in populations has historically been challenged by the ability to efficiently sample an adequate and representative number of individuals from the population. The most common approach for detecting disease agents in large populations has been to sample a statistically-representative number of individuals, and apply antibody or antigen detection assays to the individual samples or to pools of samples. Alternate approaches, including etiologic agent detection using environmental samples, such as drag swabs or hatchery papers from poultry houses, have been widely used for microbial monitoring or surveillance of flocks. Though not routinely utilized by the poultry industry, environmental air-sampling has been described for detection of selected bacterial agents, primarily *Salmonella* species. Airborne viruses, specifically Newcastle disease virus and avian influenza virus, have also been detected by environmental air sampling. In 1948, Delay, DeOme, and Bankowski (1) and in 1986 Brugh (2) first described aerosol collection in and around poultry houses to sample for Newcastle disease virus and avian influenza virus, respectively. In 2003, we utilized environmental air-sampling in poultry houses specifically for the detection of exotic Newcastle disease virus (3) and more recently for the detection avian influenza virus.

As a surveillance tool, environmental sampling and specifically air-sampling, offers some key advantages for the poultry industry. Aerosol sample collection can be completed in minutes, without direct contact or handling of birds. The air-sampling approach minimizes breaches of flock biosecurity, avoids time and labor costs associated with collecting and testing samples from individual birds or numerous environmental surface samples, and eliminates the stress on birds associated with handling them for blood or swab collection. The costs associated with air sampling, following the original investment in an aerosol collector, is limited to the cost of transporting the sampler to the selected environment plus the cost of the detection assay for a single or limited number of

environmental samples. The cost of flock surveillance for serologic sampling, swabbing, or collection of carcasses is by comparison significantly amplified by the cumulative costs associated with personnel entering the house(s), handling birds, plus collecting and testing a statistically valid number of samples.

Environmental air-sampling has seen significant growth in the past decade, primarily due to deployment of air-monitoring systems used as sentinels for early detection of air-borne biological agents in selected high-impact public venues (4). Monitoring of environmental air in public spaces, under a program initially known as Biological Aerosol Sentry and Information System (BASIS) was designed for military application in the mid-1990's, was fully deployed during the 2002 Olympics in Salt Lake City, and is currently in operation at various public sites nationally to enhance early detection of potential viral, bacterial, or toxic biothreat agents. Equipment for environmental air-sampling is commercially available for a range of industrial uses. Samplers have been variously designed to optimize air collection for a range of specific applications, typically targeting microbial/particulate or chemical agents. Once collected, the air sample can be tested for the presence of specific microbial agents or toxins using the existing range of laboratory techniques, such as standard bacterial culture, virus isolation, antigen-capture ELISA, or molecular-based diagnostics. Different air-sampling devices use different strategies for capture of target agents, and are generally classified into gravitational-style samplers and vacuum-style samplers.

Gravitational samplers (settling plates) are the simplest and least expensive means of collecting airborne microbial agents. Gravitational sampling is a passive system that relies on gravitational settling of particulates onto a nutrient agar surface, typically a Petri dish. The technique is imprecise and generally over-represents the larger particles that settle more rapidly due to their increased size and weight.

Vacuum-style samplers pull air into the device and trap airborne particulates by a variety of different methods, either onto solid or semi-solid surfaces (impaction) or into liquid (impingement). **Impinger-style samplers** draw air through a curved inlet tube where particulates are collected on the curved wall(s) of the tube and are subsequently flushed into a liquid collection media.

Impaction-style samplers collect air and deposit the airborne particulates by directing the flow of air toward a collection surface, typically nutrient agar or a membrane filter.

Cascading or sieve impaction samplers, such as those most-often used in clean-room sampling, pull air through a series of pores of decreasing size, separating the particles of different sizes sequentially by momentum differences related to the air flow through the pores. Sieve-style samplers have been used to capture viral particles onto membrane filters, from which the virus or viral nucleic acids are eluted prior to analysis. **Slit samplers** pull air through a narrow slit, directing the air flow toward a membrane filter or agar surface, typically a Petri dish placed on a rotating turn-table. The airborne microbes are spatially separated by the turntable's rotation as they impact onto the agar surface. Because bacterial colonies will disperse and be read like the divisions on a clock, slit samplers assess not only the microbes in the environment, but also the impact of air currents caused by human, animal, or equipment movement in the environment over the collection period. Like other impaction samplers, slit samples are most effective with the larger bacterial and fungal agents.

Centrifugal samplers function by creating an air-stream vortex that disperses particles having sufficient inertia to leave the air-stream. The particles leaving the vortex are impacted onto a collection surface, typically a thin layer of nutrient agar or are captured directly into a liquid media.

Electrostatic precipitator-style samplers are modeled after the ionizing air pollution filters that use electric voltage to create a charge on airborne particles passively coming within several inches of the sampler. The charged particles are then captured on a reverse-charged collection plate or nutrient media.

As noted earlier, the utility of using environmental air-sampling for rapid detection of a poultry virus was demonstrated during the 2002/2003 exotic Newcastle disease outbreak (3). At that time a cyclone wetted wall (centrifugal-style sampler) was used to collect air-samples over an eight hour period, with sub-samples collected at two and four hours. The samples were then subjected to real-time RT PCR testing and virus isolation in embryonated eggs for Newcastle disease virus, exotic Newcastle disease virus, and avian influenza virus. Exotic Newcastle disease virus was detected by realtime RT PCR and recovered by virus isolation from two naturally-infected poultry houses ranging in size from 3,000 to 8,600 birds. In one of the two flocks, the air-sample tested positive for exotic Newcastle disease virus at least two days before signs of clinical disease were detected by flock managers. In 2005, a modification of the earlier air-sampling procedure was successfully used to sample for avian influenza virus infection in commercial birds. Samples collected from multiple sites at a 20,000-bird commercial quail premise naturally infected with H6N2 avian influenza virus tested positive after just 10 to 20 minutes of environmental air sampling. Although showing no evidence of clinical disease, individual quail were confirmed positive for H6N2 avian influenza virus by

real-time RT PCR and virus isolation from tracheal swabs and tissues collected for slaughter surveillance testing.

Environmental air-sampling has been demonstrated in preliminary field work to be a viable tool for routine surveillance or targeted monitoring of poultry premises. Ongoing studies are needed to assess the detection sensitivity and specificity of environmental air-sampling, as well as to document the impact of sample collection duration, humidity, heat, flock size and density, and other management factors on the ability to recover the range of important poultry pathogens.

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SEQUENCING AS A DIAGNOSTIC TOOL

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Sequencing has gained in popularity as a diagnostic tool and many laboratories have sequencing services for a number of viral disease agents. Although sequencing could be developed for any virus, it is generally most useful in characterizing unknown viruses or for following the trends of a virus that changes frequently. In the former category, we include the truly novel viruses, viruses identified in new species and viruses for which there aren't any other tests. In the latter category, this mostly applies to RNA viruses in which mutation and recombination are normal events.

The approach to developing a sequencing protocol for a virus that has not previously been sequenced or amplified requires a certain amount of expertise in virology and molecular biology. In the initial identification process, it is very useful if an electron microscopist can classify the virus as to its type. If that service is not available, then it is very important to get as much information as possible from pathologists (presence of inclusion bodies, location and types of lesions) and virologists (isolation method used, hemagglutination, typing with antibody-based tests, etc.) and to synthesize that information into a best guess as to virus type. After the virus has been classified to some extent, sequences from closely related viruses can be aligned and conserved regions identified. Degenerate primers for RT-PCR or PCR amplification of these conserved regions can then be designed. Once the segment is amplified, it can either be directly sequenced or cloned and then sequenced.

In the cases of RNA viruses, sequencing is more likely to be used to identify mutations that change the antigenicity or pathogenicity of the virus. The strategy is very different from that described in the previous paragraph, in that the primers that are used are not usually directed at conserved regions of the genome, but rather at genes that are subject to high levels of variation. It is common to design degenerate primers in conserved regions flanking the more variable regions to amplify regions of interest. Again, once there is amplification, then the PCR product can be sequenced.

Sequencing is an important diagnostic tool that can answer a number of diagnostic questions. Perhaps the most common question that sequencing can address is "What is the relationship of this virus to other viruses?" Based on genomic sequence, a phylogenetic analysis can be performed and the relatedness of the new virus to published sequences can be determined. However, although sequencing can be used to deduce phylogentic relationships, there are some significant limitations that should be considered. Often, the question of relatedness is asked in order for the poultry producer or veterinarian to determine the type of vaccine that should be used to protect birds from virus challenge in the field. The problem is that sequencing may or may not be useful in this context because protection is based on immune responses and thus, is dependent on the presence or absence of specific epitopes. Epitopes are typically short stretches of amino acids and, in most cases, only a small part of the portion of the genome that has been sequenced. A

change of a single amino acid or nucleotide can result in a change in an epitope and thus, the ability of a vaccine to protect. So, it is entirely possible that two viruses could have nearly identical sequences and yet be serologically divergent.

Some of the RNA viruses for which sequencing is a valuable tool, not only change frequently by mutation, but are also subject to reassortment. The viruses for which this is a particularly important issue, are the orthomyxoviruses (avian influenza) and the coronaviruses (infectious bronchitis). For these virus families, it is important to note that although the immunogenic protein genes are the most relevant portions for sequencing and for comparison with other sequences, the remaining unsequenced portion of the genome may not have the same phylogenetic relationships.

I would be remiss if I didn't mention that sequencing and sequence analysis can be a time consuming procedure. The demands from clients for rapid turnaround times are increasing as tests get faster and faster. Sequencing of up to 600 bases from a virus for which you already have primers, usually takes about a week. Sequencing longer segments requires more time. In addition, the analysis of sequence data requires some level of expertise. So, if a virus is an emerging pathogen that is appearing or beginning to appear with regularity, then the better thing to do is use the sequence data you have to develop a rapid test such as a realtime PCR test. If sequencing is required as is a rapid turnaround time, such as the case with the sequencing of the Newcastle disease virus fusion gene and the avian influenza hemagglutinin to determine pathogenicity, then sequencing a short stretch of bases can reduce turnaround time.

Sequencing has been and will remain a valuable method for diagnostic laboratories. As a test, it fills a niche that other tests cannot. As discussed, sequencing is most valuable in defining novel viruses and viruses that change frequently, such as RNA viruses. Rapid tests will replace some of the need for sequencing with the availability of more and more sequences, however, in my opinion nature will always have something new in store that will require a little more work to identify.

MOLECULAR, SEROLOGIC, AND VACCINE-CHALLENGE STUDIES ON A NEW INFECTIOUS BRONCHITIS VIRUS VARIANT ISOLATED FROM BROILERS IN CALIFORNIA

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SUMMARY

A new infectious bronchitis virus (IBV) variant detected in 2004 and designated unknown/CA737/04 was isolated from broilers in California with clinical signs consistent with infectious bronchitis. Reverse transcriptase-polymerase chain reaction /restriction fragment length polymorphism analysis and sequencing of the hypervariable region in the S1 gene showed that this variant virus was similar to an isolate of IBV designated K142-02 isolated from broilers in Korea in 2002. Based on virus-neutralization testing in 10-day old embryonating eggs, the unknown/CA737/04 isolate was not related to known vaccine viruses, confirming the uniqueness of this variant virus. *In vivo* protection studies conducted in chicks indicated that a combination of IBV vaccines that includes Mass/Conn at one day of age followed by Holland/Conn at two weeks of age provided 80% protection from disease.

INTRODUCTION

In 2004, an outbreak of infectious bronchitis in broilers in California led to the isolation of a new IBV isolate designated unknown/CA737/04. The birds had typical upper-respiratory tract signs of infectious bronchitis including watery eyes, excessive mucus in the nares and trachea, swollen sinuses, tracheal rales, and sneezing. Kidney lesions were not observed. The virus was isolated from the upper-respiratory tract, and was propagated in embryonating chicken eggs. Signs and lesions produced in the embryos included stunted, hemorrhagic, and curled embryos with ureate deposits observed in the kidneys of some embryos.

In this study we typed the virus by reverse transcriptase-polymerase chain reaction /restriction fragment length polymorphism (RT-PCR/RFLP) and examined the sequence of the hypervariable region of spike gene to determine the relatedness of this new virus with other strains of IBV (2, 3, 4, 5). We conducted virus-neutralization testing in embryonating chicken eggs to determine the serologic relatedness of the virus to commonly used vaccines. And finally, we examined the pathogenicity of the virus in leghorn and broiler chicks and conducted vaccine/challenge studies to determine if currently available commercial vaccines could protect against the virus.

METHODS AND RESULTS

Viruses and virus propagation. The strain of IBV used in this study was a field virus designated Unknown/CA737/04 (CA737) provided by Dr. P. Woolcock (one of the authors) and sent to our laboratory with permission from the GA State Veterinarian (Dr. L. Myers, GA Dept of Agriculture, Atlanta, GA). Reference IBV strains include Ark-DPI (1), Conn (Dr. J. Gelb Jr., University of Delaware, Newark, DE), and Mass 41 (American Type Culture Collection, Gaithersburg, MD, VR-21). Commercial vaccine viruses were obtained from the manufacturers.

Molecular characterization. The RT-PCR/RFLP test used for typing IBV isolates was conducted on the CA737 isolate and it was found to be different from all other IBV types examined to date. The closest isolate related to the CA737 virus was an isolate from Korea designated K142-02 at 72.7% relatedness within the hypervariable region of the spike gene as determined by the nucleotide-nucleotide BLASTn and protein-protein BLASTp search analyses conducted on-line at the National Center of Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/).

Two-way cross virus-neutralization testing. To determine the serologic relatedness of the CA737 virus to standard vaccine strains, virus neutralization testing in 9-11 day old embryonating eggs was conducted. The Archetti and Horsfall relatedness values were calculated and showed that the CA737 California virus was not related to Ark, Conn, or Mass vaccine viruses.

Pathogenicity study. The pathogenicity of the CA737 virus was tested in SPF leghorn chickens. Chickens were housed in positive-pressure Horsfal isolation units. Feed and water were given *ad libitum*. The CA737 isolate was used to challenge 10 SPF leghorn chicks by eye-drop at one week of age ($3x10^4$ EID_{50}/ bird). At five days post-challenge, all of the challenged birds (10 out of 10 total) had severe clinical signs that included tracheal rales, watery eyes, swollen sinuses, nasal discharge, and sneezing. The presence of the virus in the upper-respiratory tract was determined by RT-PCR and challenge virus was detected in 10 out of 10 birds. It was not detected in the non-challenged control birds.

Vaccine-challenge study. Twenty broiler chicks were housed as above and vaccinated with B1/Mass/Conn at one day of age and with either DE072/Arkansas vaccines or Holland/Conn vaccines at 14 days of age. All of the vaccinated birds in each group were challenged with $3x10^4$ EID_{50} of the CA737 IBV isolate per bird seven days following the last vaccination (21 days of age). A non-vaccinated/non-challenged negative control group and a non-vaccinated/challenged group containing 10 birds each were also included in the experiment. All of the birds were killed and necropsies were conducted five days post-challenge. Efficacy was based on clinical signs and lesions, as well as challenge virus detection.

No clinical signs were observed and virus was not detected in the non-challenged birds. All of the non-vaccinated/challenged birds had clinical signs consistent with IBV infection and virus was detected in 100% of the birds. In the broiler chickens that received B1/Mass/Conn followed by DE072/Arkansas vaccination 90% had clinical signs and virus was detected in 90% of the birds. The broiler chickens that received B1/Mass/Conn followed by Holland/Conn vaccination had clinical signs in only 20% of the birds and virus was detected in only 20% of the birds.

CONCLUSIONS

The CA737 virus is molecularly similar to a Korean isolate of IBV and not serologically related to common vaccine strains of IBV used in the USA. It is pathogenic in SPF leghorn and broiler chickens. The best vaccine combination tested was B1/Mass/Conn at one day of age followed by Holland/Conn vaccines at 14 days of age. That combination of vaccines protected 80% of the challenged birds but it is not likely that one component of the vaccine combination afforded protection since the vaccine and challenge viruses are not serologically related. It is well known that cross-reactive antibodies can be generated when multiple vaccinations with the same serotype of IBV are given. Possibly cross-reactive antibodies were responsible for the protection observed in the birds since the Mass/Conn types (Holland is a Mass type virus) were given at both one and 14 days of age.

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THE FREQUENCY OF INFECTIOUS BRONCHITIS VIRUS ISOLATIONS FROM LAYERS AND BROILERS

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SUMMARY

The Turlock Branch of CAHFS serves the Upper San Joaquin Valley of California, which is the heart of the California poultry industry. San Joaquin, Stanislaus, and Merced counties have about 8.6 million layers on about 283 farms (1). A rough estimated number of broilers in these three counties is about 33 million birds on about 150 farms (2). These two production types are frequently in close geographic proximity to each other. Over the years, concern has been expressed on the possibility of infectious bronchitis virus (IBV) spread between layer and broiler operations. To gain some insight into this possibility, the incidence of IBV isolation over a ten year period at the Turlock Branch of CAHFS was undertaken.

Between January 1995 and December 2004, 1708 layer necropsy cases (13,000 birds) and 1334 broiler necropsy cases (11,980 birds) were performed. IBV was isolated from 67 layer cases (3.9% of total layer cases) and 340 broiler cases (25.5% of total broiler cases). Viral isolation attempts were performed more frequently in broiler cases (56%) than layer cases (33%). Further characterization of the IBV isolated was also performed more frequently when the IBV was isolated from broilers. The CAHFS avian virology section utilizes a polyacrylamid gel procedure on the IBV proteins to identify Cal-like isolates of IBV and a fluorescent monoclonal antibody procedure to identify Mass, Conn, or Ark type isolates of IBV. Positive IBV typing by these two procedures was possible on slightly less than half of the IBV isolates. The positive typing results of isolates from layers and broilers by year are listed in Table 1.

IBV infection appears to be a much more frequent problem in broilers and subsequently investigated into a greater depth. There appears to be little similarity in the frequency of IBV type identified between layer production and broiler production. The IBV type probably reflects the vaccination program utilized by these production types. A closer examination of a small subset of IBV isolates analyzed by sequencing a segment of the S1 protein will be presented.

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Table 1. IBV typing results from layer and broiler operations from isolations of IBV during 1995 through 2004.

Year		Layer IBV isolates				Broiler IBV Isolates		
	Conn	Mass41	Ark99	Cal	Conn	Mass41	Ark99	Cal
1995						5	20	9
1996			1			5	7	10
1997								8
1998				1				
1999	*	1	*	*	1	7	1	
2000					1	1		
2001				2		1		1
2002		1		2	7	1		
2003		1			10	2	1	8
2004		2			1	3	3	
Totals	0	5	1	5	20	25	32	36
* Cal/Con/Ark; variant 97								

CHARACTERIZATION OF FIELD ISOLATES OF INFECTIOUS LARYNGOTRACHEITIS VIRUS FROM ONTARIO

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A sudden increase in the number of cases of infectious laryngotracheitis virus (ILTV) occurred in the fall of 2004 when five broiler flocks in Niagara Peninsula were diagnosed with ILT. At about the same time two more ILT cases occurred in Eastern Ontario and one in a backyard layer flock in the Waterloo Region. ILT cases from Eastern Ontario were believed to be a result of adverse vaccine reactions, but there were speculations that the Waterloo Region ILT case was linked to the Niagara Peninsula ILT outbreak that the outbreak was spreading.

Objectives of this study were to 1) examine the relatedness among ILT viruses from Niagara Peninsula at a molecular level; 2) determine whether ILT viruses from Niagara Peninsula were related to other Ontario field isolates or not; 3) determine whether ILT viruses from Niagara Peninsula were related to four vaccine viruses or not. Results of molecular analysis would also provide objective data to confirm or disprove the hypothesis that the case from Waterloo Region was somehow linked to the Niagara Peninsula outbreak

In summary, we used PCR-RFLP analyses of ICP4 and glycoprotein E genes and partial sequencing of UL47/glycoprotein G genes to examine at a molecular level ten ILTV field isolates from 2004 and early 2005 and four ILT vaccine viruses. We determined that:

1) Niagara Peninsula ILT viruses were identical among themselves, because there were no detectable differences among these isolates at a molecular level;

2) Niagara Peninsula isolates were at a molecular level different from other field isolates that were examined during this study;

3) Niagara Peninsula isolates were at a molecular level different from all four vaccine viruses that were examined.

Our results also disproved the hypothesis that the Waterloo Region layer flock was somehow a part or linked to the Niagara Peninsula ILT outbreak. We demonstrated that the Niagara Peninsula outbreak was an independent cluster of ILT not related to other cases which occurred in Ontario during 2004 and early 2005. The origin of virus which caused the Niagara Peninsula outbreak remains unknown.

(A full-length article has been submitted for publication.)

AN UNUSUAL HERPESVIRUS INFECTION IN BROILERS FOLLOWING AREA VACCINATION FOR INFECTIOUS LARYNGOTRACHEITIS

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Infectious laryngotracheitis (LT) is a highly contagious respiratory disease affecting poultry throughout the United States and many other countries. Infection with LT can result in severe economic losses through the inability to export, increased mortality, and production performance losses. The poultry industry has placed major emphasis on both limiting exposure to LT and protecting against severe clinical signs by use of vaccines. While these attempts have produced some progress, LT is still endemic in some parts of the country.

LT is caused by a herpes virus and several different strains are responsible for disease in chickens. Strains vary in virulence from the extremely virulent that causes significant mortality to low virulent with mild clinical signs. Strains can be differentiated by analysis of DNA patterns with restriction endonucleases. This is important in differentiating vaccine strains from field strains, affecting U.S. export status to some countries.

Clinical signs depend upon the strain and can range from moderate with performance loss to severe respiratory disease and death. Signs are characterized by nasal discharge, moist rales, gasping, marked dyspnea and expectoration of blood-stained mucus from the trachea. Diagnosis is usually made by recognition of typical clinical signs and is confirmed by histopathology and PCR testing. Serology is less useful but elevated ELISA titers are usually found with clinical signs and a positive histopathology result.

Control of LT in chickens is difficult. Since most LT vaccines are modified live viruses, vaccine spread and reversion to virulence are a problem. Modified live LT vaccines are either of tissue culture origin (TCO) or chick embryo origin (CEO). The TCO vaccines, which are milder and less likely to spread and "heat up," are most effective when given by eye drop to each individual bird, making them less useful for mass broiler application. The CEO vaccines are more effective than TCO vaccines when given by mass application but tend to pass from bird to bird and become more virulent over time. Also, since they are herpes viruses, the vaccines given to long lived birds reside in the trigeminal ganglia and tend to recrudesce when the bird comes under stress. This is believed to be one of the reservoirs of the disease in the industry.

Broiler vaccination is typically done in an expanding grid fashion surrounding an original infected farm. Vaccination is continued in these areas until it is deemed reasonably safe to stop, depending on the epidemiological layout and the time of year, as the virus is less viable in the summer months. Once a vaccination program is discontinued, there will be no more use of the vaccine in broilers in that area unless there is another infectious outbreak.

Over the past few years, poultry veterinarians in Georgia and Alabama have been combating a pathogen that causes mild conjunctivitis in young birds. The signs consisted primarily of foamy tears and mild conjunctivitis and appeared to increase reactions to modified live Newcastle/bronchitis vaccines. Since tissue from these birds would occasionally be positive on some LT PCR tests, it was termed silent LT. The causative virus was never successfully isolated and the condition appeared to have disappeared in most areas.

In early 2002, an unusual respiratory condition appeared in a broiler flock located in a production division in Northern Florida. The disease occurred in 52 day old broilers and presented as a marked increase in mortality (six per thousand per day), corneal ulcers, severe conjunctivitis and sinusitis. The birds appeared very depressed and noise levels were very low in the houses. Blind birds refused to move and would die quickly when picked up or disturbed. Tracheas were only mildly affected, if at all, and air sacs and lungs showed no inflammation. Other internal organs appeared normal. Histopathology showed multifocal ulcers of the conjunctiva, edema and hypercellularity of the cornea with ulceration and mild to moderate degrees of lymphocytic infiltration and deciliation of the trachea. No intranuclear inclusion bodies were noted on histopathology.

Serology for Newcastle and infectious bronchitis was unremarkable but ELISA titers for LT had a mean of 615, a GMT of 158 with individual titers ranging from 0-1622. Conjunctival tissue injected into embryos resulted in pock formation on the CAMs. The virus was not successfully isolated and identified.

In late 2003 and 2004, a similar situation occurred in another broiler complex in Northern Georgia. The "silent LT" had been observed for several months when a 45 day old broiler flock with high mortality

was observed to have identical signs to those from Florida. Histopathology from tissue taken from this flock was almost identical to those from the last flock. Antibody titers were also comparable and a positive LT PCR on conjunctival tissue was recorded at the Poultry Diagnostic and Research Center in Athens, Georgia. Again, the virus was never successfully isolated. While only one flock was reported from the Florida complex, there were many subsequent affected flocks in the North Georgia complex. The condition occurred sporadically for about a year and has since disappeared.

In late 2004, the same appearing condition occurred in a complex in North Alabama. The histories, clinical signs, and lab findings were nearly identical to the last two area outbreaks. The condition persisted in this division through most of 2005 and then disappeared about as quickly as it had come. The LT PCR positive finding from the State Diagnostic Laboratory in Alabama marked the second lab that had identified the virus as a herpes virus related to LT. Interestingly, the corneal ulcers identified by histopathology were identical to ammonia toxicity. These lesions occurred in flocks that could not possibly have elevated levels of ammonia and may be the first corneal ulcers described that did not result from ammonia burn. Economic losses from decreased performance were significant, as it was in the other two divisions.

In comparing the three divisions described, it was found that they had a similar history of LT control. In every division, there had been extensive area vaccination of broilers with CEO vaccine within the last two years. Also, signs typical of "silent LT" had been noted in each division prior to the eruption of the severe signs as described. It is not known if the earlier vaccination had contributed to the sequelae of disease that followed. Had a minor atypical strain been present in the vaccine that overgrew and became dominant over time or had the virus mutated to a form with atypical signs of LT? Since we were unable to isolate the virus in these episodes, we will most likely never know. In any event, it does show us that usage of modified live vaccines in highly intensive animal agriculture can have its risks, and better and safer vaccines will continue to be needed for our industry.

MOLECULAR DATING OF THE EPIDEMIOLOGICAL EVENTS IN RELATION TO THE EMERGENCE OF VERY VIRULENT INFECTIOUS BURSAL DISEASE VIRUS

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ABSTRACT

The very virulent (vv) strains of infectious bursal disease virus (IBDV) were isolated first in Europe during the late 80s and soon after causing worldwide pandemic outbreaks in the early 90s. The enhanced virulence of vvIBDV was later proposed to be related to the unique mutations on both genome segments A and B.

Using sequence data of both genome segments collected locally during 2001-04 and available database sequences with known sampling dates, here we estimate the time of divergence of the most recent common ancestor (TMRCA) of all vvIBDV pandemic strains and its demographic history in a Bayesian framework using Markov Chain Monte Carlo sampling (BMCMC) under both strict and relaxed molecular clock methods. Based on the VP2 sequences, the TMRCA of all vvIBDV strains was estimated to be at around 1965 (1956-1975), more than 20 years before the onset of its worldwide pandemic outbreaks. The VP1 sequence of all vvIBDVs formed a mono-phylogenetic group that excludes all the other serotype I and II IBDVs, indicating that VP1 of vvIBDV may be reassorted from an unidentified reservoir and this reassortment event was estimated to be at around 1977 (1966-1979), coinciding with the TMRCA of most of the vvIBDV pandemic strains at 1979 (1975-1983).

Moreover, our estimates on the demographic behavior of vvIBDVs showed a transition from a constant population size to rapid exponential population growth at around 1982 (1978-1985), which coincided with the early reported outbreaks and its explosive transmission manner in the late 80s.

Based on these findings, we conclude that vvVP2 may have been emerged more than 20 years before the observed vvIBDV outbreaks in Europe. The rapidly worldwide spread of vvIBDV in the late 80s is likely to be related to a single epidemiological event, which is proposed to be the reassortment of the vvVP1.

(The full-length article will be published.)

ADAPTATION OF INFECTIOUS BURSAL DISEASE VIRUS TO MACROPHAGES

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INTRODUCTION

Infectious bursal disease (IBD) is a naturally occurring disease of chickens. IBD is of major importance to the poultry industry, as it causes immunosuppression that inhibits protective responses to widely used vaccines against other pathogens and renders chickens susceptible to opportunistic infections (1, 2, 3, 4, 6). IBDV has been controlled by a vaccination strategy based on immunizing hens with inactivated virus, combined with chick vaccination using attenuated virus (9, 6). However, with the emergence of antigenic variants of IBDV in the USA, the conventional vaccination programs are not always effective (7). In addition, some live attenuated IBD vaccines induce bursal lesions and immuno-suppression that compromise performance (5, 8). Immunosuppression caused by the field and the vaccine strains of IBD virus (IBDV) is a significant concern for the poultry industry. There is a need for effective vaccines that would be protective but not immunosuppressive. In the present study, we attenuated IBDV by serial passages in macrophages and examined the adapted virus as a possible candidate for a desirable vaccine.

SUSCEPTIBILITY OF MACROPHAGES TO IBDV AND ALTERED TROPISM OF THE VIRUS RECOVERED FROM MACROPHAGES

The classical (cIBDV) and variant E Del (vIBDV) strains of IBDV were serially passaged 12-13 times in NCSU cells, a chicken macrophage cell line. Both viruses replicated in NCSU cells. Virus genome was detected by RT-PCR and replicating virus was identified by immunofluorescence and immunocytochemistry. The original viruses and those recovered from NCSU cells were titrated in DF-1, an immortalized chicken embryo fibroblast cell line. As expected, the original cIBDV and vIBDV failed to replicate in DF-1 cells. However, IBDV recovered from NCSU cells after one or 12-13 passages readily infected DF-1 cells and propagated to high titers. These results indicated that IBDV changed its tropism after one passage in a macrophage cell line.

ATTENUATION AND IMMUNOGENIC POTENTIAL OF IBDV ADAPTED TO REPLICATE IN MACROPHAGES

In vivo characteristics of macrophage-adapted cIBDV (mcIBDV) and vIBDV (mvIBDV) were examined in specific-pathogen-free chickens. Groups of eighteen-day-old embryonated eggs were inoculated with 0.1 mL of virus suspension containing 2 x 10^3 $TCID_{50}$ of mcIBDV or mvIBDV. *In ovo* inoculation of either virus did not affect hatchability of eggs ($p>0.05$), caused minimal follicular damage in the bursa and did not compromise the *in vitro* mitogenic response of spleen cells. However, both viruses were highly immunogenic and induced well pronounced antibody response in chickens. Most notably, when mcIBDV was compared with a commercial attenuated live IBD vaccine virus, spleen cells from the birds exposed to mcIBDV had higher gene expression of interferon-γ than those from the birds given the commercial vaccine virus.

Upregulated interferon-γ gene indicated an enhanced cellular immune response. mcIBDV and mvIBDV protected chickens against a challenge with virulent cIBDV and vIBDV. These results indicated that macrophage-adapted IBDV was non-immunosuppressive but immunogenic and induced well-pronounced protective humoral and cellular immunity in chickens.

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FINDINGS AND PROBLEMS ASSOCIATED WITH AN IN-DEPTH LABORATORY INVESTIGATION INTO A BROILER RANCH

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This investigation was initiated based on a runting and stunting problem observed in the previous flocks on a broiler ranch. The farm under surveillance consisted of six houses with a total capacity of about 120,000 birds. Ten birds per house were selected each week during the growing period for laboratory examination. The initial focus was on virus isolation of liver, pancreas/cecal tonsil pool, tendon pool, and electron microscopy of intestine to document the possible virus involvement in the runting/stunting syndrome. In addition, serological and histological examinations were performed. Part way through the growing period, it became apparent that a bursal problem was present. Although the birds had been vaccinated for infectious bursal disease virus (IBDV) at seven and 17 days of age, damage was detected in the bursa from an early age up to the end of the grow-out period. An IBDV strain was detected by RT/PCR-RFLP which had sequences across the hypervariable VP2 region identical to the T1 strain except for one position.

The T1 strain is designated as a hot strain which causes marked atrophy of the bursa in three to four days. It also breaks through maternal immunity to IBDV produced by conventional variant and classic strains (1).

The average weight at the end of the run and the weight gain records showed no indication of a runting and stunting problem. The significance of isolating reovirus in the first three weeks, and then later reovirus and adenovirus, is unknown. Since no inclusion bodies were found in the livers, and the reovirus was isolated primarily from healthy birds, the significance of these viruses is probably minimal.

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RECENT OUTBREAKS OF HIGHLY PATHOGENIC AVIAN INFLUENZA AND ADVANCES IN DIAGNOSIS

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INTRODUCTION

Since 2002 there have been seven reported outbreaks of highly pathogenic avian influenza (HPAI) throughout the world, four caused by H7 subtype and three by H5 subtype viruses. Each of the outbreaks were unique, but in terms of size and impact, all pale in comparison to the outbreak of HPAI H5N1 that has been ongoing since 2003 in Asia and more recently in Eastern Europe. The Asian H5N1 outbreak has now involved 15 countries and caused death or destruction of more than 150 million poultry. In addition, the virus has infected more than 142 people, killing 74 (14). These events have elevated the public awareness and interest in AI to unprecedented levels, mostly driven by intense media coverage on possible emergence of an influenza pandemic virus. This presentation will provide a general overview of recent outbreaks of HPAI reported throughout the world with emphasis on the HPAI H5N1 outbreak in Asia and Eastern Europe. In addition, recent advances in AI diagnostics will be reviewed.

THE HPAI H5N1 ASIAN (BIRD FLU) OUTBREAK

Although most of the attention surrounding the outbreak of HPAI H5N1 in Asia has been focused on events that have occurred since late 2003, the origin of the problem can be traced back to 1996 when a HPAI H5N1 virus was first isolated from clinically affected domestic geese in Guangdong Province, China. The H5 goose/Guangdong/96 H5N1 virus was later shown to be a component of the precursor virus for the 1997 outbreak of HPAI H5N1 in the Special Administrative Region (SAR) in Hong Kong that infected chicken in three farms and 18 humans; six of the human infections were fatal (1). The human infections were linked to contact with infected poultry in the live poultry markets in Hong Kong. It was the event in Hong Kong in 1997 that spawned the term "bird flu" that now seems to be used frequently, albeit incorrectly, to describe most all AI infections in poultry. Between 1999 and 2003 several additional events involving HPAI H5N1 in poultry, the live poultry markets (LPMs), wild birds, and humans occurred in Southern China and/or Hong Kong, suggesting that the HPAI virus continued to circulate in the region before the outbreak in 2003. However, only the events in Hong Kong were public knowledge at the time.

The current outbreak involving HPAI H5N1 first came to the world's attention when eight Asian countries (Korea, Vietnam, Japan, Thailand, Cambodia, Laos, Indonesia, and China) reported the presence of the HPAI H5N1 virus in poultry between mid December 2003 and the first week in February 2004. Most of the infections were detected in backyard poultry and domestic ducks. Although relatively few large commercial facilities were infected with the virus, many of the commercial farms in close proximity to the infected backyard flocks were destroyed as a precautionary measure to prevent spread of disease. In August 2004, the disease had spread to Malaysia.

The lack of knowledge in the epidemiology of the H5N1outbreak in Asia was likely a major contributing factor in spread of the disease throughout the region. Early theories on methods of spread focused mainly on migratory birds. Since little could be done to prevent the spread by wild birds, limited actions were taken to enhance biosecurity in backyard flocks during the early stages of the outbreak. In addition to the wild bird theory, there was also evidence to suggest that the virus may have been disseminated through local and international markets by the movement of poultry and/or poultry products, especially domestic ducks and/or duck meat. Evidence for spread of the virus through movement of poultry meat is supported by the isolation of HPAI H5N1 virus from frozen duck meat exported from China to Korea in 2001 and to Japan in early 2003 (10, 13). The movement of live poultry from backyard flocks to local live poultry markets very likely contributed to local spread of disease during the early stages of the outbreak. As the outbreak progressed, it was found that domestic ducks remained asymptomatic even when infected with the HPAI H5N1 virus, and shed large quantities of virus in feces (15). China raises about 70% of the world's domestic ducks, of which the majority are raised out-of-doors in open rice fields. This practice has great potential for H5N1 virus contamination of the environment, thus increasing the chances for further spread of the disease to susceptible backyard poultry, wild birds and humans. Spread of disease could also have been

through activities involving game fowl (fighting chickens), an activity deeply engrained in many Asian cultures. The unrestricted movement of these birds would also have great potential to spread the virus over long distances. However, there is increasing evidence to support the theory that H5N1 virus, in some situations, was spread by wild aquatic birds. In early April 2005, thousands of wild aquatic birds were found dead in and around Lake Qinghai in Qinghai Province, China. The HPAI H5N1 virus was isolated from several species of birds, but since there were infected poultry in the region, conclusive evidence incriminating wild birds was lacking. In July and August 2005, there were numerous reports of the isolation of HPAI H5N1 from backyard poultry and wild birds in Russia (Siberia), Kazakhstan, and Mongolia, where the medium of introduction is suspected to be wild birds. Since August 2005, the disease has spread north and westward across the Ural Mountains to Romania, Turkey, and Croatia. The Ural Mountains provide a natural division between Eastern Europe and Asia. As concerns about the movement of the disease into Eastern Europe and the continued spread of the virus via migratory birds, many countries in the Middle East, Africa, and the Indian subcontinent are increasing surveillance for H5N1 in migratory birds and poultry (3, 4, 5, 6, 7).

By genetic analysis, the Asian H5N1 viruses isolated since 1996 have shown considerable genetic diversity, suggesting evolution of the virus over time, perhaps in different ecosystems. For example, the lineage of HPAI H5N1 virus that first appeared in Hong Kong in early 1997 has not been isolated since the LPMs were depopulated in December of that year. Most viruses isolated since 2003 fall into one of two major genetic clades, designated clade 1 and clade 2 (16). The majority of human isolates from Thailand, Vietnam and Cambodia are in clade 1. Viruses that comprise clade 2 are the human isolates from Indonesia as well as isolates from poultry and wild birds in China, Russia, Kazakhstan, Mongolia and Eastern Europe. The HPAI H5N1 viruses found in wild bird species in the Qinghai Lake, China are closely related to those from wild birds in Russia, Kazakhstan, Mongolia and Eastern Europe, suggesting wild birds as playing a role in the wide dissemination of the virus. However, it is not yet clear in all cases whether the wild birds or poultry are the original source of the virus in new geographical regions.

Since November 2005, several Asian countries have reported several new outbreaks of HPAI H5N1. China, for example, has reported new outbreaks in eight of the 30 provinces during November and one in December. Also, China reported the first cases of H5N1 in humans (five infections, two fatalities) in late 2005. Because of the increase in new cases in poultry and humans, China has decided to vaccinate all 14 billion poultry and waterfowl that are produced annually in the country. Vietnam has similarly initiated a country wide vaccination program for all poultry.

Surveillance for H5N1 is becoming a high priority in countries that share major migratory flyways with Asia and Eastern Europe where infected birds have been found, with the hope to detect infections early and prevent the spread to domestic poultry. The progressive spread of HPAI H5N1 virus into new regions will require proactive intervention by the countries at risk. It is important to note that wild birds dying from the infection with the H5N1 virus are most likely indicators, and not the ones responsible for dissemination of the virus. More research is needed to identify the species birds that can serve as carriers capable of spreading the virus over long distances.

The prospects for a quick resolution for the HPAI H5N1 problem in Asia is uncertain. However, the international community, including the U.S. and specifically USDA, is reaching out with assistance to countries affected by AI in hopes of controlling the spread of the virus.

OTHER OUTBREAKS OF HPAI IN THE WORLD

Since 2002, six additional outbreaks of HPAI have occurred worldwide. Two of the outbreaks were caused by H5N2 subtype viruses (USA/2004 and South Africa/2004), two by H7N3 subtype viruses (Chile/2002, Canada/2004), and two by H7N7 viruses (The Netherlands/2003 and North Korea/2005). The largest of the six outbreaks occurred in The Netherlands (8) and Canada (2) where 30 million and 19 million poultry respectively, were destroyed. The Netherlands outbreak (H7N7) was also unusual because of the 86 human infections, one involved a veterinarian who died as a result of the infection; however, most other human cases resulted in severe conjunctivitis. Two human infections (conjunctivitis) were also recorded during the Canadian (H7N3) outbreak; both individuals were engaged in depopulation activities and recovered without complications.

The 2002 outbreak of HPAI in Chile represented the first report of avian influenza in South America (12). The outbreak began as a low pathogenicity (LP) H7N3 infection in broiler breeders that mutated into a HPAI virus with 30 days. The outbreak was limited to two premises: the broiler breeder flock and a nearby turkey flock. The HPAI virus was unusual in that it did not meet the molecular criterion for HPAI as defined by the World Organization for Animal Health (OIE). The unusual characteristics of the Chile HPAI virus has lead to a major change in reporting requirements to the

OIE, namely that all H5 and H7 infections in poultry are now notifiable to the OIE. Likewise, the outbreak in Canada began as a LPAI H7N3 infection that mutated to HPAI in less than two weeks. Both the Chilean and Canadian H7N3 viruses (genetically unrelated) became highly pathogenic by the unusual process of non-homologous recombination, when a 30 and 21 nucleotide segment, respectively, of RNA was inserted near the cleavage site of the hemagglutinin protein. The 30 nucleotide segment of RNA in the Chilean virus came from the nucleoprotein gene, whereas the 21 nucleotide segment of RNA in the Canadian virus came from the matrix gene.

The HPAI H5N2 Texas outbreak was the first in the U.S. since the 1983-84 HPAI H5N2 outbreak (9). The infection was limited to a single broiler flock that supplied birds to the live bird markets (LBMs) in Houston, TX. Although the H5N2 virus met the molecular criterion for HPAI as described by the OIE the virus did not produce disease in experimentally inoculated chickens.

The HPAI H5N2 outbreak in South Africa was limited to ostriches and did not spread to poultry (OIE report).

In March, 2005 mortality in North Korean chickens was reported (OIE report). A HPAI H7N7 AI virus was isolated; however, molecular analysis of the isolate is needed to determine the origin of this virus. Subtype H7 has not previously been reported in Asia. Quick action by regulatory officials in North Korea prevented the spread of virus additional nearby flocks.

RECENT ADVANCES IN AI DIAGNOSTICS

Early detection of AI infections in poultry and rapid assessment of the virulence of AIV are critical for development of appropriate strategies to control the spread of avian influenza. Diagnostic tests that are currently used to diagnose infections of AI in poultry include isolation of the virus in embryonating chicken eggs and detection of specific antibodies in serum or yolk. In some cases, the virus can be detected in clinical specimens by the use of monoclonal antibody-based antigen capture tests such as Directigen (Beckton-Dickenson, Sparks, MD) and Flu Detect (Synbiotics, San Diego, CA), but these tests lack sensitivity for routine use and are not currently licensed for veterinary use. Most diagnostic tests are time consuming, expensive, and require a diverse set of reagents. Recently, a molecular assay was developed to detect AIV RNA in clinical specimens that can reduce the time for diagnosis to less than three hrs. This rapid test is a real time reverse transcription-polymerase chain reaction (rRT-PCR) developed by Spackman *et al.* (11). The assay is a one-step procedure that utilizes specific primers designed to amplify the target sequence. Non-extendible fluorogenic hydrolysis (Taqman) probes are used to monitor amplification of the target sequence after each PCR cycle, thus providing results in real-time. The rRT-PCR procedure is comprised of three separate assays; one that targets the matrix (M) gene and two that are subtype specific for H5 and H7 AIV's. The primers and probe for M assay are designed to detect the highly conserved M gene of AIV and, as such, will detect most strains of AIV, regardless of subtype. The H5 and H7 assays are designed to detect most North American H5 and H7 viruses. The H5 primers and probes also successfully detects the Asian H5N1 HPAI viruses (D. Suarez, personal communication).

The AI rRT-PCR assay developed by Spackman *et al.* was initially validated by testing more than 1,500 specimens collected during surveillance studies in the live bird marketing system in northeast United States. The assay was further validated with >3,500 tracheal swab specimens collected during the 2002 outbreak of low pathogenicity avian influenza (LPAI) H7N2 in Virginia. Results obtained by rRT-PCR were compared with virus isolation in embryonating chicken eggs for sensitivity and specificity. Specimens from the VA outbreak were evaluated at the specimen level and at the flock level (consensus of all specimens collected from a single flock). The diagnostic sensitivity (DxSN) and specificity (DxSP) of the assay at the specimens compared well with virus isolation (D. Senne, unpublished data). However, the test has lower sensitivity with cloacal swabs, primarily because of problems related to RNA extraction and presence of PCR inhibiting substances in cloacal swabs. Studies to improve the RNA extraction procedure for cloacal swabs are currently under study.

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A FIELD AND LABORATORY GEODATABASE FOR WILD BIRD AVIAN INFLUENZA MONITORING, ANALYSIS, AND MODELING

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SUMMARY

A basis for monitoring the dynamics and evolution of influenza A viruses in waterfowl in the US is becoming a challenge with traditional methods of data collection and reporting. With continuing complexities of new invasive species, increasing human population, changes in land use and ecological patterns throughout North America it is difficult to develop predictive models or tools to prevent or even stop potential diseases that impact our food and water supply. Traditional methods of data collection and reporting have produced erroneous or insufficient data and have sometimes been slow or have not reached appropriate officials in a timely matter.

A comprehensive geographic information data management system is being designed to aid in analyzing the phylogenetic-temporal-spatial-species relationships of the avian influenza (AI) virus. It is being constructed to handle the framework of a North America-wide data collection model for wild bird field

surveillance work for AI. The system consists of a standardized data entry system including a web-based Intranet data entry system. A geospatial relational database management system is being built using Microsoft SQL server and ESRI ArcSDE. The data entry system will support ODBC clients for authentication and connection to the SQL server. Data for use in querying, reporting, mapping and analysis will be assisted with applications using SQL and ESRI ArcIMS for web-based GIS mapping. The system is designed to promote data integrity, security and privacy of the data being collected.

Data collected with other spatial data layers will be explored using spatial analysis and geostatistic techniques for determining sensitive areas of risk associated to wild bird flyways, natural resource characteristics, properties of wild bird-origin influenza viruses and domestic poultry-origin influenza viruses, and other data sources.

Results obtained from the system will serve as a stimulus and justification to assist wildlife, poultry industry, and regulatory personnel in identifying high risk locations, risk factors, and mechanism for focusing prevention efforts. It will also serve as a planning tool for epidemiologists for determining "sensitive areas" that need additional surveillance, protection, and management. The ultimate goal is to develop predictive modeling for preventing AI in small and large flocks of poultry. Cooperative efforts of this program have been sponsored by the USDA NRI Animal Biosecurity Coordinated Agricultural Project (CAP) for research and education in "Prevention and Control of Avian Influenza in the US."

PREVALENCE AND DIVERSITY OF AVIAN INFLUENZA IN ALASKA

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Migratory birds from six continents pass through Alaska, which provides prime environments for interspecies assemblages and the introduction and transfer of pathogens such as avian influenza between members of overlapping avian migration routes. Avian influenza is a particular concern because of perceived risk for introduction and emergence of high pathogenic strains of the virus. Few previous studies have examined avian influenza in Alaska. In 2005, we initiated the largest single year sampling undertaken in Alaska to describe the prevalence and diversity of avian influenza viruses across Alaska. We sampled over 30 species and as many as 1000 birds from a single species in two ways. For all birds, cloacal swabs were preserved in ethanol for molecular screening and subtyping. Where samples were able to be maintained in a strict cold chain, matching swabs were taken into Viral Transport Media to be used in viral isolation and serological subtyping. Initial results from a sample of 528 ducks in Interior Alaska show 86 samples positive by molecular screening. Molecular screening reveals that no H5 subtypes are present in this sample which includes H3, H4, H6 and H12 subtypes. Positively screened samples that have been subtyped by both molecular and serologic methods correlate 100%. This study will use the 2005 data to refine and expand sampling in 2006.

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RESULTS OF AN AVIAN INFLUENZA VACCINATION-CHALLENGE EXPERIMENT IN DUCKS

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ABSTRACT

Ducks play an important role in Southeast Asia as a source of protein. Because of the avian influenza problems in that area, ducks (domestic and wild) are being regarded as a possible reservoir for the virus, with or without the presence of clinical signs.

This paper describes a challenge experiment carried out in four-week-old Peking ducks vaccinated with two different vaccines (two conventional oil emulsions containing AIV H5N2). A placebo group was left as control. All groups consisted initially of 13 birds, with an additional three unvaccinated ducks in the control group. In the treated groups each bird received 1 mL of either vaccine or placebo subcutaneously. Thirty days after vaccination the birds were challenged with 2.0 mL of a highly pathogenic H5N1 avian influenza isolate from Vietnam (A/dk/Vietnam/TG24-01/05, $10^{6.0}$ EID_{50}/mL). The challenge virus ($2x10^{6.0}$ EID_{50}/mL) was administered orally, intranasally, and intraocularly to each bird. Protection was assessed by the presence of clinical signs and virus shedding at 3, 4, 5, 7, 10, 14, 21, and 28 days after challenge by real time RT-PCR. For this purpose oropharyngeal and cloacal swabs were taken. Blood samples were taken from all groups seven days prior to vaccination and at 22, 29, and 58 days after vaccination (28 days after challenge), when all birds were slaughtered and necropsies were carried out. Serum samples were tested in a hemagglutination inhibition (HI) test using four hemagglutinating units and the results were given as the $\log_2$ of the dilution where hemagglutination is inhibited.

The birds in the vaccinated groups showed an excellent level of protection throughout the observation period (>98%), whereas the birds in the placebo and unvaccinated groups showed clinical signs of disease from the first observation time point, and at the end of the observation period 11 of the 16 birds in the placebo and unvaccinated group had died. Virus excretion was greatly reduced during the first week after challenge in the groups vaccinated with the conventional oil emulsions as compared to the placebo group.

In conclusion, vaccination of ducks at four weeks of age with an inactivated H5N2 oil emulsion resulted in excellent clinical protection and reduction in virus shedding as was shown in the challenge experiment, indicating that vaccination could be used as a choice in the battle against avian influenza.

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(A full report of this study will be submitted to *Avian Diseases*.)

Table 1. Percentage of clinical protection after challenge.

	Days after challenge								
Group	0	3	4	5	6	10	14	21	28
Vaccinated	100	100	100	100	100	100	100	100	100
Vaccinated	100	98	100	100	100	100	100	100	100
Controls*	100	73	67	56	50	47	48	49	52

* Placebo + 3 untreated birds

Table 2. Mean real time RT-PCR Ct values of oropharyngeal swabs (negative = 40).

	Days after challenge							
Group	0	3	4	5	7	10	14	21
Vaccinated	-	38.02	-	38.14	38.09	37.85	39.36	40
Vaccinated	-	39.71	-	38.27	38.77	38.49	40	39.73
Placebo	-	26.31	-	27.06	29.81	37.57	35.54	37.78

Table 3. Average log2 HI titer

	Days after date of vaccination			
Group	-7	22	29[a]	58
Vaccinated	0	4.9	6.2	5.3
Vaccinated	0	6.9	6.4	4.0
Controls*	0	0	0	9.0

[a] challenge one day later

* Placebo + 3 untreated birds

IDENTIFICATION OF AVIAN INFLUENZA VIRUSES FROM WILD BIRDS IN CALIFORNIA

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SUMMARY

Wild birds such as waterfowl and shorebirds are the natural reservoirs for type A influenza viruses (1). Type A influenza viruses are often characterized by the two surface glycoproteins, hemagglutinin (H) and neuraminidase (N) (2). All 16 hemagglutinin subtypes are found in these species, however the subtypes are not evenly distributed among species or locations. The virus subtype found in birds, and the percentage of waterfowl and shorebirds carrying AIV, will vary by migratory flyway and are rarely the same in consecutive years (3). Viruses of the H3, H6, and H4 hemagglutinin, N2, N6, and N8 neuraminidase subtypes are the most common subtypes isolated from waterfowl in North America (4, 5).

Avian influenza virus occurs within the gastrointestinal and respiratory system of birds and is shed in their feces and respiratory secretions. Waterfowl carry the virus in their intestinal tract and shed it in their feces (6). Susceptible birds usually become infected by contact with infected feces and virus contaminated water. The tendency of waterfowl to congregate in large numbers on lakes and wetlands, coupled with the stability of the virus in water, contribute to spread within the population. There appears to be seasonality to viral shedding in waterfowl. Dabbling duck species, particularly mallards, have the highest reported isolation rates with up to 60% of juvenile ducks being infected prior to migration in the late summer (3). In shorebirds, the greatest number of isolations has been in the spring with a second peak during the fall migration (7).

Wild birds in California are currently being sampled in order to investigate the species susceptibility, pathogenesis, and ecology of influenza type A viruses, as well as to complement an on-going avian influenza surveillance program in the United States. Cloacal swabs from live-trapped and harvested waterfowl and various species of wild birds in wildlife rehabilitation centers throughout California are being tested for influenza A viruses.

Cloacal swabs from waterfowl and both cloacal and choanal swabs from other orders of wild birds are collected. Viruses are grown in the allantoic cavity of 10-day-old embryonated chickens' eggs. Confirmation of the presence of type A influenza viruses is made using RT-PCR (reverse transcriptase polymerase chain reaction) and genetic sequencing.

Over the past four months, approximately 700 samples have been collected from a variety of species

of wild birds in California. To date, virus isolation and confirmation by RT-PCR has been performed on 50 waterfowl samples. Of these 50 samples, one influenza A virus has been isolated and is undergoing genetic sequencing. Details of the sequencing will be provided.

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CALIFORNIA LOW PATH NON-H5, NON-H7 AI CONTROL PLAN FOR CUSTOM SLAUGHTER PLANTS

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INTRODUCTION

The Avian Influenza Control Plan was created to provide standards for the monitoring of AI infections in custom slaughter poultry markets. The plan is designed as a voluntary program for the control of non-H5, non-H7 low pathogenicity avian influenza. The Avian Health Group, the California Department of Food and Agriculture (CDFA), and the University of California Cooperative Extension cooperate with markets and their suppliers to validate the program. There are currently 29 markets participating in the program in Southern California.

DISEASE PREVENTION—PLAN SUMMARY

The Control Plan relies on individual markets to design a biosecurity program for their facilities. Each market is required to adhere to Plan's disease prevention criteria. Each set of criteria is designed to prevent to movement of infectious agents throughout the market system.

In order to prevent the spread of disease between markets, delivery drivers are advised to wear protective clothing or remain outside out of the market wherever possible. Markets are asked to provide drop-off areas that will be cleaned and disinfected daily. All cages and racks are to be cleaned and disinfected prior to being loaded on trucks.

In order to limit the risk of infection, and thereby be certified, markets are required to only buy poultry from Certified Producers participating in the program. A certified producer has biosecurity protocols in place to minimize the probability of infection in his poultry production facilities and had tested negative for avian influenza prior to marketing birds on his ranches. In addition, each market is required to participate in a monthly depopulation and disinfection day. On this day, the markets must be free of all live poultry. All bird holding areas are cleaned and disinfected while empty. Representatives from the CDFA/Avian Health Group inspect the markets to assure satisfactory participation. Inspection dates are pre-determined by

the custom slaughter market owners and their suppliers.

Live birds from each market are tested on a monthly basis for avian influenza and exotic Newcastle disease. Testing involves the collection of swab samples from each supplier's birds present in the market at the time of testing.

In the event that a market tests positive for infection, all participating markets and producers are notified. The infected market must depopulate all birds, clean and disinfect all surfaces, and pass inspection by the CDFA. Each supplier to the infected market is required to have eleven birds tested by swab and blood samples as quickly as possible.

OUTBREAK RESULTS

Avian influenza testing was initiated after the program was established. One market tested positive for low pathogenic non-H5 non-H7 avian influenza. All suppliers to that market had been previously identified through the certification program. The suppliers were notified that the market was positive. Each ranch supplying that market tested eleven birds by blood and swabs for avian influenza viruses within three days of the notification. An infected supplier was identified and all of the markets he supplied, stopped taking his birds. The positive market depopulated after which, all markets receiving birds from the infected supplier were retested. Two more markets were found to be positive and both were depopulated. After the final round of depopulation, all markets and suppliers were tested and found to be negative. The infected supplier has depopulated his production flocks and does not plan to repopulate.

GENETIC VARIATION AMONG THE POLYMERASE GENES OF LOW PATHOGENICITY AVIAN INFLUENZA VIRUSES ISOLATED OVER THREE DECADES

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INTRODUCTION

There have been a number of outbreaks caused by avian influenza viruses with low pathogenicity (LPAI) in the United States over the years. Although the LPAI viruses do not cause high mortality, infections in commercial poultry flocks may result in considerable economic losses and there is the possibility that LPAI viruses of the H5 or H7 subtypes may become highly pathogenic viruses as has been observed (1, 2).

Avian influenza virus carries three of its own polymerase genes, PB2 (basic polymerase II), PB1 and PA (acidic polymerase). These polymerases form hetero-trimers, and are involved in many aspects of viral replication (3). PB1 is the core of the polymerase complex and account for the polymerase activity. The polymerases interact with host factors and, therefore, play roles in host specificity (4, 5). A single amino acid in PB2 was identified in determining the host range of influenza A viruses (4). The acidic polymerase, in addition to the role in RNA replication, it may have an unrecognized role in assembly or release of influenza virus virions, perhaps influencing core structure or the packaging of viral RNAs or other essential components into nascent influenza virus particles (6).

Since the polymerases are internal proteins, they are not under the strong selective pressure of the host's immune system as the two surface proteins, hemagglutinin and neuraminidase are. This makes the polymerase genes and their proteins excellent candidates for evolution analysis and the understanding of host adaptation.

To understand genetic background of the variations, we analyzed the three viral polymerase genes of LPAI viruses. We selected 19 viruses isolated from poultry in California and Texas in the last 25 years. They covered a wide range of serotypes, avian hosts, and geographic regions. We present the results of phylogenetic relations of PB2, PB1 and PA proteins

of our isolates and some archived isolates. These analyses of LPAI viruses may help our understanding of the association of gene mutations and changes of host range and virus evolution from LPAI to HPAI.

MATERIAL AND METHODS

Viruses. Virus isolates were obtained through 1979 to 2005 from poultry in California and Texas. They were isolated from various hosts, including turkeys, ducks, chickens, emus, pheasants and quail.

RT-PCR. Viruses were propagated in 10 to 11 day-old SPF chicken embryos at 37.5°C with approximately 40% humidity. Allantoic fluids containing viruses were harvested 48-72 hours post inoculation. Viral RNA was extracted using viral RNA extraction kit according to manufacturer's instruction (InVitrogen, Carlsbad, CA). Reverse transcription was conducted by incubating 500μg of Uni12 primer, (Hoffman, 2001) and 7μL of each RNA preparation at 70°C for 5 minutes. And cDNA was produced by incubating the mixture with MLV-RT at 42°C for 1.5 hours. The RT reaction was terminated at 75°C for 10 minutes. Full length individual influenza genes were amplifies using gene specific primers (Hoffman, 2001) using Pfu (Stratagene, La Jolla, CA) or Ex Taq (TaKaRa, Japan).

DNA sequencing. Two to three microlitters of RT-PCR product was used to clone each gene fragment into pCR2.1 (InVitrogen, Carlsbad, CA). Clones were screened for insert by PCR, and confirmed by restriction enzyme digestion. Three candidate clones of each gene from each isolate were submitted for sequencing using T7 and M13R primers by Davis Sequencing Inc (Davis, CA).

Sequence analyses. For each polymerase gene from individual isolate, three sequence results were aligned using ContigExpress (InVitrogen, Carlsbad, CA). Nucleotide and amino acid sequences were compared among isolates and to the archived sequence data from GenBank. Phylogenetic trees were built using Vector NTI (InVitrogen, Carlsbad, CA).

RESULTS

In this study, the amino acid sequences of three influenza polymerases, PB2, PB1, and PA were analyzed. These polymerase proteins demonstrated relatively less variations as indicated by the similarities of >97%, >98%, and >96% for PB2, PB1, and PA amino acid sequences, respectively, among the 19 isolates.

Phylogenetic analyses suggested that the polymerases shared similar sequences with the viruses circulating during the same period of time and in the close vicinity, regardless of their subtypes and the hosts from which they were isolated. Sequence comparison showed that fewer mutations occurred within the functional domains and more in the structural regions. It also showed that the polymerase subunits of California isolates, the H6N2, and sometimes H10N9, have changes that are different from those observed in the non-functional domains of other subtypes.

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SURVEILLANCE FOR END AND AI IN CALIFORNIA: TARGETING RISK

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Why Californians are concerned about poultry disease surveillance. California's position as a Pacific Rim and South American trading partner, the State's large, diverse and mobile ethnic population, and the Pacific flyway compound the risk of highly pathogenic avian influenza (HPAI) or exotic Newcastle disease (END) being introduced into large populations of broilers, layers or turkeys in this State. Poultry producers, private practice veterinarians and university faculty have joined efforts with Federal and State officials to reduce the risk of an introduction of HPAI or END, and detect and respond to these diseases should they evade disease barriers. While the approach to protect California's poultry is multi-faceted, disease surveillance plays a major role with regard to preparedness, risk communication and market protection.

"Systems" approach to surveillance. The 2001 outbreak of low pathogenic avian influenza (H6N2) in California poultry initiated new, in-depth analysis of several commercial poultry production to market systems in order to identify the equivalent of disease "critical control points." During the large 2002-2003 outbreak of END in Southern California, epidemiologic investigations further illustrated the complexity of poultry market segments and potential for spillover of disease between segments. Recently, studies have been expanded in order to better understand specialty poultry market patterns in different areas of the State, including Northern and Central California. Also, the California Department of Fish and Game is helping to identify areas where wild waterfowl and domestic poultry have increased risk of virus exchange, and UC Davis is conducting wildlife avian influenza surveys.

The result of this collaborative effort is targeted disease surveillance with the goal of identifying END or avian influenza (AI) as near to the point of introduction/adaptation to domestic poultry as possible. Other surveillance goals, such as demonstrating a population of birds is free of disease for export purposes, still exist, but the over-riding animal health goal is early detection of poultry-adapted AI or END based on an understanding of the poultry marketing systems in California.

While surveillance for END and AI in California poultry populations is still evolving, current efforts focus on several populations that may provide early warning of disease introduction as described below. Both active and passive surveillance is being used with the highest frequency of sampling ideally occurring in populations most likely to reflect disease status.

Turkeys, broilers, layers. Employee movement and shared equipment, including processing plants, feed trucks, manure trucks, breeding crews, vaccination crews and rendering trucks can contribute to the spread of disease. Active surveillance is primarily targeted to test just prior to bird movement, during routine health monitoring or at slaughter. Sampling is determined by logistics, probability of infection and consequence of moving an infected bird to a particular location. Passive surveillance also occurs based on clinical signs.

Custom slaughter markets. These markets and their suppliers cater to specialized, often culturally based demands for poultry products. Different types of birds are gathered from multiple sources. People and delivery trucks may travel from market to market. In California, there is rarely a wholesaler involved, and the markets are all under a State or Federal food safety inspection program. Because of the mixing of suppliers entering these markets, potential exposure of some of the species to wild birds, and overlap between suppliers to these markets and other commercial sectors, surveillance is conducted at least monthly at all markets and at most suppliers. State and Federal animal health officials complete much of the testing.

Swap meets, auctions, feed stores. These venues often involve gathering a wide range of poultry from very diverse communities and dispersal to naive populations of birds with potential overlap into commercial poultry systems via people, rendering, feed delivery, or proximity. Surveillance is random and conducted by State and Federal animal health officials. Testing for AI and END in this sector is considered particularly valuable for early disease detection, but can pose trace-back challenges.

Pet and hobby flocks. While these birds are often housed in a fairly isolated manner, they may travel to shows and competitions under stressful conditions which can lead to rapid spread of disease. Owners sometimes help one another with sick birds, further contributing to the spread of disease. Analysis also indicates that there is potential overlap between this population of birds and others primarily due to

proximity and people. Surveillance in this sector is often passive and stimulated by outreach efforts, but there is also an active component targeting game fowl breeders, animal control agencies, and fair coop-in exams.

Summary of AI and END surveillance in California. Implementing an AI and END surveillance plan in California has been important to industry, human health agencies, animal health agencies, and the public. A systems approach and clear surveillance goal have made it easier to communicate the significance of positive and negative tests and react appropriately. This approach has also allowed cooperators to allocate scarce resources to surveillance activities that will provide the most useful information.

In 2005, California laboratories processed tests from approximately 118,000 domestic poultry in accordance with the targeted surveillance plan, and have not detected H5/7 AI or END. Looking forward, collaborators plan to expand the systems analysis, evaluate data and continue to modify and formalize testing goals in each segment of the poultry population.

Related initiatives. Related initiatives include development of rapid testing capacity, automation of information collected during the sampling process, and database development that allows for meaningful evaluation of surveillance results.

The California Animal Health and Food Safety Laboratory (CAHFS) is working to expand rapid testing capacity in this State. Current collaborators include Lawrence Livermore National Laboratory and the National Veterinary Services Laboratory. Some technologies being developed or expanded include real-time polymerase chain reaction with multi-plex capability and new modes of environmental sampling. CAHFS is also collaborating with the California Department of Food and Agriculture (CDFA) and the United States Department of Agriculture (USDA) to automate field sample data collection and implement universal messaging so that the information can be shared between data management systems. Some data collection technologies being used in the field include bar coding, electronic pens, and notebooks. In order to ensure surveillance is adequate in the State, epidemiologists must look at test results in the context of populations. Over the past several years in order to accomplish this analysis, CDFA developed a data management system that can be used to evaluate poultry surveillance data. USDA is now collaborating with CDFA to incorporate user requirements into their Generic Database giving all States access to a more robust system yet enabling each State to maintain separate, secure data.

OVERVIEW OF THE H5/H7 LPAI PROGRAM FOR THE LIVE BIRD MARKETING SYSTEM

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SUMMARY

Over the past decade, recurring cases of low pathogenicity avian influenza (LPAI) in the live bird marketing system (LBMS) and commercial poultry industry have become an increasing poultry health concern in the United States and internationally. Concerns specifically are based on the persistence of an H7N2 LPAI subtype virus in the Northeastern LBMS, the ability of H5 and H7 LPAI viruses to mutate to highly pathogenic (HPAI) viruses, the ability of HPAI viruses to, in some cases, directly infect humans, and the extensive trade sanctions against U.S. poultry exports that have followed limited LPAI or HPAI infections in commercial poultry.

Consequently, the USDA has developed an H5/H7 LPAI monitoring and control program that is a cooperative effort between state and federal government and associated poultry industries. The goals of the program are to: (1) diagnose, control, and prevent LPAI H5/H7; (2) help participants to improve biosecurity, sanitation, and disease control in their operations; and (3) minimize the effects of LPAI in the U.S. commercial poultry industry. The program consists of two components – the commercial poultry industry and the LBMS.

The LBMS segment of the program has been addressed through the development of a Uniform Standards document effective October 2004. This document defines uniform guidelines for all participants in the LBMS, including the producers/suppliers, dealers, haulers, auction markets, wholesalers, and the retail live bird markets in the areas of licensing, AI testing, recordkeeping, sanitation, biosecurity, surveillance, inspections, and response to positive facilities. Participation in this program is voluntary for the States with administration and

enforcement at the state level. Participating States must have requirements for licensing or registration of all businesses within the LBMS and may enact regulations to require the participation of their live bird markets, producers, and distributors.

The H5/H7 LPAI program benefits the poultry industries through increased response and control of LPAI infections when they occur, particularly in the live bird marketing system. Our expectation is that increased monitoring in the LBMS and early detection and response to LPNAI infections is critical to preventing the development of HPAI infections for the benefit of both poultry and human health. This is consistent with the new Code Chapter on Notifiable Avian Influenza (NAI) adopted by the international committee of the OIE in May 2005 and effective January 2006.

COMPARISON OF THE HEMAGGLUTINATION INHIBITION TEST AND THE ENZYME LINKED IMMUNOSORBENT ASSAY FOR ANTIBODY TO NEWCASTLE DISEASE IN NONCOMMERCIAL LAYERS RAISED IN THE BACKYARD OF POOR FAMILIES AND A COMMERCIAL FARM FROM ARGENTINA

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SUMMARY

A commercial Newcastle disease virus (NDV) ELISA and the HI test were performed to serum samples for the detection of NDV antibodies. The layers belong to a social program called *Pro-Huerta* in which chickens are given to poor families. At the same time sera from a commercial layer farm were obtained. Since Argentina has been declared free of velogenic NDV with vaccination in 1997 and backyard birds can be a potential source of the virus, this study was not only to check for the presence of antibodies, but also to compare the results obtained between the ELISA and the HI tests. Due to the economic situation of the country, the possibility of running an ELISA as was done in previous years is somehow complicated and the HI could be another way of helping sanitary controls.

DEEPLY MULTIPLEXED RT-PCR ASSAYS FOR HIGH-THROUGHPUT SURVEILLANCE OF ANIMAL DISEASES

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A critical challenge facing US Agriculture in detecting and responding to outbreaks of animal disease is the availability of rapid, validated diagnostic assays for the detection of multiple diseases or multiple strains of the same disease in a single assay. Laboratory methods currently used to detect animal diseases are generally single agent and can be time-consuming, labor-intensive, and difficult to scale up to meet diagnostic demands in the event of an outbreak.

The Bioassays and Signatures Group (BSG) of the Chemical and Biological National Security Program (CBNP) at the Lawrence Livermore National Laboratory (LLNL) has developed rapid, reliable and sensitive assays geared towards enhancing the National Security of the United States. As such, LLNL is involved in a number of projects with multiple collaborators developing TaqMan, Multiplex, Microarray, and other assays for the early detection of Infectious Disease Threats. For example, a TaqMan assay developed at LLNL in collaboration with the California Animal Health and Food Safety Laboratory

(CAHFS) at UC Davis was used to combat the 2002 exotic Newcastle disease outbreak in California.

One of our most promising current areas of research is the development of deeply multiplexed nucleic acid assays. These assays can detect multiple genome regions of many pathogens in a single tube assay simultaneously and with great sensitivity & selectivity. We have developed two panels of such assays, one targeting human pathogens, and another targeting mammalian agricultural pathogens. We have demonstrated the ability to simultaneously extract and amplify both DNA and RNA targets with a high degree of efficiency. With the help of our many collaborators, our assays are being implemented into the National Animal Health Laboratory Network (NAHLN), and the Laboratory Response Network (LRN).

The currently multiplexed assay can detect 17 distinct genomic regions from a panel of agricultural foreign and domestic viruses that are clinically indistinguishable from Foot and Mouth Disease virus in cattle, sheep and pigs. The 21-plex assay that we have developed is run in a single tube and in addition to the 17 genomic signatures, includes four internal controls. This assay can be run in a 96-well format using standard laboratory instrumentation which is commercially available. We can use the capabilities of the BSG to develop any number of different multiplex panels with a theoretical "plex ceiling" of 100.

Our hope is that with the help of new collaborators with expertise in avian diseases, we can extend the multiplex approach to the development of new assays. We hope to develop both assays for the diagnosis of multiple avian diseases in a single tube (ILTV, *Pasteurella multocida*, FPV, APV, duck viral enteritis, HEV, and APV) as well as assays for the detection and characterization of infectious pathogens whose genetic diversity is beyond the current capabilities of single TaqMan assay detection. Such genetically diverse agents could include but are not limited to infectious bronchitis virus (IBR), avian leukosis virus (ALV), and avian influenza (AI).

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ADVANCES IN THE MICROBIOLOGICAL DIAGNOSIS OF BACTERIAL DISEASES OF POULTRY IN THE PAST DECADE

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INTRODUCTION

A primary role of a poultry bacteriology laboratory is to identify potential poultry pathogens involved in disease processes. Included in this role is the identification of new and re-emerging pathogens, identifying bacterial pathogens to strain level when required for epidemiological purposes, and determining pertinent pathogen properties such as antimicrobial susceptibility patterns. Greater emphasis on food safety issues, bioterrorism and agroterrrorism, and international trade requirements have impacted all clinical veterinary bacteriology laboratories including those with a poultry emphasis. Advances in the microbiological diagnosis of bacterial diseases of poultry during the last decade have been driven by the need to improve laboratories' capabilities in order to fulfill aforementioned roles and to address new challenges introduced during the last decade. In this overview advances in diagnostic bacteriology capabilities are outlined according to the current major roles of clinical bacteriology laboratories.

SUMMARY

Identification of bacterial pathogens. Identification of bacterial pathogens is central to the clinical bacteriology laboratory. There are a number of methods and technologies available to achieve this goal. Proper use of different methods and technologies is critical to arriving at a correct conclusion. *Classical bacteriology methods, commercial identification systems and molecular-based methods* all play a role at some level in identifying poultry pathogens.

Classical bacteriology methods are still employed at some level in most poultry laboratories. In fact, some core tests for any bacteriology laboratory such as the Gram stain have changed little over the years and remain an integral part of overall laboratory testing approach. New selective and differential media developed over the last decade such as various chromogenic culture media and systems for measurement of pre-formed enzyme activity have improved diagnostic capabilities and time to identification when using classical methods. For some common poultry pathogens, the classical methods still

remain the most cost effective and accurate means to reach a desired result.

Commercial identification methods have advanced substantially over the last 30 years. Manual systems based on phenotypic properties and derived numerical profile databases for identification are still commonly used. Today there are commercial systems available that incorporate automated loading, incubating, reading and interpretation of identification cards for bacterial identification. These have evolved to minimize hands on time. Such commercial systems may be well suited for some poultry pathogens such as the *Enterobacteriaceae*, *Staphylococcus* and *Streptococcus*, yet have limitations that must be recognized. Some of these systems' shortcomings include (1) absence of many veterinary/poultry pathogen profiles in these systems' databases, (2) that the fastidious nature of some poultry pathogens makes them unsuited for use in these systems, and (3) that some commercial systems use identification characteristics that are not able to discriminate between closely related veterinary/poultry pathogens because of the tests employed. The expense of fully automated systems may also preclude their use in some laboratories.

Molecular-based methods have found a substantial role in veterinary bacteriology laboratories during the past decade. Numerous molecular-based assays have been described but few have been commercialized because economics do not support their development. A molecular based identification system for identification of selected poultry *Mycoplasma* serotypes is the most common commercially-available, molecular-based system in use today. Many molecular-based assays used in poultry bacteriology are dictated by individual laboratory requirements and have been either developed in-house or optimized in-house by adapting procedures from published literature. As an example, a PCR assay for identification of *Avibacterium paragallinarum* is currently used at the California Animal Health and Food Safety (CAHFS) laboratory system to compliment phenotypic testing for identification of the agent of fowl coryza. This method has been well validated in the literature, as well as in-house. Unfortunately, validation studies for many of molecular-based methods are currently lacking. In addition, a good number of these procedures have not been standardized throughout the veterinary laboratory community. In recent years, more and more of these PCR-based identification assays have been transitioned from a standard PCR format to a real-time format, which should decrease turnaround time and chances for cross contamination.

Since the late 1990s DNA sequencing for identification of specific bacterial isolates has also become a more common procedure in a number of veterinary bacteriology laboratories. Commercially available nucleic acid extraction kits and reagents, centralized or commercial sequencing facilities, publicly available and searchable databases, decreased overall associated costs and turnaround time have made DNA sequencing a powerful tool for the clinical bacteriology laboratory. Currently it is not unreasonable to expect to have a 48-72 hour turnaround time from the time an isolate is in hand until sequence results are available and analyzed. The selection of primers and target genes are critical for DNA sequence analysis to be useful. Currently the 16S rRNA gene is the gene most commonly used for identification of bacterial isolates, although for specific organisms such as *Mycobacterium*, other genes (65 kDa heat shock protein gene) may be more suitable. Sigma factor or RNA polymerase beta-subunit encoding (rpoB) genes may be alternatives to the 16S rRNA gene although current databases for these genes are not very extensive. Sequencing is also useful for identifying clinical fungal isolates where morphologic features are not sufficient for identification. Regions of the large subunit of rDNA or the 5.8S rDNA and flanking spacer regions are most commonly used. Commercially available sequencing kits and databases are available, however, they are expensive and some of the databases have limited species or strain entries. Public databases are free and have a large number of submissions but search results must be closely scrutinized because deposited sequences are not reviewed for accuracy. Public databases that are adapted specifically for bacterial identification and that eliminate inaccuracies found when using sequences in general public databases are being developed. With the increasing availability of commercial or centralized sequencing facilities, minimal equipment (electrophoresis equipment and a thermocycler) is required from the bacteriology laboratory. In our laboratory our first recognition of some newly described pathogens has been through DNA sequencing results. Examples of avian pathogens identified in our laboratory by DNA sequencing have included *Pelistega europea* and *Volucribacter psittacicida*. It is now also possible to identify fastidious or non-viable pathogens directly from tissue samples without prior isolation by directly amplifying genes to be sequenced from a clinical sample. This requires using samples that are normally sterile and/or primers specific for the pathogen of interest.

Whole genome sequencing has been completed for over 200 bacterial species to date. This information should provide new targets for identification of isolates at species and strain levels. Advances in sequencing technology such as the use of microfabricated high-density picolitre reactors and pyrosequencing

technology hold promise that whole genome sequencing may be a feasible diagnostic tool in the future. Currently available systems claim an entire genome can be sequenced by a single technician in three days.

Addressing taxonomy changes. Changes in bacterial taxonomy and the naming of new or previously undescribed pathogens present an ongoing issue for the clinical bacteriology laboratory. In order to provide the most current and accurate information, bacteriology laboratories must incorporate taxonomic changes and newly described taxa into their identification schema. It is important to ensure laboratory end-users are familiar with taxonomic changes in order to be able to relay meaningful laboratory information. Improvements in genetic analysis capabilities over the last decade have resulted in a more logical taxonomic ordering of poultry pathogens although the process has sometimes been painful. The end result is that more logical taxonomic assignment of pathogens results in more meaningful information for individual disease situations and analysis of disease trends. A number of changes to previously well-accepted species and genera of a number of avian pathogens, as well as descriptions of new genera and species have occurred in the last 10-15 years. Some of these changes have included changing *Bordetella avium*-like to *Bordetella hinzii*, *Pasteurella anatipestifer* to *Reimerella anatipestifer*, Bisgaard Taxon 33 to *Volucribacter* sp., combining *Pasteurella anatis*, "*Pasteurella haemolytica*," "*Actinobacillus salpingitidis*" to form *Gallibacterium anatis* biovars and *Haemophilus paragallinarum* to A*vibacterium paragallinarum*.

Also during the last 10-15 years a number of new avian pathogens have been recognized and new clinical presentations identified or at least clarified. Some examples include the identification of *Brachyspira alvinipulli* in intestinal spirochetosis, *Ornithobacterium rhinotracheale* in respiratory disease in turkeys, *Pelistega europaea* in respiratory disease in pigeons, the recognition of an unidentified "*Neisseria*"-like species causing bronchopneumonia in turkeys, and identification *Mycoplasma gallisepticum* causing epornitics in free-ranging finch.

Antimicrobial susceptibility testing. Antimicrobial susceptibility testing using either micro- or macro- broth dilution, agar dilution or agar diffusion methods is well established. These standardized test methods following the guidelines of the Clinical and Laboratory Standards Institute (CLSI), formerly the National Committee for Clinical Laboratory Standards (NCCLS), have been available for human isolates for a number of years. In the last decade a number of events have impacted and improved antimicrobial susceptibility testing in veterinary bacteriology. In 1997 "Tentative" Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals (M31-T) were adopted through CLSI. These standards were advanced to "Approved" status (M31-A) in 1999; providing veterinary bacteriology laboratories, including those working with poultry pathogens, specific guidelines for antimicrobial susceptibility testing, interpretation and reporting. These guidelines provided standardized methods for veterinary bacteriology laboratories to insure the quality and accuracy of results reported. All laboratories performing antimicrobial susceptibility testing for poultry isolates should currently be following these standardized methods and interpretations.

Increased interest in the effect of antimicrobial use in livestock and poultry on human health and food safety has impacted veterinary bacteriology laboratories. Some examples where poultry or livestock pathogens have been suspected as the source of resistant strains for human infections have included the use of avoparcin and its purported link to the development of vancomycin resistant enterococci (VRE) in poultry and humans in Europe, resistance of *Salmonella* isolates from cattle to ceftriaxone and the relation to ceftiofur use, and the increase in resistance of *E. coli* and *Campylobacter* to quinolone antimicrobials used in poultry that ultimately resulted in the withdrawal of the approved use of all quinolones for poultry in 2005. Additionally, the National Antimicrobial Resistance Monitoring System (NARMS), established in 1996, has been collecting annual data on antimicrobial resistance for trend analysis. Sources for isolates tested by NARMS include public health laboratories, healthy animals on farms, and raw products collected from slaughter plants. Also the American Veterinary Medical Association recently published guidelines for the "Judicious Use of Antimicrobials for Poultry Veterinarians." Included in these guidelines are recommendations that historical laboratory susceptibility data be used as a basis for future empirical treatment.

Concerns about antimicrobial resistance, new monitoring practices by various agencies and groups, and more specific guidelines for antimicrobial use have all impacted the laboratory's role in reporting antimicrobial susceptibility results. Current reporting of antimicrobial susceptibility results should include whether a specific antimicrobial belongs in an approved or extra-label category for a particular animal species and ensuring that results for those antimicrobials prohibited under the Animal Medicinal Drug Use Clarification Act are not listed in laboratory reports. Improved reporting provides additional information and guidance for clinicians and producers

in prudent antimicrobial use. Data generated by antimicrobial susceptibility testing for individual premises should also be used by clinicians and producers to perform their own premise antimicrobial susceptibility trend analysis.

Strain typing/fingerprinting of bacterial isolates. Phage typing, antimicrobial susceptibility pattern determination, restriction fragment length polymorphism (RFLP) analysis, ribotyping, and protein profiling have all been used in the past to fingerprint poultry pathogens. During the past decade numerous additional techniques for identifying bacteria to the strain level have been introduced and optimized for laboratory use. These have included randomly amplified polymorphic DNA (RAPD) analysis, repetitive extragenic palindromic PCR (REP-PCR) analysis, pulsed field gel electrophoresis (PFGE), multi-locus variable number tandem repeat analysis (MLVA), and multi-locus sequence typing (MLST). Improvements in molecular-based technology and commercialization of many of the reagents and kits used have made these techniques more suited for use in a clinical laboratory setting. These new methods and commercially available reagents and typing systems have extended clinical laboratories' abilities to provide more detailed information about pathogens recovered. Methods likely to be used in the future for strain analysis include MALDI-TOF-MS, DNA microarrays and whole genome sequencing.

Whether a particular fingerprinting technique is proper to use or not is influenced by a number of factors including the particular organism under investigation and the time period of interest for which strain differences must be detected. As it becomes more common for clinical veterinary laboratories to employ these techniques for outbreak investigations, strain tracking on individual premises or ranches, and differentiation of vaccine strains, it is critical that the appropriate method be used and validated for the intended purpose. Therefore, comparison of bacterial strains should be performed with caution to prevent erroneous conclusions. The importance of ensuring that the proper methodology is used became evident when investigating strains of *Salmonella* Enteriditis phage type 4, which proved to be highly clonal. Use of many fingerprinting techniques for differentiation of phage type 4 strains proved unusable or invalid. Some currently used techniques such as RAPD analysis of *Mycoplamsa gallisepticum* and RFLP analysis of *Pasteurella multocida* strains have provided useful information for epidemiologic analysis at the ranch level, for comparative analysis of geographically related strains and for vaccine strain identification.

Use of bioinformatics for interpretation of laboratory data. Bioinformatics deals with methods for storing, retrieving, and analyzing biological data, such as nucleic acid (DNA/RNA) and protein sequences. Incorporation of molecular-based techniques for identification of bacteria at the genus, species, or strain level requires that technical staff be familiar with the use of computer applications for data analysis and management. Because of the volume and complexity of data currently being generated, bioinformatics has become an integral part of the clinical bacteriology laboratory. The use of bioinformatics to manage large and complex databases has resulted in the enhanced capabilities of clinical laboratories and has subsequently improved and expanded diagnostic capabilities.

Participation in laboratory quality assurance programs. In addition to the guidelines previously described for antimicrobial susceptibility testing, there are current efforts in all areas of veterinary diagnostic medicine to insure that adequate quality control, method validation, document control, and measurement of staff competence to perform testing are in place to insure that laboratory results are accurate and defensible. This is an obvious benefit to the end-user of laboratory data. As of 2007, the American Association of Veterinary Laboratory Diagnosticians (AAVLD) accreditation process will require all accredited laboratories have a documented quality assurance program in place. This is part of an overall effort to assure that accredited laboratories meet standards of the World Organization for Animal Health (Office International des Epizooties). These processes along with proficiency tests administered by the AAVLD are steps to help improve microbiological diagnosis in poultry laboratories that are members of AAVLD. Having this additional layer of assurance regarding laboratory results should benefit the poultry industry at all levels including the area of export trade.

Food safety. In bacteriology laboratories that deal with any part of production medicine, food safety has become a substantial component of those laboratories' overall responsibilities. In the 1990s the Food Safety and Inspection Services (FSIS) moved from a visual-based inspection system to a microbial based detection approach resulting in more laboratory testing of bacterial loads on carcass surfaces. In July 1996, the Pathogen Reduction; Hazard Analysis and Critical Control Point (HCAAP) Systems ("Mega Regs") for meat and poultry processors and federal inspectors was enacted. This new system was directed towards preventing contamination on meat and poultry. Because the poultry industry is very integrated, it is a food chain that is fully traceable and readily able to implement such controls.

Interest in detection of food safety pathogens has resulted in a number of the technological advances already discussed in the section on identification of specific poultry pathogens. As previously mentioned,

antimicrobial resistance trends of poultry isolates considered important food pathogens and the role of poultry products as vehicles for introduction of antimicrobial resistant pathogens to human is also of substantial interest. Clinical laboratories can provide producers with important information about trends in order to recognize potential changes in susceptibility patterns before they become widespread or of public health concern.

As an example of the importance of clinical bacteriology laboratories' role in these food safety issues, the CAHFS laboratory was first to recognize *Salmonella* Enterititdis (SE) phage type 4 in the United States from clinically affected birds and was closely involved in understanding the epidemiology of this organism. Nationally veterinary bacteriology laboratories have played a major role in SE control at a number of levels including traceback investigations, voluntary control programs and research in areas of epidemiology, strain variation, and pathogen detection.

Role in agroterrorism and bioterrorism preparedness. Since the events of 911 and the anthrax bioterrorism event on the east coast in 2001, bioterrorism and agroterrorism have been at the forefront of concern at a number of levels. Some bacterial poultry pathogens are included as potential agents of bioterrorism. These include *Salmonella* serotypes, *Chlamydophila psittaci*, and *Clostridium botulinum* and its toxins. In fact, *Salmonella* Typhimurium was used in one of the most successful bioterrorism attacks to occur in the United States in 1984 in Oregon. Although most of the serious agents of agroterrorism are viruses, introduction of some bacterial agents of poultry such as *Salmonella* Pullorum or some of the pathogenic poultry mycoplasmas could have substantial economic consequences if purposely introduced into a commercial operation.

Because of expertise with many of the potential bioterrorism agents, clinical veterinary bacteriology laboratories are finding themselves more and more involved in laboratory networks in order to be able to address issues of national concern either from a terrorism event or naturally occurring epidemic. Thus a number of veterinary laboratories, including some routinely dealing with poultry diagnostics are becoming members of these national laboratory networks that have been organized over the last five to six years. These networks include the National Animal Health Laboratory Network, the Food Emergency Response Network and the Laboratory Response Network.

CONCLUSIONS

Over the last decade or so a number of technological advances as well as regulatory, global, and political issues have directly impacted clinical veterinary bacteriology laboratories' capabilities and how laboratories function overall. Bacteriology laboratories performing poultry diagnostics are included among those affected. Many of the technological advances have expanded laboratories' capabilities to perform testing for and identification of poultry pathogens that would not have been feasible 10 years ago. This has provided producers and clinicians with more detailed and accurate information from which to make informed management decisions. The role of the clinical veterinary laboratory has also expanded to include areas of food safety and bio- and agro-defense. Globalization, standardization of methods, and greater emphasis on laboratory quality assurance programs have further enhanced the capabilities of clinical veterinary bacteriology laboratories and how they provide diagnostic support to poultry clinicians and producers.

PREVALENCE OF *CAMPYLOBACTER* AND *SALMONELLA* ALONG THE FARM TO FORK CONTINUUM IN SPECIALTY MARKET POULTRY

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INTRODUCTION

Very little is known about food safety risk factors for specialty poultry products. Specialty poultry products include squab (young pigeon), duckling, quail, poussin (young chicken), guinea fowl, and free-range chickens. However, there is a complete lack of species-specific data concerning the prevalence and origin of microbial pathogens within these commodities. Infection status of the host population can be an important factor in the contamination status of the final food products. Transport of broilers to the processing plant was shown to increase the prevalence of birds positive for *Salmonella* and *Campylobacter* due to fecal contamination of skin and feathers by neighboring birds during shipping (5). Processing has been shown to increase contamination by *Salmonella*

and *Campylobacter* in studies comparing on-farm prevalence to final product prevalence (3, 4, 6). The objective of this study was to define the incidence of *Campylobacter* and *Salmonella* throughout the continuum from farm to final product. This information could be used to identify potential CCP for microbial contamination that could be incorporated into commodity specific HACCP plans for ensuring food safety.

MATERIALS AND METHODS

Three flocks from six specialty types of poultry were sampled on farm, after transport and at processing. The three processing plants were federally inspected. Plant A processed squab, poussin, and quail; Plant B processed duck and guinea fowl; and Plant C, free-range chickens. Sampling stations common to all flocks were farm, post transport, picker, and prepackage. The other two to three stations in the processing plant varied by commodity. All testing occurred during the summer months. The number of samples taken at the farm, post-transport, and within the processing plant was determined by estimating the prevalence of *Campylobacter* spp. in each group. A minimum of 40 samples and a maximum of 80 were obtained per station. Forty samples were obtained if previous studies indicated that the prevalence was high in the species. An intermediate number of 60 samples were collected if the management system on the farm, or if the processing methods, were similar to broiler production. Eighty samples were obtained if the prevalence was low for or if no information was available for the species. Fecal samples were obtained at the farm and post-transport by cloacal swab using sterile cotton-tipped swabs. Processing plant samples were obtained by swabbing the skin of the carcass along the length of the body, avoiding the cloacal area. Data collected from sampling stations that were common to all six commodities were evaluated by block logistic regression to test the influence of each processing step on the prevalence of *Campylobacter* and *Salmonella* on the final product. This analysis generated the odds ratio for being positive at the pre-packaging (final) site, given a bird was positive at a previous sampling station. The SPSS univariate function, with Tukey's multiple range test, was used to evaluate the percentage of positive samples for on-farm, at post-transport, and post-transport 2 (PT2).

RESULTS AND DISCUSSION

The prevalence of *Campylobacter* and *Salmonella* among the six commodities was highly variable. On the farm, the prevalence of *Campylobacter* in the six commodities are as follows from highest to lowest: poussin > free-range > quail > duck > guinea fowl > squab. Additionally on the farm, the prevalence of *Salmonella* was highest for both poussin and free-range. Both quail and guinea fowl yielded no *Salmonella* on the farm, but duck and squab did carry low levels of the bacteria. *Salmonella* species recovered included *S. infantis*, *S. typhimurium*, *S. heidelberg*, *S. seftenberg*, *S. kentucky*, *S. agona*, *S. siegberg*, *S. hadar*, *S. montevideo*, and *S. cerro*. Prevalence of *Salmonella* positive birds in the two chicken commodities was variable by flock, ranging from 0 to 23%.

Campylobacter prevalence ranged from 5 to 25% in guinea fowl flocks and 14 to 41% in quail flocks. No *Salmonella* was isolated from either species. The similarity of these results were not due to similar management or environment. Guinea fowl were raised on litter in houses similar to broiler chickens and marketed at 73 days, while quail were grown in wire cages approximately three feet above the ground and marketed at 56 days of age.

Poussin and free-range chickens were the two groups that had the highest prevalence of *Campylobacter* positive birds, with poussin 80 to 97% positive and free-range 32 to 68% positive. The extremely high prevalence in poussin, with a market age of 28 days, agreed with a prospective epidemiologic study conducted in broilers of similar age. Although free-range chickens have access to outside soil and water, which could provide exposure to additional vectors of infection, we observed higher prevalences in the poussin flocks which were reared in enclosed housing. Possible explanations may be: the effect of bird density and increased success of fecal-oral transmission, strain differences in the birds, immune status of the flocks, strain differences among the *Campylobacter* isolated, in addition to the role of environmental factors.

Squab had very low prevalences of *Campylobacter* spp. at all three farms (10%, 0, 0). *Salmonella* spp. prevalence was lower than the other commodities at 1.2%, 2.5%, and 0. The low on-farm prevalence of these bacteria in squab was consistent with our earlier work (1). Prevalence results from our duck sampling did not agree with previously published reports (2) stating 100% prevalence at eight days of age. We found the prevalence of *Campylobacter* spp. in ducks at the market ages of 69 to 84 d to be much lower (27%, 2.5%, and 60%).

Free-range chicken was the only commodity that exhibited an increase in the prevalence of *Campylobacter*. The largest increase in positive swabs occurred at the PT2 sampling. Prevalence of *Campylobacter* increased 27% to 87% in flock one from PT to PT2, 73% to 80% in flock two, and 52% to 72% in flock three.

In this study, each commodity had a slightly different processing format, therefore sampling was arranged to accommodate these differences while capturing data from core stations similar to all. When prevalence's were combined across species for these core stations, odds ratio analysis failed to identify any of the core stations (post-transport, post-picker, post-evisceration, pre-packaging) as significant contributors to the bacterial status of the pre-packaged carcass. This is an important finding because it supports commodity specific HACCP, not generic HACCP plans for specialty poultry products.

Deriving food safety recommendations from larger industries, with different management practices, processing techniques, and access to veterinary care and resources, may be largely irrelevant. Some species of birds appeared to have lower prevalence's of both *Campylobacter* and *Salmonella* than others. With labor, time, and financial constraints, reducing bacterial pathogens on the farm and in the processing plant remain difficult tasks. The results of this study clearly demonstrate that critical control points for reducing bacterial contamination are not the same across all species or commodities and suggest that HACCP plans for *Campylobacter* and *Salmonella* control may need to be specifically designed to accommodate these differences.

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COLONIZING CAPABILITY AND SKIN RECOVERY OF *C. JEJUNI* GENOTYPES FROM LOW PREVALENCE AVIAN SPECIES IN BROILER CHICKENS

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INTRODUCTION

The handling and consumption of *Campylobacter jejuni* contaminated poultry has been epidemiologically linked to, and remains a primary source of, *C. jejuni* related illnesses and outbreaks in humans (3). Low prevalence of *C. jejuni* in some non-chicken species may be a result of variation in host susceptibility to infection, superior clearing mechanisms, or low carriage and colonization potential of the *C. jejuni* strain. The first study was conducted to determine if *C. jejuni* isolated from low prevalence host species (squab, duck) would have reduced colonization and carriage rates when compared to chicken-derived isolates. The objective of the second study was to measure the probability of detection (sensitivity) for *C. jejuni* using skin swabbing as a sampling method followed by enrichment in an aerobic semi-solid media. The sensitivity of this sampling methodology was compared between commercial broilers, retail ducks, and squab. Large differences in the sensitivity of detection for *C. jejuni* on poultry skin may help explain the extreme difference that has been observed in the on-farm prevalence of *C. jejuni* in commercial broilers compared to retail ducks and squab.

MATERIALS AND METHODS

Study 1. There were two trials in study 1. One hundred and fifty, day-old, Cornish cross broiler chicks were obtained from a commercial hatchery on the day of hatch. To confirm *C. jejuni* negative status, birds were cultured two days prior to the start of the trial at 18 days of age. On day 20, the birds were divided into four treatments consisting of 36 birds and dosed with a 1 mL of inoculum. The inoculating strains of *C. jejuni* were obtained from cloacal swabs of ducks, squab, and

broiler chickens. Stock solutions of *C. jejuni* isolates were serially diluted to reach a final concentration of 10^3 cfu/mL. Inoculum concentrations were confirmed by spread plating and colony counts. The *C. jejuni* isolates were grown on blood agar (BA) plates at 42°C for 48 h under microaerophilic conditions (5% CO_2, 10% O_2, and 85% N_2). The inoculum concentration was intended to mimic *C. jejuni* concentrations found in poultry house litter and feces (4). Group one was challenged with a strain recovered from a duck; group two was challenged with a squab isolate; group three was the positive control and received a strain isolated from a broiler chicken. Group four was the negative control and received a dose of 1 mL PBS. The ileo-cecal junction (12) from 12 birds in each group was removed and cultured at 2, 4, and 10 d post-inoculation. Each sample of intestine was placed into a separate sterile bag and weighed. A 1:10 dilution was made based upon weight, using 0.01M PBS. Samples were stomached, serially diluted, and spread plated onto CampyFDA agar (12). Sample plates were incubated at 42°C for 48 h under microaerophilic conditions. Suspect *Campylobacter* colonies were identified by gram stain, catalase, and oxidase tests. *C. jejuni* was further differentiated from *C. coli* by hippurate hydrolysis testing. One colony per sample was saved and stored at -80°C until processed for DNA extraction. The isolates recovered at necropsy were verified as identical to the inoculum by polymerase chain reaction—restriction fragment length polymorphism (PCR-RFLP) of the *flaA* gene.

Study 2. One *C. jejuni* isolate previously collected from the skin of a commercial broiler chicken was used for the duration of the study. Prior to each assay, a bead was streaked onto blood agar (BA) incubated under microaerophilic conditions (5% CO_2, 10% O_2, and 85% N_2) at 42°C for 48 h. Square sections (1 in^2; 2.5 cm H 2.5 cm) of skin were cut from the breast and backs of retail broiler chickens, squab, and duck carcasses. Each skin sample was rinsed with sterile distilled water and dried with new paper towels before inoculation. To confirm negative status prior to inoculation, each skin sample was swabbed using a sterile, cotton-tipped swab. Each swab was immediately placed in a tube containing semi-solid enrichment media. Five skin samples were tested at each concentration of the bacterial inoculum. Each skin sample was inoculated with 0.1 mL of inoculum diluted to deliver 10, 100, 1000, 10000 cfu/in^2 skin and held for a contact time of three minutes. A time of three minutes was estimated to mimic the line speed, and the amount of time passed between processing stations, in a small- or medium-sized poultry plant. Inoculum concentrations were confirmed by spread plating. Beside each set of skin samples, a negative control skin sample was dosed with 0.1 mL of PBS. At the end of three minutes, the inoculated skin was swabbed with a sterile cotton-tipped swab and the swab was immediately placed in semi-solid enrichment media. The isolation and identification of *C. jejuni* from samples is described in Study 1. The sensitivity of detecting ≥1 cfu *C. jejuni* per in^2 of skin was modeled using logistic regression.

RESULTS AND DISCUSSION

Study 1. No clinical illness was reported in any of the treatment groups, and negative control birds remained uncolonized for the duration of both trials. Significant differences between treatments ($P \leq 0.05$) were observed on day two PI, where the group inoculated with the squab isolate had fewer *C. jejuni* (cfu/g ceca). The number of *C. jejuni* (cfu/g) recovered in the, positive control group (chicken-isolate) was significantly different from the duck and squab groups by day four PI in the second replicate of the trial ($P \leq 0.05$). By day 10 PI, no significant differences in the percent of birds colonized or number of *C. jejuni* (cfu/g ceca) were present between the squab and duck treatment groups ($P \leq 0.05$). The isolates recovered from birds at necropsy were found to be identical to the inoculating strains.

Campylobacter species are not host specific as demonstrated in our study. In the second replicate of this trial, the positive control (chicken isolate) did not colonize as quickly as the duck isolate but in both trials 100% of the birds given either duck-origin or chicken-origin isolates were colonized by day 10 PI. This probably represents normal variation between sets of birds used in the studies. Chickens, ducks, and pigeons seem to be ideal hosts for *Campylobacter* with body temperatures near optimal for *Campylobacter* growth (42°C, 42.1°C, and 42.2°C, respectively) (5). The squab strain appeared to take longer to become established than either the duck or chicken isolates, based on 2 d PI recovery rates, but the number of bacteria recovered on day four PI and day 10 PI were comparable to both chicken and duck. The fact that commercial squab have repeatedly demonstrated a low *Campylobacter* prevalence in field studies may have more to do with their behavior and housing than with host resistance (1, 2). Further research of this topic may support this statement.

Study 2. The probability of detecting skin contaminated with *C. jejuni* was significantly higher for broiler chicken compared to retail duck or squab at low levels of contamination. Thirty-three and 100% of skin samples were detected as contaminated with *C. jejuni* at 10 or 100 cfu/in^2 skin for broiler chickens, respectively. Our method of using skin swabs and enrichment with semisolid media had almost 100% probability of detection for concentrations of 1,000 or

10,000 cfu/in^2 skin regardless of species of poultry. The higher probability of detecting *C. jejuni* from chicken skin may have considerable impact on the incidence of positive samples and therefore prevalence rates reported for *C. jejuni* on processed chicken carcasses as compared to other poultry commodities. Differences in chicken skin as compared to other poultry commodities may also influence the success of skin contamination during transport and holding prior to slaughter and of cross contamination during processing. The possibility exists that because the inoculating *C. jejuni* was of chicken origin, it may have been more adapted for attachment to chicken skin versus duck or squab. It would be interesting to test *C. jejuni* from other poultry commodities in this experimental design.

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VALUE OF US EGG QUALITY ASSURANCE PROGRAMS TO PROTECT CONSUMERS FROM *SALMONELLA* ENTERITIDIS

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SUMMARY

The introduction of voluntary Egg Quality Assurance Programs (EQAPs) during the 1990s together with related post-harvest preventive measures resulted in a significant reduction in *Salmonella* Enteritidis infection in consumers attributed to eggs and egg products. The plateau in incidence rates during the past five years is associated with persistence of infection in flocks. Inappropriate selection of the age of sampling and the inherent insensitivity of manure drag swabs which are the basis of industry-mandated EQAPs will result in perpetuation of SE infection in both cage-free and in-line operations. Adoption of more intensive sampling based on the epidemiology of SE infection as proposed by FDA and embodied in the Pennsylvania-EQAP will lead to a reduction in flock prevalence and ultimately, lowered incidence rates in consumers of eggs.

METHODOLOGY

Epidemiologic data relating to *Salmonella* Enteritidis* (SE) infection in the US population as published by the Centers for Disease Control was assembled and analyzed. Surveillance procedures comprising manure drag swabs in egg-producing flocks required by three US Egg Quality Assurance Programs (EQAPs) were reviewed. The frequency of sampling in relation to the age of the flock was compared and evaluated in relation to risk of transmission of SE to consumers.

RESULTS & DISCUSSION

The emergence of SE in consumers in the USA during the mid-1980s (12) and the subsequent attribution to eggs contaminated by the vertical route (3) resulted in control measures embodied in voluntary EQAPs. Compliance required defined biosecurity procedures and surveillance for SE based on manure drag swab assays (8). The advent of EQAPs was associated with a decline in the incidence rate of human infection (9). The annual changes in incidence during the preceding three years before the advent of EQAPs were +12%, +15%, and +30% in sequence

followed by declines of -16%, -10%, and -7% in the subsequent three years.

The impact of the Pennsylvania EQAP is confirmed by the decline in SE positive flocks in that State from 38% in 1992 to 13% in 1995. In the initial year of the program it was estimated that 50% of flocks excreted SE at some stage of their production cycle. The prevalence rate was reduced to 15% in 1996. The prevalence of SE on 133 farms in California averaged 10.5% during the period 1998-2000 based on examination of 2128 drag swabs which yielded a 1.1% recovery rate based on sampling of individual rows (2, 6).

Surveillance of SE in consumers in the US is based on the Public Health Laboratory Information System which initiated reports of isolates in 1973. In 2001 the Electronic Food borne Outbreak System was established to enhance recognition of outbreaks of SE through the Food borne Diseases Active Surveillance Network. This CDC initiative with collaboration from the FDA and the USDA covered a population base of 36 million in 2003 in a total of ten locations and states. Epidemiologic data has shown a decline in incidence of confirmed SE outbreaks from 81 in 1990 to 45 in 2000, with a continuing decline to a plateau of 30 during subsequent years. The number of cases of documented SE infection has declined from 2,800 to 1,000 reports over the same period. From 1975 to 2002, 960 outbreaks of SE, involving 32,000 cases were confirmed. A vehicle was determined in 45% of outbreaks and 79% of these which were investigated were attributed to eggs or egg-containing dishes. In 2002, 50% of cases were associated with consumption of eggs. The FDA attributes a mid-range estimate of 66% of SE cases to eggs. Assuming that 16% of SE infections are acquired outside the USA, and that 38 cases occur for every confirmed diagnosis, there are approximately 120,000 incident cases of SE annually in the USA (11). FoodNet documented an SE incidence of 2.32 cases/100,000 in 2002 and 1.82/100,000 compared to a five-year mean of 2.0/100,000 indicating persistence of reservoirs and the emergence of new vehicles including produce. In 2003, incidence rates among the ten cooperating States ranged from 3.92/100,000 in Maryland to 0.98/100,000 in Tennessee.

The Presidential Executive Order of August 25th, 1998 established the Council on Food Safety. This body has developed guidelines (Healthy People 2010) to reduce food borne infections, including salmonellosis, by 50% from the then existing levels. The resulting FDA initiative was formalized in the proposed rule "Prevention of *Salmonella Enteritidis* in Shell Eggs During Production" detailed in the Federal Register 69:183 of September 22, 2004. In the preamble FDA acknowledge a reduction in incidence following introduction of voluntary EQAPs but note the non-uniform administration and lack of comprehensiveness of programs are responsible for continued attribution of SE to consumption of eggs. The "cornerstone" of the proposed rule is a "requirement that producers test the environment for SE in poultry houses." The proposed FDA rule was supported by data collected by USDA-APHIS National Animal Health Monitoring System (NAHMS) on Table Egg Layer Management ("Layers ´99"). In 1994, 16% of houses with more than 100,000 hens yielded SE from environmental samples.

The current EQAPs which cover between 50% and 60% of shell egg production in the USA have variable requirements for the frequency and the age of sampling flocks during production (Table 1).

Delaying the first sampling age of layers to within two weeks before depletion exposes consumers to the possibility of vertical egg-borne infection. Detection of infection will only be possible in most cases when flocks are close to 100 weeks of age, having passed through the critical post-peak and molt periods when systemic infection and vertical transmission are most likely to occur. The industry-driven, voluntary surveillance programs in place in States, other than Pennsylvania, are inadequate to detect SE. Prompt identification of infected flocks, especially on multi-age in-line farms with over one million hens, is necessary to implement appropriate control measures. The present approach exemplified by the UEP program is self-serving in that no specific action is required by producers in the event of detection of SE other than decontamination of layer housing. The inherent variability and lack of sensitivity of the manure drag-swab (1, 7, 10) assay under conditions of non-controlled field application is a second restraint to prompt diagnosis of SE. It is therefore probable that the prevalence of SE in commercial flocks is higher than the levels disclosed by the NAHMS survey and published reports. The relatively low but constant incidence rate of egg-borne SE among consumers is associated with the inherent low rate of transovarial and transoviductal transmission (4) especially in vaccinated flocks (5), the obligatory storage and transport of eggs at 7ºC, rapid rotation of inventory, the use of pasteurized egg products for institutional products, and enhanced training of food preparers in commercial and domestic kitchens.

The application of the more realistic program of environmental monitoring as proposed by FDA will increase the probability of detecting infected flocks and will provide a higher level of protection to consumers. The program will require environmental sampling using approved methods at 40-45 weeks of age and 20 weeks after completion of molting if flocks are held for a second cycle.

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Table 1. Egg Quality Assurance Programs: Frequency and sampling requirements.

United Egg Producers "5-Star Program"	2 to 3 weeks before depletion 2 drag swabs per row or 2 drag swabs/belt of each row
Ohio EQAP	2-10 weeks before depletion sample manure pits
Pennsylvania EQAP	29-31 weeks of age 44-46 weeks of age 5-7 weeks post-molt 2 samples beneath each cage row
California EQAP	2-3 weeks before depletion drag swabs from 32 lengths of 30´ beneath cages

EPIDEMIOLOGICAL SIGNIFICANCE OF GENETIC FINGERPRINTING OF *ORNITHOBACTERIUM RHINOTRACHEALE* BY M 13 AND ERIC 1R PCR

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SUMMARY

Ornithobacterium rhinotracheale (ORT) is a gram-negative bacterium which has been identified as an emerging respiratory pathogen in turkeys and chickens. The organism has also been isolated from wild birds. There are 17 serotypes of ORT so far reported viz: ORT serotypes A through Q. The distribution of ERIC (Enterobacterial Repetitive Intergenic Consensus) elements on prokaryotic genome has been examined by polymerase chain reaction (PCR), which was shown to provide an efficient technique to differentiate species and strains in gram-negative bacteria. Moreover, serotype identification of many bacteria using M 13 PCR yielded discriminatory DNA fingerprints between unrelated strains. The objective of this study is to address the question whether PCR fingerprinting technique could be used to differentiate serotypes of ORT. For this we selected two PCR fingerprinting techniques: one using a rep primer ERIC 1R and another with M 13 to differentiate serotypes of ORT.

Fifty eight isolates of ORT were fingerprinted in this study. Eight of them were reference isolates of ORT for serotypes A, C, D, E, F, I, J and K, kindly provided by Dr Van Empel, Netherlands. Forty-nine isolates of ORT used in this study were isolated from turkeys in and around Minnesota, over the last four years and one was a field isolate from infected chickens in Iowa. All the ORT isolates were serotyped by agar gel precipitation test (AGP test). Stock solutions of bacterial DNA were adjusted to a concentration of 100ng/5µL for PCR. Two primers ERIC 1R (5'ATG TAA GCT CCT GGG GAT TCA C) and M 13 (5'TAT GTA AAA CGA CGG CCA GT) were used to amplify 100ng of purified ORT genomic DNA for each PCR fingerprinting reaction. The PCR was performed using a modified protocol of Amonsin et al. (1997). The reactions were carried out in a thermal cycler and to confirm the reproducibility of the technique, two separate reactions were conducted with each isolate.

Among the 50 field isolates used for this study, thirty two isolates were serotyped as ORT serotype A, eleven as serotype C, and seven as ORT Serotype I by AGP test. All the eight Reference ORT serotypes A, C, D, E, F, I, J and K tested gave different fingerprint patterns with M 13 PCR. The ERIC 1R PCR fingerprints for eight reference serotypes gave only five different fingerprint patterns. The ERIC 1R fingerprint patterns were different for ORT serotypes C, D, E, and K but ORT serotypes A, F, I and J gave similar fingerprints. Differences in the fingerprint patterns within each serotypes of ORT tested viz; A, C and I were also found. From fifty eight isolates of ORT that included reference strains fingerprinted belonging to eight serotypes, twelve distinct and different fingerprint patterns were obtained with M13 PCR fingerprinting and six distinct and different fingerprint patterns were obtained with ERIC 1R fingerprinting. This suggests that M 13 PCR is more discriminatory than ERIC 1R PCR in ORT fingerprinting. A combinatorial approach of looking at both ERIC 1R and M 13 fingerprints will be more helpful in differentiating more ORT isolates. Though by ERIC 1R and M 13 PCR fingerprinting alone we could not differentiate all serotypes of ORT in the present study, they helped us to differentiate most of the serotypes. We could differentiate and group ORT isolates based on their fingerprint patterns alone or within each serotypes. This will help us in epidemiological studies in ORT outbreaks.

PERITONITIS IN TABLE EGG LAYERS: DEFINING THE FOUR POSSIBLE SOURCES THAT COULD INITIATE THIS DISEASE

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OVERVIEW

Peritonitis in commercial table egg layers as well as in breeders causes significant negative economic consequences in the US and most likely in other parts of the world.

To date, there is no published information that documents the total economic impact to the egg industry or examines the cost to the producer when flocks experience high mortality and/or the reduction in egg production due to this disease.

Peritonitis is a serious problem for the table egg industry. It appears that this problem has been kept out of the research scope, and people who experience this problem only talk about it to close associates. People are reluctant to share their experiences with the challenge, so there is little information about the onset and process of the disease in the field and the successes or failures of treatments implemented to reduce mortality.

The only way we are going to understand the sources that initiate the disease process of peritonitis in commercial egg layers and to prevent the problem is by reviewing published information, collecting anecdotal information, comparing treatments used, conducting an epidemiology study, and ultimately, initiating research projects that can test hypothesis and field experiences.

Some of the best information available comes from the descriptions that are presented by producers and professionals who have been involved directly or indirectly with mortality, reduction in egg production and/or who have had an increased cost of production, due to peritonitis problems in flock(s).

Their explanations in combination with published information, others and my own field experiences and observations for the possible sources that induce peritonitis or are the catalyst of the problem include lesions due to peritonitis on any hen during any stage of its egg production can be due to a variety of causes and/or factors.

Characteristic peritonitis lesions produce an inflammatory response. Serous and edematous exudates tend to accumulate in the ceolomic cavity. Exudates undergo cessation to form a firm, dry, yellow, irregular, cheese-like mass. Time determines the extent and size of the exudates.

Peritonitis can be initiated in an organ and affect other organs. Organs that can be affected are ovaries (oophoritis), oviduct (salpingitis), air sac (airsacculitis), intestinal tract (enteritis), and systemic (septicemia) infection. The most common bacterium isolated from peritonitis lesions is *Escherichia coli* (*E. coli*), and in a lesser frequency other bacteria types including *Enterococcus*, *Pasteurella*, *Salmonella*, *Staphylococcus*, and *Streptococcus*.

E. coli, a gram-negative bacterium normally present in the intestinal tract of poultry and other animals, is the most common bacterial isolate in poultry worldwide. The most common pathogenic *E. coli* in poultry have been O1, O2, O35, and O78 serotypes. New serotypes have been linked to colibacillosis in poultry.

Lesions by *E. coli* should not be referred to by the name *E. coli* alone without the descriptor "coliform" being added because other opportunistic bacteria can behave similar to *E. coli* in secondary infections. The following describes the known or suspected factors that increase host susceptibility to *E. coli* infections in poultry.

GENERAL FACTS KNOWN ABOUT PERITONITIS

- It is the most common cause of mortality in commercial layers and breeders.
- Can affect other types of female birds in egg production (e.g. broilers, ducks, and geese).
- Airsacculitis, salpingitis, and septicemia can be present in conjunction with the peritonitis lesions.
- Peritonitis appears to be an acute problem.
- A flock does not appear to be sick or in distress.
- Peritonitis is common in layers, but not in pullets prior to the onset of egg production.
- In general, egg production is not affected, but is reduced due to mortality.
- Daily bird mortality appears normal a day prior to sudden mortality.
- Birds do not look sick. Affected birds are often in good physical condition, having full crops and in good egg production.
- Peritonitis is the inflammation of the peritoneum. The condition is marked by exudations in the peritoneum membranes and any of the organs within the abdominal cavity.

Abdominal cavity. The peritoneum is a serous membrane lining the abdominal-pelvis (ceolomic) cavity walls and investing the viscera inside them. This strong colorless membrane with a smooth surface forms a double-layered sac. The potential space between the parietal and visceral peritoneum is called the peritoneal cavity.

The ceolomic cavity is the largest area of the body of the hen. This cavity contains among others the largest air sacs (abdominal sacs), most of the digestive tract organs (proventiculus, gizzard, and the entire small intestine, part of the large intestine, liver and pancreas), genital-urinary systems (kidneys, ureters, ovary, most of the oviduct and atrophied right oviduct) and the spleen.

POSSIBLE SOURCES OF PERITONITIS

The following are four possible sources (immunological, digestive, respiratory, and/or reproductive) for the presence of peritonitis lesions in egg producing hens. These four factors can induce the syndrome in isolation or in combination with each other.

Immunological. Presence or challenges from Marek's Disease, IBD, mycotoxins, molting procedures among others.

Digestive. Presence or challenges from enteric disturbances, *Eimeria* spp. challenges, ration changes, contaminated water, among others.

Respiratory. Presence or challenges from infectious bronchitis virus (IBV), Newcastle disease virus (NDV), infectious coryza (*Haemophilus paragallinarum*), avian influenza, *Mycoplasma gallisepticum*, *Bordetella avium*, *E. coli*, dry and dusty environment, high ammonia levels, inadequate ventilation, temperature extremes, among others.

Reproductive. Large egg size, low body weight, inadequate skeletal development, significant fat accumulation in abdominal cavity, light intensity, length (hours) of light, hormone mechanisms, among others.

Others. Incorrect beak treatment, old cage equipment, rough handling during bird transfers, bird density, bird nervousness, among others.

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FIELD RESULTS OF A NEW NECROTIC ENTERITIS TOXOID VACCINE IN ANTIBIOTIC-FREE CHICKENS

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Necrotic enteritis is an enteric disease of poultry that is caused by the enterotoxins of *Clostridium perfringens*. The clinical form is an acute disease resulting in high mortality with friable, distended intestines and classic pseudomembranous lesions. A milder subclinical form affects performance parameters and has been estimated to have an economic cost of $0.05 per bird.

The standard approach in the poultry industry to protect against this disease has been through the use of in feed sub-therapeutic antibiotics and/or antibiotic ionophores. As the poultry industry has moved to minimize the use of in feed sub-therapeutic antibiotics the incidence of necrotic enteritis has in-creased. Additionally, necrotic enteritis is a major concern for companies that produce antibiotic free birds.

This paper will discuss a new approach in preventing necrotic enteritis in broilers through the use of a necrotic enteritis toxoid vaccine developed by Schering-Plough Animal Health. The toxoid vaccine was developed against *C. perfringens* alpha toxin (Type A) and adjuvanted with water-in-oil emulsion.

A total of 82,800 of replacement pullets from a company that produces antibiotic free chickens were vaccinated with a necrotic enteritis toxoid vaccine via subcutaneous injection at 10 and 18 weeks of age. Chicks from breeder hens vaccinated with the necrotic enteritis toxoid vaccine were segregated to allow for placement on select farms. Weekly mortality from pure house flocks of chicks from vaccinated flocks will be compared against chicks from non-vaccinated hens.

EFFICACY OF INOVAPURE® AND *LACTOBACILLUS* SP. ON THE CONTROL OF CLOSTRIDIAL NECROTIC ENTERITIS IN BROILER CHICKENS

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ABSTRACT

Clostridium perfringens type A causes both clinical and subclinical forms of necrotic enteritis in domestic avian species (1). Lysozyme (inovapure®) was found to inhibit *Clostridium perfringens* (CP) type A and its α-toxin production (2). A strain of *Lactobacillus* species (LAB) isolated from healthy chicken gut also showed inhibition against CP in an agar spot test. Further study revealed that H_2O_2 and acid produced in the medium were two major inhibitory factors against CP. In an *in vitro* co-culture system, synergistic antimicrobial effect was observed between lysozyme and LAB against CP. A floor pen study using a well-developed necrotic enteritis (NE) challenge model showed that both inovapure and the inovapure/LAB combination worked as well as BMD in reducing the lesion score and NE mortality, and they were significantly better than the challenged, non-medicated control. Inovapure/LAB treatment worked better than inovapure alone in feed conversion and weight gain.

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(The full-length article will be submitted to the journal of *Avian Diseases* or *Journal of Applied Microbiology*.)

A CASE OF BOTULISM IN COMMERCIAL BROILERS

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INTRODUCTION

Clostridium botulinum is a spore forming, gram-positive, anaerobic bacterium. It is usually found in decomposing animals and occasionally in plant material. Botulism is a poisoning of the nervous system that causes mortality, caused by a toxin (1). Birds, with the exception of vultures and flamingos, are susceptible to all types of botulism toxins. The predominant cause of mortality in birds is type C toxin. Botulinum toxins act by interfering with the supply or action of Ca^{++}, which interferes with the release of acetylcholine resulting in a flaccid paralysis (2).

Birds can get exposed via different routes: they ingest preformed toxin or their intestinal tract gets colonized by *C. botulinum* with subsequent toxin production. A third opportunity is a possible *C. botulinum* growth in a wound, also leading to toxin production (3, 4).

Over time, there have been several reports of cases of botulism in broilers from various parts of the world (5, 6, 7, 8). In this report, an overview on a case of botulism in a commercial broiler production unit will be given, the course of the disease on the ranch and the diagnostic approach of the laboratory are described and preventive measures are discussed.

CASE REPORT

During the summer of 2005, a commercial broiler producer started to lose birds in a subunit of six houses that belong to a complex with more than 50 houses. In the index case, nine 26-day-old broilers were submitted

to the Turlock branch laboratory of the California Animal Health and Food Safety Laboratory System (CAHFS) due to increased mortality and a 5-10% ill thrift. No remarkable lesions were found during necropsy and all the birds tested positive for botulism toxin C in the mouse assay. No *C. botulinum* was found in bacteriology cultures, and the problems were limited to one house.

In the next subsequent run, the problem occurred again – this time with higher losses. Finally, the mortality was increased in four out of these six houses of the subunit, and reached more than 20%. Three more sets of birds were submitted; one from the index house and two from neighboring houses, with slightly variable, but basically comparable findings. Based on the clinical picture, a preliminary diagnosis of botulism was made and later confirmed by the mouse assay. Additionally, an avian paramyxovirus type I was isolated from a tracheal swab, most likely related to a previous vaccination with a live vaccine. Additionally, the birds had antibodies against various pathogens (IBV, NDV, and reovirus), but were always negative against avian influenza, *Mycoplasma gallisepticum*, and *Mycoplasma synoviae*. Low numbers of coccidial oocysts were found by direct mucosal scrapings of the guts.

In an attempt to find the source of intake of botulism toxin, a visit to the ranch was made. The most striking finding was the typical clinical signs of botulism in literally dozens of birds in various stages of disease spread all over the houses: from closed eyelids and inability to stand to recumbency with extension of the neck. Also, there was a sudden increase in the mortality curves of several houses.

The houses were typical side-curtain houses with a soil floor and deep litter. Feeder and drinker units were state of the art, and there were very few areas found with caked litter. The houses had concrete walls that were approximately two feet high. The external environment of the houses was very clean, and inside the houses, the mortality was removed at least twice a day to avoid a carry-over of *C. botulinum* by rotting carcasses.

One interesting factor noted was that the soil level inside the houses was comparatively low: the explanation for this was that scrapers are used to remove used litter, and to take out as much litter as possible, a certain amount of soil gets taken away every time. This, over time, led to the removal of approximately 10 to 15 inches of top soil. Consequently, soil samples from four different locations from three houses were taken, but all tested negative for the presence of the toxin. No attempts to culture *C. botulinum* from the soil samples were made.

No source of intake of *C. botulinum* into these flocks was found. This led to the speculation that the litter removal might have led to the exposure of a soil layer that was previously contaminated with *C. botulinum* spores, dating back to times prior to the construction of the ranch. Therefore, in an attempt to solve the problem, the litter was removed, the top soil sprayed with PLT® (Jones-Hamilton Co, Newark, CA), and fresh soil from a clay pit was brought in and compacted. The spraying was repeated, and a total of up to 15 inches of soil was added. The birds of the first run on fresh soil in these houses did not have any health problems, and there was no sign of a reoccurrence of botulism.

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APPLICATION OF A RELATIVE RISK CALCULATOR IN A BIOSECURITY AUDIT

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Population disease risk can be ranked using a relative risk calculator that utilizes quantitative data on transmission risk factors and the proximity of susceptible animals to disease reservoirs. The reservoir represents the available microbial load (a quantity), derived from the mass of the contaminant, the percent available for dissemination, the initial microbial content of the contaminant and its age and half life. The proximity measurement uses distance from the reservoir to calculate an area over which the microbial exposure might be spread. Dividing the reservoir by the proximity measurement, one obtains a relative risk measurement that is significantly correlated with veterinarians' perceptions of risk (Spearman's rank correlation 0.8910, $p<.01$) (1). The formula is written Relative Risk = Log_{10} (Mass of contaminant) (Percent available for transmission) (initial Titer of the pathogen) $(0.5^{\text{Age of contaminant/Half life}})/ (\pi)$(distance squared). Abbreviated RR = Log_{10} $M*P*T*0.5^{A/H}/\pi *R^2$ (2).

The relative risk ranking can be used to rank population disease risks associated with events and practices in animal production. The advantages of the relative risk calculator are that it derives a relative risk measurement from available objective information, it provides a way to compare disparate sources of disease transmission risk, it can be modified for specific diseases, it can be used to audit farm biosecurity, and it provides a foundation for developing and evaluating mitigation strategies. From the relative risk measurement, mitigation strategies and available resources can be focused appropriately to prevent or control disease. Biosecurity programs and disease control measures can be directed at those areas of greatest risk for spreading disease.

From a survey of 90 farms participating in an avian metapneumovirus control program ten survey responses were chosen at random. Nine survey questions related to proximity to alien poultry; proximity to wild waterfowl; proximity to alien manure; and proximity to trucks hauling live birds, dead birds, and manure were selected for application of the relative risk calculator. Relative risk measurements were calculated for each farm for each of those areas.

To make the calculation, certain assumptions were made: a neighboring farm was assumed to have 20,000 turkeys, mortality for the life of the flock was assumed to be 10%, and a body of water was assumed to harbor 100 ducks. Titer of the contaminant was set at 10^6 generic microbes per gram and age of the contaminant was set at 0. The resulting logarithmic numbers were rounded to the nearest whole number and expressed as a relative risk measurement (Table 1). The different relative risk measurements and the sums for each farm illustrate the different levels of risk that can be attributed to proximity to alien poultry, wild waterfowl, alien manure, and trucks hauling dead or live birds or manure.

Table 1. Relative risk measurements for proximity to alien poultry; proximity to wild waterfowl; proximity to alien manure; and proximity to trucks hauling live birds, dead birds, and manure.

Proximity		Farms									
Measurements		A	B	C	D	E	F	G	H	I	J
Poultry	a	4	4	6	4	4	4	4	4	4	4
	b	0	5	5	0	0	5	5	5	0	5
Wild waterfowl	a	4	4	4	2	2	4	4	3	4	4
	b	6	6	0	0	0	6	6	0	6	6
	c	3	3	3	3	0	3	3	0	3	3
Manure		0	8	8	0	0	8	8	8	0	8
Trucks	a	5	5	5	0	5	5	0	5	0	0
	b	4	4	4	4	4	4	4	4	4	4
	c	0	6	6	0	0	6	0	6	0	6
Sum		26	45	41	13	15	45	34	35	21	40

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FOOD/AGRICULTURE INFRAGARD – A NATIONAL SECURITY NEXUS CONTAINING THE VETERINARY COMMUNITY AND COMMERCIAL POULTRY

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The Federal Bureau of Investigation (FBI) and the intelligence community have identified vulnerabilities to many infrastructure components in the United States, including the food distribution and agricultural production systems. Specific evidence has emerged indicating an interest by both international and domestic terrorist groups in conducting attacks, so as to damage the economy, foment public unrest, and cause death and disease.

"Food/Agriculture InfraGard" is a new national security program designed to better protect the United States agricultural production system and food supply. The program, which is sponsored by the Federal Bureau of Investigation (FBI), is designated as a "Special Interest Group" (SIG). The SIG is sub-component of the FBI's larger Infragard program, which is the national security program, also sponsored by the FBI, designed to assist in the protection of "Critical Infrastructures" as designated by Homeland Security Presidential Directive/HSPD-8 - December 17, 2003 (Whitehouse, 2003), and more specifically in the case of agriculture and food, as designated by Homeland Security Presidential Directive/HSPD-9, Defense of United States Agriculture and Food - January 30, 2004 (Whitehouse, 2004). Infragard, first begun in the Cleveland FBI office eight years ago, is regionally based and currently includes 85 Chapters, which contain over 12,300 FBI-vetted U.S. citizen volunteers.

InfraGard has been a vital program to the success of the FBI in its mission to protect the critical infrastructures of the United States. The program was created in an effort to develop trusted partnerships between the FBI and industry, academia, and state and local government. Subject matter experts have partnered with the FBI through the program and have greatly enhanced the Federal Government's ability to successfully investigate cases within these experts' infrastructure sectors. The liaison contacts and partnerships created through InfraGard have also helped the FBI to better understand business models and operations within various industries, which has been essential to increasing the investigative response time to criminal and terrorist acts affecting our nation's critical infrastructures. Many criminal investigations have been opened as a direct result of the program.

Food/Agriculture InfraGard's overarching goal is to better protect commercial agriculture and the U.S. food supply and strengthen overall national security by providing a nexus of cooperation between industry, academia, and the FBI. Administrative oversight responsibilities for the program are jointly held by the Counterterrorism and Cyber Divisions, which are based in FBI Headquarters in Washington, D.C.

Designated tasks for Food/Agriculture InfraGard include:

1. Identification of subject matter experts in commercial agricultural production (plant and animal) and food/beverage processing, distribution and retail sales.
2. Enhance information sharing capability among public and private sector stakeholders. The stakeholders may on occasion be called upon to assist the FBI in detecting, deterring, assessing, and preventing malicious attacks (criminal and terrorist origin) on the food production, processing, and agriculturally oriented chemical industry sectors.
3. Provide expertise through interaction with the recently established FBI field office Agroterrorism Working Groups (AWG's). AWG is a component of the FBI's Food and Agriculture Security Program, which is based in the Weapons of Mass Destruction (WMD) Domestic Terrorism Operation Section of the FBI.

Oversight responsibilities for Food/Agriculture InfraGard are met at the local/regional level through the assignment of Infragard Coordinators, who are FBI field agents, assigned in part or fully to the Infragard program, through the field office. In all, the FBI contains fifty-six field offices. It is at this level that veterinary professionals or other qualified volunteers can participate in the Food/Agriculture InfraGard program. Among the critical needs for the group are veterinarians trained in foreign animal diseases and/or weapons of mass destruction or those which work directly with commercial agriculture (all species). Other needs include professionals embedded in all levels of agricultural production (animal and plant), transportation, warehousing and distribution, wholesale, and retail sales.

Applicants interested in participating in the Food/Agriculture InfraGard SIG must be U.S. citizens and must first be enrolled in the Infragard program. Currently active Infragard members can join the SIG, by applying through Infragard's secure website, located at www.infragard.net. Access to the secure website will require the use of VPN client software, which is provided to the program members. Other individuals, not previously members of Infragard must first make application to Infragard. Applications are publicly accessible at www.infragard.net. The application can be filled out on line, but once completed in full, must be printed out and submitted by mail to the appropriate field office Infragard Coordinator. All applicants will be vetted with a background check. Any follow up or clarification of submitted information, if needed will be made by the local Infragard Coordinator.

Benefits for those joining Infragard include: 1) no cost to user; 2) networking opportunities at the local, regional, and national level; 3) access to DHS/FBI Threat Alerts, Advisories & Warnings; 4) training initiatives and opportunities; 5) direct access to an FBI Agent responsible for program coordination at the members' local FBI Office; 6) valuable contacts and access to data; 7) discussion groups and specifically focused listserves; 8) seminars and conferences; and 9) national partnerships.

Once approved, new members will be given access to the secure Infragard website, whereby application to the Food/Agriculture InfraGard SIG can be made, as previously described. Membership is permissible in more than one SIG, so long as the applicant's occupational responsibilities are appropriate to the group. Members of Infragard and the Food/Agriculture InfraGard SIG are required to abide by standards as designated in the Code of Ethics available at: http://www.infragard.net/membership/pdfs/code_of_ethics.pdf, the Interviewing and Publications Policy available at: http://www.infragard.net/membership/pdfs/ipolicy.pdf, and the Infragard National Members Alliance Bylaws as amended by Congress 2004, which is available at: http://www.infragard.net/membership/pdfs/nat_by_laws_2004.pdf. Violation of any of these standards could result in the removal of membership.

Subject matter experts in the Food/Agriculture InfraGard SIG are encouraged, but not required to submit original articles and informational content. When submitted, the material will be reviewed by the discussion group moderator and disseminated as appropriate to the group as a whole. Members will also occasionally receive information from FBI, which is deemed appropriate for dissemination in a sensitive but not classified format. Members can also participate at the local level in all Infragard activities, including those which involve members from other SIGs. Specific chapter information and application forms are available at the non-secure website, located at: http://www.infragard.net/index.htm.

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DETECTION METHODS FOR ENTERIC VIRUSES OF POULTRY: AN OVERVIEW AND GLIMPSES OF THE FUTURE

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ENTERIC VIRUSES OF POULTRY: JUST WHAT YOU NEED TO KNOW

Similar to all economically important species, including people, enteric viruses are abundant in poultry. Rotaviruses, reoviruses, coronaviruses, adenoviruses and astroviruses are among the most commonly identified virus families in the intestinal tracts of both healthy and sick chickens and turkeys. Each of these virus families represents a diverse group of viruses, most of which have been minimally characterized.

Enteric disease, including runting stunting syndrome of broilers (14), poult enteritis complex, poult enteritis mortality syndrome (3, 4), and unclassified conditions, can have a major economic impact on commercial poultry production. Overt disease characterized by diarrhea, depression, and in severe cases high mortality is sporadic and often seasonal. Although enteric disease has been observed in most poultry producing regions of the US, it tends to be more common and severe in the Southeast US. Enteric disease also tends to be more common and severe in turkeys than in chickens. The economic impact of enteric disease is primarily due to production losses, where the affected birds do not gain weight and in some cases losses due to culls can be high. Production costs may also be increased due to the use of therapeutic treatments such as antibiotics. Definitive causes for these conditions are not always identified and although the focus here is viruses, bacteria are likely to be involved in many cases; enteric disease of poultry is considered to have a polymicrobial etiology.

Identifying the roles of specific viruses in inducing enteric disease has been challenging for several reasons. First, reproducing disease in the laboratory with a single agent has been difficult, and minimal work has been done in evaluating concomitant infections. Secondly, numerous viruses are found in healthy and sick birds and frequently more than one virus is detected in a given specimen. Thirdly, the gut is a highly complex environment where the interaction of the bacterial flora, viruses, and bird physiology will affect disease presentation.

Because the precise causes of enteric disease in poultry have been unclear, determining which viruses to control, therefore which viruses to target diagnostically has been difficult. Thus, diagnostic methods for enteric viruses have been relatively slow to be developed and are often developed in the context of research and in-house tests. In some cases, such as with avian reovirus (ARV), the available commercial diagnostic and detection tests have been developed because the primary diseases caused by viruses from those families are economically important and well defined, but not necessarily enteric.

HOW DIAGNOSTIC METHODS ARE USED FOR ENTERIC VIRUSES OF POULTRY

Because the viral causes of enteric diseases in poultry have been unclear, and formal industry-wide control methods have not been developed, the application of diagnostic tests for enteric viruses varies. Enteric disease is generally not treated in an agent specific manner, but therapeutically, to minimize the impact of the virus on disease. Testing for enteric pathogens is probably applied as much for epidemiological information as for direct disease control.

One exception for which diagnostic testing has had the most clear application in the field is probably turkey coronavirus (TCoV), where the direct role of the virus in disease production losses has been most clearly established (8, 19, 45). Numerous detection methods have been developed for TCoV (6, 12, 15, 25, 31-33, 37, 40). Commercial turkey flocks may be screened for TCoV and control measures may be implemented if a flock is found to be positive. However, unless there is a problem, most commercial poultry flocks are not routinely screened for enteric pathogens.

UNIQUE CHALLENGES TO ENTERIC VIRUS DETECTION

For a variety of reasons enteric viruses have also been relatively difficult to work with as targets for diagnostic tests. Virus isolation in cell culture or embryonating eggs is generally not reliable as many enteric viruses grow poorly, if at all, outside of the animal. In addition, specimen types for enteric viruses, i.e. intestinal contents or tissue, are relatively "dirty" with a high bacterial load, which further complicates

isolation. Additionally, it is not uncommon to identify multiple viruses in a single specimen, making cultivation of a pure culture in any non-selective system such as embryonating eggs difficult. Finally, because of the difficulty with identifying important viruses, few commercially available reagents have been developed for enteric viruses of poultry (i.e. monoclonal antibodies).

The advent of molecular detection methods has greatly aided the diagnostic efforts for enteric viruses, as they have with all viruses, because they are fast, highly sensitive, and very specific. However, molecular methods have short-comings of their own. First, they can be too specific; variants of an agent may not be detected. And although molecular tests can handle numerous sample types, as with classical methods, the dirty or biologically complex samples used for enteric virus detection can be problematic. Substances which are inhibitory for RT-PCR are common and numerous nucleic acid purification methods have been investigated to minimize the possibility for false negatives (16, 34, 38). With samples containing intestinal contents, the high concentration of nucleic acids from the bacterial load, in addition to host cellular nucleic acids, can decrease nucleic acid extraction efficiency and RT-PCR efficiency and may increase the chances of non-specific cross reactions.

ENTERIC VIRUS DETECTION METHODS OF THE PAST AND PRESENT

Almost all virus diagnostic techniques have been applied to enteric virus detection at some time; however, this discussion will focus on the most commonly used tests for the most commonly targeted viruses. This is not intended to be a comprehensive list, but an overview of common methods.

Although not completely definitive, direct visualization by EM of negative stained specimens is widely used and thus is probably as close as one can get to a gold standard for many enteric viruses (36). Electron microscopy has relatively low to moderate sensitivity with a detection limit of 10^5-10^6 virions/mL and specificity is somewhat dependent upon the skill of the microscopist. Immuno-electron microscopy (IEM), which requires antibody specific for the virus, increases specificity and is somewhat more sensitive. Avian rotavirus, avian reovirus, avian astroviruses, and the ubiquitous "small round virus", now thought to be astrovirus, are all frequently observed by EM in intestinal contents. Electron microscopy continues to be a valuable tool for enteric virus detection.

Indirect immunofluorescence assay (IFA) for TCoV has also been widely used (31, 32). IFA for TCoV may be used to detect viral antigen in intestinal tissues from affected flocks directly, or may be used to evaluate the intestines of turkey embryos inoculated with material from infected flocks which allows for some enrichment of the virus. Since coronaviruses are among the more difficult virus families to identify by EM, this test has been quite valuable.

Electropherotyping is a relatively simple method that has been used with the double stranded, segmented RNA viruses, reovirus and rotavirus (27, 35). Electropherotyping involves the extraction of RNA from a specimen, often feces, and simply running the RNA on an agarose gel. When sufficient levels of virus are present in the sample the viral RNA segments can be directly visualized on the gel. Some strains within a virus family can be differentiated by their electropherotype pattern. Since a high level of virus is needed, as with EM, this method is most useful during the acute phase of infection when high titers of virus are being shed into the feces.

In recent years the application of PCR based tests, including reverse-transcription PCR and real-time PCR, have become common in veterinary diagnostics. Development of PCR methods for enteric viruses of poultry has proven to be beneficial due to the speed, sensitivity, low cost, and specificity of these tests, despite the previously mentioned disadvantages. An additional advantage of PCR based methods is that they can be performed on cloacal swab samples, therefore it is not always necessary to sacrifice birds unlike tests which require tissue. Polymerase chain reaction based methods, including reverse-transcription and real-time PCR methods, have been reported for TCoV, turkey astrovirus type 2 (TAstV-2), avian astroviruses, ARV, and type 1 and type 2 avian adenoviruses (6, 9, 17, 22, 37, 40, 42). Most of these tests are directed to enteric viruses in turkeys.

Virus may also be directly detected by the antigen capture (AC) ELISA. An AC-ELISA has been reported for avian astrovirus (42). The AC-ELISA can process similar sample types as EM, and has high specificity but low sensitivity.

In addition to the direct detection of a target virus, exposure may also be demonstrated by the presence of antibody (Ab). However, by the time antibody can be detected, the acute infection is generally over and recovery has begun; therefore, the value of detecting antibody for direct virus control is limited. Because of limited application to the field, few antibody detection methods are available for enteric viruses of poultry. There are commercial ELISA kits for chickens for avian reovirus antibody and adenovirus type 2 in turkeys (hemorrhagic enteritis virus). The agar gel precipitin tests have also been used for both avian reoviruses and adenovirus. For viruses which can be cultured *in vitro* virus neutralization assay is an option.

LESSONS FROM ENTERIC VIRUSES OF OTHER SPECIES

Many species share enteric viruses, or at least are susceptible to similar viruses from a few families. Probably more important for diagnostics, sample types are similar across species. Viruses from humans are probably the best characterized for diagnostic purposes. Not surprisingly, the detection of human viruses in general, is frequently technologically ahead of poultry viruses. Also, with human enteric viruses there is an added element of diagnostics directed to virus detection in water and food, since they are considered a food safety issue. Additionally, higher sensitivity is demanded as the tests are validated for individuals instead of groups or flocks. That being said, the current standard detection methods used for human enteric viruses are similar to those being used for poultry; EM, IEM, PCR based methods, and antigen detection methods including AC-ELISA and latex agglutination (reviewed in (11, 29). The biggest difference is the availability of numerous commercial assays. In fact, these detection methods are essentially the standards for enteric viruses for livestock and other domestic animals as well. The newest technologies are still in the research lab, and as will be discussed, are mostly advances in nucleic acid detection based methods and antibody based antigen detection methods.

THE FUTURE FOR VIRUS DETECTION, DARE TO DREAM…

What is currently in development for the most important diseases in human medicine is a good predictor of what may be on the distant horizon for poultry enteric virus detection and really detection of any viral pathogen. Some of the most exciting techniques which have been reported, at least experimentally, are the use of genetically engineered "reporter cell lines," nanoparticle technology, bio-sensors or "lab-on a chip," and microarray technology.

Reporter cells lines are probably one of the most innovative novel virus detection methods in development. Reporter cell lines are cell lines that have been genetically engineered to be susceptible to and subsequently respond to infection with a target virus by expressing a "reporter" gene. The reporter gene encodes something that can be easily detected, such as beta-galactosidase or chloramphenical acetyltransferase. Alternatively, infection may trigger a growth effect in the cells, essentially engineered cytopathic effects. Reporter cells lines have been reported for human immunodeficiency virus, human T-cell lymphoma virus (HTLV) type-I and HTLV-II (reviewed in 30). A commercially available reporter cell line kit for herpes simplex virus, the enzyme linked virus inducible system or ELVIS (diagnostic Hybrids Inc., Athens OH), is even available.

Nanoparticle probes and quantum dot based methods can be used to detect either nucleic acid with DNA probes or antigen with antibody and vice versa. There are numerous variations on the exact format of this technology, the details of which are reviewed elsewhere (23, 28, 43). Briefly, DNA probes, antibodies, or proteins may be conjugated to gold nanoparticles. When the particles bind the analyte, they become cross-linked resulting in a measurable change in a physical property which can be colorimetric, fluorescent, or a change in melting temperature. In theory this type of technology can be extremely sensitive, with a potential detection limit of a single virus particle. Of course the sensitivity and specificity are only as good as the antibody or probe used. What sample types are feasible has yet to be determined. Other advantages are that this technology can be very inexpensive, very rapid, it can be highly multiplexed ("DNA barcodes"), and it can be performed point-of-care or "pen-side". At this time nanoparticle based detection tests have only been reported for respiratory syncytial virus (1). In general, the use of nanoparticles in cell biology has been better established for cancer and genetic disease diagnosis than for viral diagnostics.

The use of microarray technology has also recently been applied to virus detection (20, 41). However, sample processing and high cost may make high through-put and application to veterinary diagnostics impractical at present. Microarrays do offer several interesting advantages over other nucleic acid detection methods; they can identify multiple agents simultaneously (5, 7, 47) and they can differentiate genetic lineages of viruses and serotypes (13, 21, 24, 26), and can even detect the presence of drug resistance associated genes (10). All of this information is available upon the initial detection of the virus.

Bio-sensors which use various chemistries and platforms to detect an analyte are also in development for virus detection (2, 18, 39, 44, 46, 48). Although they have been successfully used for chemical detection, their successful application to viruses has yet to be shown. Based on the available platforms, the application of biosensors to virus detection will likely be antibody based, therefore will have similar limitations to other antibody based assays.

Basically there are only three things which can be detected when determining exposure to a given virus: 1) the virus protein or antigen itself, 2) viral nucleic acid, or 3) antibody. Most of the newest cutting edge technology involves the use of novel technology to

more sensitively detect what has always been used. What is important for enteric viruses of poultry is to target viruses which cause disease, and therefore virulence markers and not broad virus families.

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MOLECULAR CHARACTERIZATION OF ASTROVIRUSES AND ROTAVIRUSES DETECTED IN TURKEY FLOCKS FROM HATCH TO 12 WEEKS OF AGE

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Phylogenetic analysis was performed with sequence data obtained from astroviruses and rotaviruses detected during a survey done to determine the prevalence of enteric viruses on eight commercial turkey farms. Although there were differences in production parameters, these farms were considered normal. Intestinal contents were collected from poults prior to placement and at 2, 4, 6, 8, 10, and 12 weeks of age. The samples were screened for astrovirus, rotavirus, reovirus, and coronavirus by RT-PCR, and for Type II adenovirus by PCR. The samples collected before placement of the poults on the farms were only positive for rotavirus (44% of samples examined). All of the farms at all other time points were positive for rotavirus and astrovirus. Of the 96 samples examined 89.5% were positive for astrovirus and 67.7% were positive for rotavirus. All samples were negative for coronavirus and reovirus at all time points and positive for adenovirus Type II or hemorrhagic enteritis virus at six weeks. These results are similar to previously published reports on the prevalence of enteric viruses in commercial turkeys in that astroviruses and rotaviruses are the most frequently identified viruses in turkey flocks (6, 7, 10, 11). However these earlier studies were done using electron microscopy and electropherotyping techniques which are not as sensitive as the molecular techniques used today for viral diagnosis. Thus, the prevalence of enteric viruses in healthy flocks, as demonstrated by RT-PCR, is likely much higher than previously thought.

Molecular characterization of the detected viruses was performed by partial sequence analysis of both the polymerase and capsid genes of the astroviruses and the NSP4 gene of the rotaviruses. Three types of avian astroviruses were detected; turkey astrovirus 1 (TAstV-1), turkey astrovirus 2 (TAst-2), and avian nephritis virus (ANV) were identified. The most commonly detected astrovirus was TAstV-2, which had a high level of genetic variation, particularly in the capsid gene, with more than one genotype detected at the same time in the same farm. This indicates that possibly more than one serotype of TAstV-2 is circulating in turkey farms (4). Detection of TAstV-1 has not been reported in turkeys since it was first isolated in 1985 (5, 8), and this is the first time ANV, which is has previously only been isolated from chickens, has been detected in turkeys although antibody to this virus has been previously reported in turkey flocks (1, 3).

Phylogenetic analysis of the NSP4 gene of the detected rotaviruses showed that at least three different genotypes appeared to be circulating in the farms, although this gene was fairly conserved with 90% nucleotide identity among isolates. The electropherotype pattern of these rotaviruses was similar to that reported for rotavirus group D, also referred to as rotavirus-like viruses (2, 7, 9).

In conclusion, the presence of rotaviruses and astroviruses in commercial turkeys is more common than previously reported, and multiple genotypes may co-circulate on a given farm. Further investigations to determine the role of these viruses in enteric diseases of turkeys are needed.

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CHARACTERIZATION OF CHICKEN ASTROVIRUSES FROM FLOCKS EXPERIENCING RUNTING-STUNTING SYNDROME

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ABSTRACT

Chicken astroviruses (CAstV) were isolated from young broiler flocks experiencing a runting stunting syndrome in the winter of 2005. Initially, PCR primers specific for the polymerase and capsid genes of turkey astrovirus 2 (TAstV-2) were used to screen intestinal tissues and contents for astroviruses. Samples were negative using these primer sets. However, using degenerate primers to the astrovirus polymerase gene, numerous positive samples were detected. Sequence analysis identified viruses that were unlike any of the reported turkey astroviruses and most similar to a previously reported avian nephritis virus (ANV). ANV has recently been classified as an astrovirus. The capsid gene of the viruses was amplified using newly constructed primers and sequenced for analysis. Amino acid sequence analysis of the capsid gene revealed a high similarity to ANV.

The viruses were propagated in SPF embryos. Specifically chicken embryos inoculated at 16 days of embryonation (doe) via the yolk sac had moderately distended intestines four days post inoculation. Embryos inoculated at six doe via the yolk sac were hemorrhaged by 12 doe. Embryo intestines from these passages were submitted for negative stain electron microscopy. Viral particles in the range of 27-33nm were observed. However, no hallmark structural characteristics were seen. Embryo intestines were also positive for astrovirus by RT-PCR. The viruses do not appear to replicate in primary chicken embryo liver or kidney cells or in primary chicken kidney cells. This contradicts reports that ANV replicates and causes cytopathic effect in primary chicken kidney cells prepared from certain breeds of birds. Additional cell lines are currently being evaluated for CAstV replication. The embryo-propagated virus was sucrose purified and homogenized intestines inoculated via gavage into groups of one-day-old SPF and broiler chicks. Body weights and lengths will be taken as well as tissues for histopathology.

EVALUATION OF CURRENT VACCINATION METHODS FOR aMPV INFECTION IN TURKEYS

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SUMMARY

A live attenuated avian metapneumovirus vaccine is presently used in turkeys in Minnesota to prevent pneumovirus infection. The objective of this study was to compare two commonly used vaccination protocols (spray and eyedrop vaccinations) for their comparative efficacy. We evaluated immune response, reduction in clinical disease, and virus shedding post challenge. Turkeys were challenged with aMPV three weeks post first and second vaccinations. Following the first vaccination challenge, birds in the spray vaccinated group showed clinical signs almost similar to the non-vaccinated control group whereas birds in the eyedrop vaccinated group showed only minimum clinical signs. However, birds in both the spray and eyedrop vaccinated challenged groups showed only minimal signs post second vaccine challenge. Birds in the vaccinated and non-vaccinated groups sero-converted by 14 days post-challenge following first vaccination. There was a significant increase in the GMT scores of birds in the vaccinated groups upon challenge post second vaccination. Birds in the non-vaccinated and spray vaccinated challenged groups showed significant virus shedding post first vaccine challenge whereas there was a considerable reduction in the virus shedding in the eyedrop vaccinated group. There was a significant reduction in the virus shedding in both the eyedrop and spray vaccinated challenged birds following the second vaccination.

INTRODUCTION

Avian metapneumovirus (aMPV) is a member of the Metapneumovirus genus of the family *Paramyxoviridae* (7). It is a negative sense single stranded non-segmented RNA virus with an envelope. In turkeys, the virus causes an acute upper respiratory tract infection. The clinical signs of aMPV infection include nasal discharge, swollen sinuses, coughing, sneezing, tracheal rales, and foamy conjunctivitis (5, 8, 10).

Infections with aMPV are a serious concern for the turkey growers of Minnesota. Strict biosecurity measures adopted to control infection appear to have not reduced its incidence in the state (2). Prevalence studies indicate an increase in the incidence of aMPV in Minnesota turkey flocks over the years (4). A live attenuated virus (6) marketed as Pneumomune (Biomune, Lenexa, KS) has been used in Minnesota turkeys to prevent aMPV infections.

The objectives of the study were first to determine the efficacy of the vaccine by analyzing clinical signs, virus shedding and serology post-challenge and second to compare two vaccination methods, low volume spray and eye drop vaccination, for their effectiveness in reducing the severity of infection and virus shedding. Currently there are a number of live viral vaccines for use in poultry. They are being administered via spray or drinking water.

MATERIALS AND METHODS

Birds. Two hundred and forty female turkey poults (Large White, Nicholas) from an aMPV naïve breeder flock were used in this study.

Vaccine. A commercially available live attenuated aMPV vaccine (Pneumomune, Biomune) was used in this study. This vaccine was originally developed by serial passage of aMPV (aMPV/Minnesota/Turkey/1a/1997) on Vero cells (6).

Vaccination methods. Three treatment groups of turkeys were used to evaluate the two different methods of vaccine application; eyedrop and spray vaccinations. Turkeys in group one received the vaccine by eyedrop method at one and five weeks of age, and turkeys in group two received the vaccine via a low volume sprayer (80-100 um particle size) at one and five weeks of age. Turkeys in group three served as non-vaccinated controls.

Challenge virus. A Minnesota isolate of aMPV (aMPV/Minnesota/Turkey/19/2003) was used (10) to challenge vaccinated turkeys. This virus first propagated in chicken embryo fibroblast (CEF) was further propagated in two-week old-turkeys. Nasal turbinates were collected at 6 days post-infection (PI) and a 20% homogenate was prepared. This preparation

designated as MN 19 TH 20, was used as the challenge virus.

Experimental design. Two challenge studies were conducted at four and eight weeks of age on turkeys three weeks after the first and second vaccinations respectively. Ten turkey poults were randomly selected from each vaccine treatment group and from the non-vaccinated control group. These thirty poults were mixed together and kept in an isolation room. Four such replicates were used in the study with a total of 120 birds for the first and another 120 for the second vaccine challenge studies. Three weeks after the first vaccination, poults were challenged with CEF aMPV MN 19 20% nasal turbinate suspension. Fifty microliters of the virus suspension was instilled in each eye and nostril using a micropipette. A total volume of 200 µl of the challenge virus was inoculated oculonasally.

Choanal swabs were collected from each bird on 6 and 21 days post challenge (dpc) for RT-PCR analysis for viral RNA. Two milliliters of blood was collected from each bird on 14 and 21 days post challenge to look for the levels of aMPV antibodies by aMPV-ELISA.

Clinical signs. Clinical signs in turkeys challenged with aMPV were recorded on alternate days. A clinical sign scoring system was followed to monitor and score the severity of the disease expression in the birds (10). The total score for each bird was added and expressed as the fraction out of the total number of birds showing clinical signs.

Serology. Serum samples were collected from challenged birds at 14 and 21 days post challenge following both first and second vaccinations. Sera were examined for the presence of antibodies against aMPV by aMPV-ELISA using anti-turkey IgG conjugate as the secondary antibody (2).

Virus shedding. Virus shedding from the birds in both the non-vaccinated challenge and the vaccinated challenge groups were assessed by testing the choanal swabs collected from the birds by RT-PCR (9).

RESULTS

Clinical signs. Birds in the non-vaccinated challenged group showed clinical signs on 2, 4, 6, 8, 10, and 12 dpc following first vaccination. The major signs included nasal discharge and swelling of the infraorbital sinus. Birds in the low volume spray vaccinated challenged group also showed clinical signs almost similar to the non-vaccinated control groups whereas birds in the eyedrop vaccinated challenged group showed only minimum clinical signs. Birds recorded a maximum average clinical sign score of 2.44 on 8 dpc in the non-vaccinated control groups. Birds in the low volume spray vaccinated challenged group showed a maximum average score of 2.05 on 8 dpc. In the eyedrop vaccinated challenged group, birds showed a maximum average score of 0.15 on 8 dpc.

Birds in the non-vaccinated challenged group showed clinical signs on 2, 4, 6, 8, 10, and 12 dpc following second vaccination. The major signs included nasal discharge and swelling of the infraorbital sinus. Birds in the low volume spray vaccinated challenged group and birds in the eyedrop vaccinated challenged group showed only minimum clinical signs. Birds recorded a maximum average clinical sign score of 1.05 on 8 dpc in the non-vaccinated control groups. Birds in the low volume spray vaccinated challenged group and birds in the eyedrop vaccinated challenged group showed a maximum average score of 0.025 on 4 dpc.

Serology. Birds in both the vaccinated (spray and eyedrop) and non-vaccinated groups sero-converted by 14 dpc following first vaccination. The geometric mean titer (GMT) was 62 for the non-vaccinated challenged birds. The eyedrop vaccinated challenged and the low volume spray vaccinated challenged birds recorded a GMT of 127 and 77, respectively. There was no significant increase in the antibody titer in birds in any of the groups when sera were tested 21 dpc. The GMT score for non-vaccinated challenged birds remained at 62 on 21 dpc whereas there was a slight decrease in the titer of birds in the eyedrop vaccinated challenged group. The titer was reduced to 110 from 127 on 21dpc in the eyedrop vaccinated group. There was a slight increase in the GMT score for birds in the low volume spray vaccinated challenged group where the titer increased to 82 on 21 dpc.

There was a significant increase in the GMT scores of birds in the vaccinated groups upon challenge post second vaccination. The non-vaccinated challenged birds had a GMT of 47 and 62 on 14 and 21 dpc, respectively. The eyedrop vaccinated challenged birds had 473 and 433 GMT scores on 14 and 21 dpc, respectively. Birds in the low volume spray vaccinated challenged group recorded a GMT of 249 on 14 dpc and a score of 237 on 21 dpc.

Virus shedding. Birds in the non-vaccinated challenged group showed the presence of viral RNA when tested by RT-PCR. There was a significant reduction in the virus shedding upon challenge in birds that were eyedrop vaccinated. Interestingly, birds in the low volume spray vaccinated challenged group also showed significant virus shedding. Five birds each in the first three replicates and seven birds in the fourth replicate showed the presence of viral RNA when 10 birds each were tested from each replicate. On average, 85 % of the birds showed virus shedding in the non-vaccinated challenged group, 5 % in the eyedrop vaccinated group and 55 % in the low volume spray vaccinated group.

The non-vaccinated challenged birds showed virus shedding on 6 dpc following second vaccination when tested by RT-PCR. There was a significant reduction in the virus shedding in both the eyedrop vaccinated challenged and in the low volume spray vaccinated challenged birds. In the non-vaccinated challenged group, 80-90 % of the birds were positive for aMPV RNA. In the eyedrop vaccinated group, 12.5 % birds were positive for viral RNA whereas in the low volume spray vaccinated challenged group, only 5 % of birds showed the presence of viral RNA.

DISCUSSION

A single spray vaccination of turkey poults against aMPV infection at one week of age did not provide protection upon challenge. However a second booster spray vaccination at five weeks of age was protective against aMPV infection. This finding explains to some extent the inconsistencies observed in Minnesota turkeys upon vaccination with live attenuated vaccine against aMPV infection. Development and evaluation of a live attenuated vaccine against aMPV infection was reported previously (6). In that study the authors evaluated the vaccine given oculonasally or orally in turkeys. The present study investigated the comparative efficacy of spray and eyedrop vaccinations in protecting birds against aMPV infection. The study and the results are relevant in the context that the majority of farms in Minnesota vaccinate birds against aMPV infection through spray vaccination. The serological response and virus shedding also support the observation that the initial spray vaccination was ineffective in providing protection against aMPV challenge. It appears that initial spray vaccine sensitized turkeys for a response to the second booster vaccine. There can be various reasons for the inability of spray vaccination to elicit an efficient immune response. Dose of vaccine and time required for spray vaccination may be of prime importance. A study to compare different vaccination methods to combat Newcastle disease showed that spray vaccination was ineffective in protecting birds against challenge (3). Their findings are similar to the present results that spray vaccination is inferior to ocular vaccination.

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DIFFERENCES IN PATHOLOGY BETWEEN EARLY AND RECENT ISOLATES OF AVIAN METAPNEUMOVIRUS IN TURKEYS

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SUMMARY

Four different preparations of early and recent isolates of the aMPV were analyzed for their pathogicity in turkeys. The specific objective was to compare the disease causing potential of these viruses. We examined clinical signs and histopathological changes in turkeys post-infection. Briefly, seventy two-week-old turkey poults were divided into five groups. Group 1 was kept as non-infected controls. Group 2 was inoculated with Vero cell propagated early isolate (aMPV/MN 2a/1997) oculonasally. Birds in group 3 were inoculated with Vero cell propagated recent isolate (aMPV/MN 19/2003). Groups 4 and 5 were inoculated with allantoic fluid propagated early isolate (MN2a) and chicken embryo fibroblast (CEF) propagated recent isolate (MN19) respectively. On day six post-infection (PI), five birds from each group were selected randomly to collect choanal swab and blood for RT-PCR and aMPV-ELISA, respectively. Tissues such as nasal turbinate, trachea and lungs were collected from the necropsied birds. Clinical sign scoring of infected turkeys demonstrated a more pronounced clinical disease in birds inoculated with MN 19. Nasal turbinate and trachea showed histological changes in birds inoculated with MN 19-Vero, MN 19-CEF, and MN 2a-Vero. Birds infected with MN 2a-allantoic showed histopathological lesions only in the nasal turbinates. The findings of the present work indicated that the recent isolate produced more severe clinical signs and histopathological changes in the infected turkeys compared to the early isolate.

INTRODUCTION

Avian metapneumovirus (aMPV) produces an acute upper respiratory tract infection in turkeys and is also involved in the etiology of swollen head syndrome in chickens. The disease in turkeys is characterized by oculonasal discharge, coughing, sneezing, tracheal rales, foamy conjunctivitis, and swelling of infraorbital sinuses. In layers, aMPV causes a drop in egg production and paleness of the eggshell. Uncomplicated cases have low mortality (2 to 5%) but infections with concurrent bacterial infections can result in up to 25% mortality (4, 5).

The disease was first identified in South Africa in 1978 in the form of an acute sinusitis affecting three to four week old poults. Later, the disease was detected in the United Kingdom, France, Spain, Germany, Italy, Netherlands, Chile, Israel, Dominican Republic, Hungary, Austria, Greece, Taiwan, Brazil, Mexico, and Asia. The United States was considered free of aMPV infection until 1996, when an outbreak of upper respiratory tract infection occurred in some of the turkey farms in Colorado. In the following year, the disease was identified for the first time in Minnesota. Since then, the incidence of the disease has increased in Minnesota despite biosecurity measures apparently similar to those used in Colorado (2, 3, 4, 5, 6).

The specific objectives of this study were to compare the early and recent isolates of aMPV for their potential to cause infection in turkeys and to analyze their pathogenicity and virulence. The disease causing potential of these viruses was assessed by analyzing clinical signs and histopathological changes in turkeys inoculated with different virus preparations.

MATERIALS AND METHODS

Turkeys. Seventy two-week-old female turkey poults (Large White, Nicholas) from an aMPV-naïve breeder flock were used.

Viruses. Four, differently processed preparations of aMPV were used in this study as detailed below.

1. **MN 2a-Vero**. This virus was originally isolated from the nasal turbinates of 11-week-old male turkeys showing respiratory disease in 1997 from Minnesota (2). The virus was designated as aMPV/Minnesota/turkey/2a/97. This virus was first passaged in CEF cells and later adapted to Vero cells. The virus at 12^{th} passage with a titer of 10^5 $TCID_{50}$ / mL was used.

2. **MN 2a-allantoic**. This preparation was obtained from Dr. Darrel Kapczynski, Southeast

Poultry Research Laboratory, USDA, Athens, Georgia. This preparation was made by passaging aMPV/Minnesota/turkey/2a/97 in turkey embryos by inoculating the virus through the allantoic cavity route. The allantoic fluid with a virus titer was 10^6 EID_{50}/mL was used for inoculation. This preparation was also titrated on Vero cells to analyze the tissue culture infective dose.

3. **MN 19-Vero**. A Vero cell-propagated, recent isolate of aMPV (aMPV/Minnesota/Turkey/19/2003) was used (8). The virus was originally isolated from nasal turbinates of 8-week-old turkeys with acute upper respiratory tract infection. Six blind passages were performed on CEF followed by six passages on Vero cells. The virus having a titer of 10^5 $TCID_{50}$ / mL was used as the inoculum.

4. **MN 19-CEF**. This preparation was made by blind passage of aMPV/Minnesota/Turkey/19/2003 on CEF cells for 7 passages. When titrated in Vero cells, the virus had a titer of 10^4 $TCID_{50}$ / mL.

EXPERIMENTAL DESIGN

Seventy two-week-old turkey poults were divided into five groups, groups 1 through 5. Group 1 with 10 birds was kept as non-infected control. Group 2 with 20 birds was inoculated with MN 2a-allantoic. Birds in group 3 with (10 birds) were inoculated with MN 2a-Vero. Groups 4 (with 20 poults) and 5 (with 10 poults) were inoculated with MN 19-CEF, and MN 19-Vero, respectively. As a standard procedure, a total of 200 µL of the virus was inoculated oculonasally; 50µL of the virus suspension was instilled in each eye and nostril using a micropipette.

On day six post-infection (PI), five birds from each group were selected randomly. Choanal swabs and blood were collected from these birds for RT-PCR (7) and aMPV-ELISA (1), respectively. Nasal turbinate, trachea, and lungs were collected for RT-PCR. A subset of these tissues was also collected in 10% buffered neutral formalin for histopathology and immunohistochemistry.

RESULTS

Clinical signs. Birds in all four groups inoculated with different preparations of aMPV showed clinical signs on 2, 4 and 6 days PI. The major signs included nasal discharge and swelling of infraorbital sinuses. Birds inoculated with MN 19-Vero showed severe clinical signs with the highest clinical sign score of 2.7 on 6 day PI. Based on clinical sign scoring, the severity of infection was in the following order: MN 19-Vero followed by MN 19-CEF, MN 2a-Vero, and MN 2a-allantoic. Comparison of aMPV MN 19 and aMPV MN 2a based on clinical sign scoring showed that birds inoculated with aMPV MN 19 produced more clinical signs than birds inoculated with aMPV MN 2a. Birds in the non-infected control group did not show any clinical signs.

Histopathology. Birds inoculated with MN 2a-allantoic showed lesions only in nasal turbinates and not in the trachea or lungs. Nasal turbinates and tracheas showed histological changes in birds inoculated with MN 19-Vero, MN 19-CEF, and MN 2a-Vero. 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 and individual necrotic cells in the epithelial lining were also observed. One important feature in the nasal turbinates from birds inoculated with aMPV MN 19 (both MN 19-CEF and MN 19-Vero) was the presence of a multifocal loss of cilia. The main lesions in trachea consisted of an increased infiltration of lymphocytes, macrophages, and heterophils in lamina propria. The lung tissues showed no histopathological changes in any of the birds inoculated with any of the virus preparation. None of the tissues from non-infected birds showed any histopathological lesions.

RT-PCR. Viral RNA was detected on 6 day PI in nasal turbinates from all of the birds inoculated with MN 19-Vero, MN 19-CEF, and MN 2a-Vero. Only forty percent of the birds inoculated with MN 2a-allantoic were positive when tested by RT-PCR. In the tracheas, viral RNA could be detected in 60% of the birds infected with MN 19-Vero and MN 19-CEF while only 40% of the birds infected with MN 2a-Vero showed viral RNA by RT-PCR. The lung tissues did not reveal the presence of viral RNA in any of the infected groups. No viral RNA could be detected in any of the three tissues collected from birds in the non-infected group.

Immunohistochemistry. Immunohistochemistry revealed the presence of aMPV antigen on 6 day PI in the nasal turbinates and the tracheas. Viral antigen could be detected on the apical surface of the ciliated epithelium of the nasal turbinates. Seventy-five to eighty percent of the birds showed aMPV antigen in turbinates on day 6 PI when inoculated with MN 19-Vero, MN 19-CEF, and MN 2a-Vero. Tracheas from 60% of the birds were positive for viral antigen when inoculated with MN 19-Vero and MN 19-CEF, whereas only 20% of the birds were positive when inoculated with MN 2a-Vero. Viral antigen could be detected in the mucosal epithelium of the trachea. Immunohistochemistry did not show the presence of aMPV antigen in lungs on day 6 PI in any of the infected birds. Birds in the non-infected group were negative for the presence of aMPV antigen by immunohistochemistry.

Serology. Sera of birds collected from the treatment and control groups on 6 days PI were negative for antibodies against aMPV.

DISCUSSION

We examined four different preparations of aMPV for their disease causing potential in turkeys. The four preparations were selected based on various considerations, specifically, prior knowledge on the virulence of the isolate in our pilot experimental studies (8). The 1997 isolate of aMPV/Minnesota/2a has been extensively studied and used in several studies. A Vero cell propagated as well as a turkey embryo propagated MN 2a were used in the study. The turkey embryo-propagated virus (MN 2a-allantoic) was used with the hope that propagation of the virus in the natural host embryo would improve the pathogenicity of the virus.

All the four virus preparations were titrated on Vero cells. The Vero cell - virus titers for MN 2a-allntoic, MN 2a-Vero, Mn 19-CEF and MN 19-Vero were $10^{2.5}$, 10^5, 10^4, and 10^5 $TCID_{50}$/mL, respectively. It is possible that CEF and embryo adapted preparations may not replicate as well on Vero cells as they do in CEF and embryos. There fore it is possible that these titers do not accurately reflect the amount of virus present. Clinical signs score and virus detection in tissues by RT-PCR and IHC were lowest in the MN 2a-allantoic group. The MN 2a-allantoic virus preparation had the lowest titer in Vero cells even though it had a titer of 10^6 EID_{50} in embryos.

Sera from birds from all the treatment and control groups were negative for antibodies against aMPV by aMPV-ELISA. This was to be expected because the time point of serum collection was too early for the development of a detectable level of antibodies.

The results of this study clearly indicate that the 2003 isolate of aMPV produced more severe clinical signs and histopathological changes in infected turkeys than those by MN 2a. These results suggest that aMPV is becoming more pathogenic with time and that the new 2003 isolate could be an ideal candidate for the development of challenge models of aMPV infection in turkeys.

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CHICKEN INFECTIOUS ANEMIA VIRUS (CIAV) IN COMMERCIAL BROILERS

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Chicken infectious anemia virus (CIAV) or (CAV) was first isolated in 1979 in Japan by Yuasa *et al*. The virus is now classified in the family *Circoviridae* and genus Gyrovirus. The virus is non-enveloped with single strand negative sense DNA. The virus is highly resistant to disinfectants, heat, chemical, and environmental degradation. There is now world wide distribution with CAV being present in all poultry rearing facilities.

Adult chickens and pullets typically show no signs of disease but they are potential vertical and horizontal shedders to their progeny and will likely have a varied degree of immunosuppression themselves resulting in a poorer immune response to vaccinations and increased susceptibility to secondary bacteria during times of stress.

The classic clinical disease caused by CAV is seen in broiler chicks 9-18 days of age as anemia, subcutaneous and muscular hemorrhages (blue wing), dermal necrosis, thymic and bursal atrophy, retarded growth, depression, and some mortality. The surviving chicks from a clinical course of CAV may also be permanently immunosuppressed leading to increased susceptibility to secondary bacterial infections as the chicks become older. The clinical disease is seen in chicks with inadequate maternal antibody at hatch through the first five days or so of life. The clinical aspects of CAV in broilers can be easily prevented by exposing breeder pullets and males to CAV through either natural exposure to CAV or by vaccination with a commercially available CAV vaccine by wing web or drinking water.

If the breeder hens pass on adequate CAV specific maternal antibody to their progeny the CAV will be neutralized thus preventing clinical disease in the chicks. As soon as chicks hatch they are at risk from either vertical or horizontal exposure or both to CAV and CAV neutralizing antibodies are critical for preventing clinical disease.

The virus is transmitted both vertically and horizontally and any chicken regardless of age can be infected with CAV. The cycle of transmission begins at the parent and pullet stage and is bolstered by environmental contamination leading to additional horizontal exposure in the broiler house. Latency is a common occurrence in adults, embryos, and chicks with the virus residing in the reproductive organs. CAV infections can be latent or active in the reproductive organs of hens and roosters regardless of the CAV antibody status and vertical transmission can lead to either active or latent infections in the embryo. There are multiple regulators that play a role in the promotion or inhibition of viral replication. Recent findings suggest sexual hormones such as estrogen and testosterone play a role in the activation of viral replication. This is supported by identification of replicating virus in the embryo only during times of sexual organ development and the common occurrence of seroconversion to CAV in breeder hens during the onset of egg production.

The age at which a flock acquires seroconversion as a result of natural exposure to CAV varies dramatically between houses on the same farm, between farms, between complexes and companies, breed, and seasonally. The exposure is frequently uneven with in a house creating a high % CV and a large minimal and maximum range of titers. The titers also vary significantly during the production cycle of the flock which is reflected in the progeny as uneven and low CAV maternal antibody titers. It is difficult to predict how a naturally exposed flock will seroconvert and perform. Vaccination is typically administered between 10-12 weeks of age with high titers occurring in nearly all birds around nine weeks post vaccination. The titers to CAV obtained through vaccination are maintained through lay with minimal to mild variations during a flock's production cycle thus providing protective maternal antibody to progeny which is more uniform and predictable verses naturally exposed flocks.

Since most of the hens in the USA are serologically positive to CAV classical CAV induced blue wing is not nearly as common as the subtle effects on performance and the reduced ability to "fight" off secondary bacterial infections. The most common and greatest losses related to CAV infections are not due to the clinical blue wing but are rather a consequence to the subclinical immunosuppression which occurs to some degree in nearly 100% of broiler flocks in the USA. Immunosupression results from CAV replication in actively dividing host cells such as hemocytoblast in the bone marrow and any tissue with a population of T-lymphocytes, in particular, the thymus. With viral replication comes the death of the host cell with

resulting anemia and or decreased T-lymphocytes. Decreased antigen specific cytotoxic T-lymphocytes and platelets also leads to decreased macrophage function and ultimately reduced ability to defend against bacterial infections and reduced responses to vaccinations. Co-infection with a combination of Marek's, infectious bursal disease virus (IBDV), or reovirus will increase the severity of the immunosuppression leading to a greater percentage of secondary infections and reduced performance.

The transient or permanent depletion of cytotoxic T-lymphocytes by replicating virus is the major factor leading to a reduced ability to fight off bacterial infections which leads to retarded growth, increased feed conversion, mortality, and condemnations. Generally mortality is only slightly increased between 10-14 days but can be greatly increased beyond 35 days of age. Typically, this mortality is due to bacterial infections leading to osteomyelitis, (*E. coli* or *Staphylococcus* spp.), peritonitis or septicemia (*E. coli*), or gangrenous dermatitis caused by *Clostridium* spp. or *Staphylococcus aureus*. Increased susceptibility to respiratory viruses is also common following an infection with CAV. Classical CAV induced blue wing is not nearly as common as the subtle effects on performance and the reduced ability to "fight" off secondary bacterial infections.

During the last seven years there has been a noticeable yet slow increase in the number of flocks overtly experiencing the adverse effects of CAV in both pullets and broilers. Some of the clinical occurrences experienced in pullets include delayed seroconversion of pullets until sexual maturity at which time vertical transmission of CAV to progeny and early infection (two to six weeks of age) related to increased mortality and stunting prior to eight weeks of age. In broilers there has been an increased number of clinical CAV infections in broiler flocks across geographic regions and companies as a result of vertical transmission of CAV leading to replication of CAV in the embryo and after hatching and an increase of secondary bacterial infections have dominated many broiler operations suggesting immunosuppression caused CAV, IBDV, Marek's, reoviruses, etc. It is likely the most common and costly effect of CAV is also the most subtle and CAV is rarely associated as a root cause. These effects are reduced feed conversion, increased condemnations, reduced daily gain, poorer response to vaccination, increased adverse reactions to respiratory vaccines, and increased mortality. The reason(s) for the increase in CAV infections has not been confirmed, but it is likely multifactorial and may be related to prolonged periods of litter usage, short out times, and higher stocking densities leading to increased CAV levels on the farm, dynamics of the percentage of breeds and breed crosses being reared, genetic changes in the breeds, changes in the virulence of CAV in the field, changes in the nutritional formulations and the rate of gain, and a combination of infections with other viruses such as Marek's, IBDV, reovirus or unknown viruses or factors.

Serologic surveys indicate that all broiler operations have exposure to CAV and are therefore susceptible to infection. The serologic data also show that the level of exposure to CAV does vary between flocks, houses, farms, complexes, and companies. This variable exposure is one reason it is difficult to associate performance issues and disease with CAV infections. The exposure to and replication of CAV and serologic responses in breeder hens also varies through out the life of a flock. The variations occurring in the hen flocks is a major source of the variations detected in the broilers. Although additional work needs to be done for confirmation, the early evidence suggests that broiler flocks with the lowest maternal antibody levels and the highest CAV titers at processing also have the poorest performance.

Maternal antibodies to CAV play a crucial role in preventing viral replication and the clinical effects of CAV in broilers. However recent data show that the maternal antibodies are depleted rapidly under current field conditions and CAV is able to replicate creating infections and or immunosuppression in broilers. The level and duration of maternal antibodies to CAV must be extended beyond 10 days of age to prevent the associated adverse effects on performance. The control of IBDV, reoviruses and Marek's are also required to prevent the clinical effects of immunosuppression.

ADAPTATION OF NEW TECHNOLOGIES TO DETECT CONTAMINATION OF LIVE AVIAN VACCINES WITH CHICK INFECTIOUS ANEMIA VIRUS (CIAV)

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SUMMARY

Recent infection of Australia's only SPF egg supply flock with CIAV necessitated attempts to rescue bulk and finished vaccines manufactured from potentially CIAV contaminated substrate. Some unique approaches were employed including spiking product with CIAV and delineating limits of detection with PCR. Preservatives used in the manufacture of a live coccidiosis vaccine were evaluated for their ability to inactivate CIAV. Bulk Newcastle disease (ND) vaccines were tested for evidence of CIAV contamination by three different detection methods. Evidence of freedom or contamination obtained from these studies was presented to the Australian national registration authority. The vaccine manufacturer requested approval to supply these vaccines to poultry companies. An interim measure to import SPF eggs was not approved by the Australian government.

INTRODUCTION

Contamination of Australian SPF egg production flocks used for live avian vaccine production with CIAV has been reported sporadically in the 1980s and 1990s but was not subsequently reported until 2005. Prior to the establishment of a national vaccine registration authority in 1995, vaccine manufacturers could negotiate with State regulatory agencies on the sale of vaccines from potentially CIAV contaminated substrate. Australian SPF egg producers had, in the past, voluntarily destroyed infected flocks due to difficulties with the marketing of live vaccines derived from those flocks. More recently, Australia has largely adopted *European Pharmacopoeia* (EP) standards and vaccine supply from potentially CIAV contaminated substrate is now strictly governed by those conditions.

In September 2005, the only Australian SPF egg supply flock reported serological evidence of CIAV infection in its only flock that was supplying SPF eggs to manufacturers of avian vaccines. This paper describes actions taken by one Australian vaccine manufacturer to test and validate bulk and finished products derived from SPF eggs supplied immediately before notification of the CIAV infection.

CIAV IN SPF FLOCKS

The EP (3) requires vaccine manufacturers to declare as unsatisfactory any product produced from SPF flocks that show evidence of the presence of a slow spreading agent, such as CIAV, during the four-week period immediately proceeding the date on which the positive sample was detected. However, the EP does allow use of CIAV-positive material in vaccines for use in birds from seven days of age and for the production of inactivated vaccines. The Australian regulatory authority took a more conservative approach and required a six-week period of quarantine of product immediately preceding the detection of a positive serum sample. The basis for these exclusion times are dependent on evidence of limited vertical transmission times in commercial breeding flocks based on virus isolation studies (5). However, other studies (2) have indicated that the presence of antibodies can no longer be considered a true indication of CIAV status and that long-term vertical transmission of viral DNA, detected by nested PCR, can occur from antibody positive and negative hens (1, 6). Consequently, it has been suggested that eradication of CIAV on infected SPF sites may be difficult because viral DNA can be transmitted vertically and can be reactivated during the laying cycle (7).

The Australian SPF egg producer has attempted eradication of CIAV through slaughter of the affected flock, decontamination of the site and testing of an adjacent separately-housed replacement flock for antibody by ELISA and for viral DNA extracted from blastodisc samples by nested PCR. To date, no evidence of CAIV has been found in the adjacent SPF production flock and vaccine manufacturers have been supplied with eggs for live avian vaccine production.

TESTING OF POTENTIALLY CONTAMINATED BULK AND FINISHED VACCINES

Following an inventory check of vaccines produced during the six-week period immediately preceding the reporting of positive CIAV serology by the SPF egg producer, both bulk and finished live coccidiosis vaccine ("Eimeriavax 4M") and bulk ND vaccine destined for production of Vaxsafe® ND were identified as being derived from the potentially CIAV contaminated SPF eggs.

As Eimerivax® 4m is the only live coccidial vaccine produced in Australia, some urgency was placed on the testing of this product. In the absence of a specific monograph on live coccidial vaccines, some modifications of EP monographs for Avian Viral Vaccines were considered together with Veterinary Services Memoranda 80081, 800.89 and 800.109 for Bursal Disease Vaccine of Chicken Bursal Origin, Chicken Anemia Virus and Master Seed and Master Cell Stock Test, respectively. It was decided to undertake three tests to establish freedom of bulk and finished product from CIAV, as follows:

a) CIAV spike test using MSB1cell lines with serial amplification to demonstrate that the processing steps in Eimerivax 4m production reduce or eliminate CIAV. In this test, a commercial culture of *E. acervulina* was inoculated with a high dose of CIAV at the first stage of oocyst processing. Some expectations of freedom were anticipated because the processing methodology involved the use of potassium dichromate and sodium hypochlorite treatment, serial dilution and storage in low levels of formaldehyde solution.

b) CIAV antigen detection in bulk and finished product by IFA following serial passage in MSB1 cells. This test was undertaken in accordance with EP 5.0 Section 2.6.25 "Avian Viral Vaccines: Tests for Extraneous Agents in batches of Finished Product" (4).

c) Chick inoculation test extended to 21 days post vaccination to detect CIAV antibody. This test was modification of the EP Directive 81/852/EEC as amended. Appendix A "Tests for extraneous agents - A3 – Test for Chick Anemia Agent (CAA) in SPF Chickens. The 42 bulks from each of two batches were pooled and inoculated into SPF chicks.

Bulk ND antigens were subjected to three test procedures to detect CIAV contamination as follows:

a) PCR for CIAV on DNA extracted from the bulk antigens using the Qiagen kit (QIAamp Blood Kit) (8).

b) CIAV antigen detection in bulk product by IFA following serial passage in MSB1 cells. This test was undertaken in accordance with EP 5.0 Section 2.6.25 "Avian Viral Vaccines: Tests for Extraneous Agents in Batches of Finished product" (4). Samples from ND bulks 1-20 and 21-42 were separately pooled, neutralized with NDV specific antiserum. Neutralized vaccine was then inoculated into five separate flasks of MSB1 cells and passaged eight times before testing for CAV IFA.

c) Chick inoculation test extended to 21 days post vaccination to detect CIAV antibody. The same procedure was undertaken as that described above used for Eimeriavax 4m.

RESULTS

Table 1 provides evidence that the *Eimeria* bulks and final product were free of CIAV contamination. The data also indirectly provided evidence that the manufacturing process used during purification of the coccidia oocysts inactivates CIAV. In contrast, there was clear evidence that the ND bulks were contaminated with CIAV using three different detection methods. Some discrepancies in the results of the PCR and tests for extraneous agents using tissue culture and SPF chicks were evident. Reports of these procedures together with test laboratory results have been prepared and submitted to the Australian regulatory authority requesting advice as to whether or not the quarantined products can be processed and marketed. Advice has recently been received that Eimeriavax products have been approved for release.

DISCUSSION

More recent evidence of the unreliability of serological testing for CIAV as an indicator of the true status of SPF flocks has caused considerable concern to manufacturers of live avian vaccines. While this concern generally remains, in this case there was clear evidence of the vertical transmission of CIAV following evidence of seroconversion of the flock resulting in contamination of ND bulk antigens. The duration of vertical transmission was fortunately curtailed following the decision of the SPF egg supplier to destroy the flock. However, prior vertical or later horizontal transmission to an adjacent progeny flock in a separate building caused major concern to vaccine manufacturers as they had no alternate SPF egg supply at that time. Despite the great concern expressed by Australian vaccine manufacturers over CIAV contamination of Australia's only SPF supply flock, the Australian government and industry

stakeholders were reluctant to immediately lift restrictions on the importation of SPF eggs.

Evidence for CIAV freedom of processed *Eimeria* vaccine bulks and finished product may well allow these products to be treated in a similar fashion to inactivated vaccines. The recent decision by Australian regulatory authorities to permit release of the Eimeriavax products provided some relief from the current restrictive SPF egg importation policy.

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Table 1. Test results on potentially contaminated products were as follows:

Product	Test Procedure	Detection Method	No. +ve / No. -ve
Eimeria bulk antigens	CIAV spike	Serial passage on MSB1 cells + IFA	0/1
Eimeria bulk antigens	Extraneous agents in seed lots	Serial passage on MSB1 cells + IFA	0/2
Eimerivax 4m finished vaccine	Extraneous agents in seed lots	Antibody in SPF chicks tested by IFA	0/1
ND bulk antigens (42) from two batches	CAV PCR on DNA extractions	PCR	0/20; 2/22
ND bulk antigen (42) from two batches	Extraneous agents in seed lots	Serial passage on MSB1 cells + IFA	2/2
ND bulk antigens (42) from two batches	Extraneous agents in seed lots	Antibody in SPF chicks tested by IFA	2/2

REOVIRUS PROGENY STUDIES—A COMPARISON OF DAY OF AGE ELISA PROFILES AND CHALLENGE PROTECTION

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INTRODUCTION

In two studies broiler progeny from a total of eight 50-60 week-old broiler breeder flocks were challenged. The goal was to see how well the reovirus maternal antibody status of day of age broilers could predict their susceptibility level to reovirus challenge. The first progeny challenge study was reported last year (WPDC, 2005) in which two sister flocks with very different maternal antibody profiles had significantly different protection levels. A second study was conducted to see if this challenge model was repeatable as well as to provide more data for the interpretation of reovirus serology in breeders and broiler progeny.

MATERIALS AND METHODS

All broilers were housed in Horsfal isolator units. Twenty chicks from each flock were bled and sacrificed shortly after hatch for reovirus serology (Idexx ELISA). At three days of age, 20 birds per flock were challenged with reovirus malabsorption strain 2408 by intratracheal (IT) gavage. Another group of 20 remained as non-challenged controls, while at 10 days of age the remaining birds were challenged by foot pad (FP) inoculation. All dosages were titrated at 4.0 $logs_{10}$ (chick ID50). At three weeks of age all birds were weighed and sacrificed. FP inoculated birds were lesion scored using the following system: none (0), mild→ half of FP swollen (1), moderate→ entire FP swollen (2) and severe→ swelling extends into the shank/hock +/- the opposite FP/hock (3).

RESULTS

Flock A/A had by far the lowest geometric mean titer (GMT = 1,216) and the highest percentage of chicks in titer groups 0-1 (45%) and 0-2 (80%). This was also the only flock that had a significant weight suppression (29%) after the three-day IT challenge as well as the highest incidence of viral arthritis (VA) after the 10-day FP challenge. Conversely, Flock C had the highest GMT (2,880), no chicks in titer group 0-1, and the fewest chicks in titer groups 0-2 (20%). They also suffered no weight suppression and the lowest incidence of VA lesions (20%). The other six flocks ranged from 1,815-2,516 on GMT, 0-15% in titer groups 0-1 and 30-57% in titer groups 0-2. None showed signs of weight suppression (Flock F was not significant) and the incidence of VA lesions ranged from 30-44%.

DISCUSSION

Reovirus infections and their possible sequellae have received increasing attention in the past few years. Generally there are two clinical conditions that can result from reovirus infections of broilers. The main difference between them is the window of time that the broiler is susceptible. Reovirus infections by four to five days of age can result in malabsorption and runting/stunting. Infections occurring as late as 10-12 days of age can result in ruptured tendons at processing. Femoral head necrosis, brittle bones and loss of capsular integrity have also been described. The challenge model developed here was designed in order to measure susceptibility to both of these disease conditions.

Because there is little antigenic diversity in the current live priming vaccines and inactivated products, we felt there was a good chance that maternal antibody titer levels would correlate well to protection levels against the reference reovirus malabsorption strain 2408. While the standard serological parameters of GMT and %CV were analyzed in this study, a focus on the incidence of the lowest titer groups seemed to be the best predictor of protection from either the three- or 10-day challenge.

The flock with the lowest GMT (1,216) had the highest incidence of VA (65%) and was the only one with significant weight suppression (29%). Conversely, the flock with the highest GMT (2,880) had the lowest incidence of VA lesions (20%). However, there was no strong trend in the remaining groups with GMT's in between (1,815-2,516) as their VA protection rates ranged from 30-44%. The 29% weight depression in Flock A/A suggests that the GMT may be a good screening tool to flag the most vulnerable flocks by setting a tolerance threshold. Based on these two studies, a broiler GMT of less than 1,500 would be a conservative "line in the sand." The interpretation of the 10-day FP challenge is problematic. It is difficult to say yet what should be an acceptable incidence level. Over time, we may find that 50% protection should be considered good—similar to the IBDV progeny challenge model—but this will require future correlations to field performance.

Of the three serological parameters used, the coefficient of variation (%CV) was the least insightful. Flock B's %CV was as high as the poorest protected flock A/A (68 vs. 70, respectively), yet Flock B's VA incidence (40%) was the same as flock F, which had the best or lowest %CV. Perhaps this is because the %CV is an index of the spread of *all* titer groups in a flock, whereas early reovirus susceptibility is most likely limited to birds in the *lowest* titer groups. For example, a flock with a 5,000 GMT and 90% CV would have fewer birds with group titers 0-2 and thus would be preferred over a flock with a 1,500 GMT and 50% CV – even though the %CV is substantially lower.

The incidence of birds in the lowest titer groups correlated the best with susceptibility to challenge. In the eight flocks tested, a sizable number of birds with no or very low antibody levels (titer groups 0-1) was necessary to see susceptibility to a three-day IT challenge. In the first study, 45% 0-1's resulted in a significant weight depression while 15% did not. The critical threshold would thus seem to be somewhere in between the two. The strongest correlation was seen between the incidence of titer groups 0-2 and the incidence of VA lesions after the 10-day FP challenge. In fact, other than flocks A/A (15% difference) and A

(27% difference) the rates are within five percentage points of each other (see Table).

If one allows for 50% transfer of maternal antibody levels to the progeny, then the breeder reovirus titer profiles should be about twice the level as the broilers. Based on the results presented here, a conservative goal for breeder flocks would be to have a GMT of at least 3,000 and no more than, say, 25% of a flock in titer groups 0-2 to prevent broiler susceptibility to runting and stunting caused by classic reovirus strains. Because maternal immunity wanes during production, a peak GMT at 23-26 weeks of 5,000-6,000 would likely be necessary to maintain this 3,000 minimum for the life of the flock.

These studies demonstrate a minimum level of maternal immunity that is necessary to avoid weight suppression due to a controlled reovirus challenge. How well this model approximates relative protection levels in the field is not exactly clear, though. IT inoculation of reovirus is a natural route of exposure but the starting levels on a broiler farm may not be as high as the progeny challenge inoculum until a cycle of bird-to-bird transmission has taken place. However, this could easily occur in the first four to five days necessary for weight suppression. These studies thus suggest that it would be worthwhile to establish minimum serological standards in order to limit the potential economic losses due to reovirus challenge in broilers.

Table 1. Comparison of broiler reovirus serology at hatch and 2408 challenge results at three weeks.

Group information		Reovirus serology information at hatch				Results after challenge via: IT at 3 days	FP at 10 days
Study	Flock ID	GMT	%CV	No. in titer groups 0-1	No. in titer groups 0-2	Weight suppression?	No. birds with VA lesions
#1	A/A	1,216	70	45%	80%	29%	65%
	B/A	1,981	41	15%	30%	No	35%
#2	A	2,162	62	0%	57%	No	30%
	B	2,516	68	0%	39%	No	40%
	C	2,880	45	0%	20%	No	20%
	D	1,815	45	6%	29%	No	30%
	E	2,428	58	0%	47%	No	44%
	F	2,246	35	5%	40%	5%	40%

DEVELOPMENT OF REAGENTS FOR THE STUDY OF RETICULOENDOTHELIOSIS VIRUS IN THE ENDANGERED ATTWATER'S PRAIRIE CHICKEN

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INTRODUCTION

Reticuloendotheliosis virus was first reported in the endangered Attwater's prairie chicken in 1993 at Texas A&M University (4). The resulting disease subsequent to infection has caused depletions in numbers in these birds and thus, potential loss of genetic variability. Previous REV infections have been described in poultry (6), as well as several other avian species with the resulting disease severity ranging from relatively low mortality rates in chickens, although many are culled prior to death, to development of extensive lymphomas (3, 7). Understanding the disease caused by this virus in the endangered prairie chicken population is critical for designing future methods of prevention.

Initial research of REV in the prairie chicken involved development of a set of reagents capable of identifying the virus, and prairie chicken specific antibodies and lymphocytes. Unlike the chicken, commercial reagents do not currently exist specifically for the prairie chicken. Commercial monoclonal antibodies recognizing chicken CD4 and CD8 antigens

have been identified that cross reacted with prairie chicken CD4 and CD8 positive lymphocytes (1). However, monoclonal antibodies identifying prairie chicken IgY have not been identified nor developed. Future studies involving the investigation of prairie chicken antibody responses to REV require access to reagents capable of identifying prairie chicken antigens.

Additionally, reagents and procedures characterizing REV infection have been limited. Chicken anti-REV antisera (Charles River Laboratories, Wilmington, MA) was commercially available, but antibodies specific for REV antigens necessary for establishing standardized assays for the detection of REV were not. A purified REV protein was also necessary for establishing standardized controls, as well as generating REV antibodies.

We describe the development and purification of reagents that identify REV antigens and prairie chicken antibodies. Following the purification of these reagents, enzyme-linked immunosorbent assays (ELISA) were developed for detection of both virus and prairie chicken antibody.

RESULTS

Purification of REV gag protein and generation of anti-REV gag polyclonal antibody. REV gag polypeptide was expressed in bacteria as a fusion protein with a carboxyl 6X histidine tag and purified by nickel affinity chromatography under native conditions (Qiagen, Valencia, CA) (8). Purified gag polypeptide was visualized as a band of approximately 25 kD on an SDS-PAGE gel. Following purification, polyclonal antibodies specific for the gag polypeptide were produced in rabbits (Robert Sargeant, Ramona, CA). Western blot analysis indicated that whereas normal rabbit sera, serving as a negative control, did not react with the REV product, the rabbit antibody generated after inoculation of the recombinant gag polypeptide was specific for the gag polypeptide.

REV antigen ELISA. An ELISA was developed to detect and quantify virus in REV infected samples. Unlabeled rabbit anti-REV gag antibody was immobilized on a standard ELISA plate (5). Virus samples were loaded into wells with 0.5% Triton X-100, such that the gag protein could bind to the immobilized antibody. Two-fold serial dilutions from 1:16 to 1:512 of both purified REV gag polypeptide in PBS and REV prairie chicken isolate R92 in culture media were analyzed and absorbance at 630 nm was compared. Uninfected culture media, also serially diluted and used as a negative control, was treated in the same manner as the infected samples. Purified REV gag polypeptide and prairie chicken REV isolate R92 were both readily detected by the ELISA and show absorbance values over 1.0 in dilutions at least up to 1:256 (Fig. 1). Absorbance of negative control sera from uninfected prairie chicken were four to five times lower than those of the gag and three to four times lower than those of the whole virus.

Western blot analysis of rabbit polyclonal antibody specific for prairie chicken IgY. Prairie chicken IgY was purified from serum and injected into rabbits for production of polyclonal antibody (Robert Sargent, Ramona, CA). Purified REV gag polypeptide was electrophoresced by SDS-PAGE and transferred to a nitrocellulose membrane before reacting with either chicken or prairie chicken sera. Both the generated rabbit anti-prairie chicken IgY and goat anti-chicken IgY were used to detect the presence of antibodies against the IgY. Prairie chicken anti-REV sera positively labeled the gag antigen as detected by the rabbit anti-prairie chicken IgY, whereas sera were diluted 1:500 to 1:1000 in 5% milk. No cross reactivity was observed between the prairie chicken IgY and the goat anti-chicken IgY. Chicken anti-REV sera also positively labeled the gag antigen as detected by the goat anti-chicken IgY. However, cross reactivity was observed between the chicken IgY and the rabbit anti-prairie chicken IgY secondary antibody. Secondary antibody alone did not react with the gag polypeptide.

DISCUSSION

An ELISA identifying REV envelope proteins has been previously described for domestic chickens (2). In this study, the detection systems developed targeted the generally more conserved nucleocapsid and capsid proteins of the gag gene although neutralization and now genome sequencing studies suggest that there is little variation among REV strains. Since antigenicity of these proteins does not depend on eukaryotic expression, large stocks of viral protein made in bacteria served as a convenient antigen for generation and detection of virus specific antibody (5).

The generated anti-gag polyclonal rabbit antibodies reacted with the purified recombinant gag polypeptide, as well as antigen on virus grown in productively infected cell culture. Therefore, the reagents proved to be useful in detection of REV specific antibody and for detection of REV infection. Although not tested, the conserved nature of the retroviral gag and the conservation of the REV genome among various isolates strongly suggest that the ELISA will be useful for other REV isolates. Cui *et al*. (2) has demonstrated cross-reactivity among REV subtypes with an envelope ELISA for the domestic chicken. Although cross reactivity of antibodies recognizing chicken immunoglobulin with prairie chicken IgY was not observed, it is interesting that the anti-prairie chicken antisera did react with domestic chicken IgY.

Both quantification of virus and the antibody response are basic essentials for determining the pathogenesis of infection. This study, along with the recently developed nest PCR, has provided the tools necessary to examine the host immune response to REV infection in the prairie chicken and to evaluate potential vaccine strategies that will control infection in these endangered birds.

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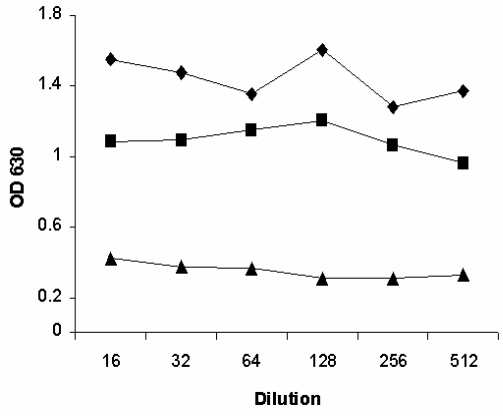
Figure 1. REV antigen ELISA with varying dilutions of purified REV gag (◆) in PBS, REV isolate R92 (■) whole virus preparation from tissue culture media and a blank control (▲). Stock concentration of REV gag was 10 µg/mL. All wells were coated with polyclonal rabbit anti-REV gag prior to the addition of antigen.



SEVERE SOFT SHELL EGG PROBLEM AND MORTALITY RELATED TO VITAMIN D_3 DEFICIENCY

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Nine houses containing 800,000 egg layers were affected by severe soft shell egg problems with an increase in mortality. Signs of a respiratory disease were not reported from any of the flocks. Chickens submitted for necropsy from two houses had lesions of soft, fragile, and crooked breastbones. The ribs were soft and caved in. Rupture of ovarian follicles and associated egg yolk peritonitis was present in all dead birds necropsied. Choanal swab samples tested from two affected flocks were negative for AI and NDV by RRT-PCR. Analysis of vitamin and mineral supplement received was found to be missing vitamin D_3. The egg production loss and mortality continued for three weeks. Excess mortality of 7,144 hens and a loss of 4,412 cases of eggs were present as result of vitamin D_3 deficiency.

A RETROSPECTIVE STUDY OF VITAMIN A DEFICIENCY IN TURKEYS

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Data on vitamin A deficiency in turkeys submitted to the California Animal Health and Food Safety Laboratory System, Fresno branch were reviewed for the years 2004 and 2005. The criteria for diagnosing vitamin A deficiency were based on gross and microscopic lesions and analysis of liver, serum, and feed for vitamin A. Twenty submissions from three commercial companies were selected for the study.

Age of the turkeys submitted with vitamin A deficiency ranged from three to 10 weeks. Clinical signs in the turkeys included ruffled feathers, anorexia, weakness, depression, ocular discharge, and increased mortality of 3 to 5 % per week. Mortality as high as 70 to 85 % over three to five weeks were seen in some flocks. Most common gross lesions were pale yellow small nodules or whitish pseudomembrane on the mucosa of the proximal esophagus, oral cavity and tongue. Other lesions included pale yellow caseous exudate in the conjunctiva, sinuses, turbinates, trachea, and bursa of Fabricius. Microscopically there was squamous metaplasia sometimes associated with keratin formation of the mucosa of the esophagus, oral cavity, tongue, larynx, conjunctiva, and the third eye lid. Glands such as submucosal glands of the esophagus and oral cavity and salivary glands were also commonly affected, but lacrimal and gland of Harder were occasionally affected. Other organs similarly affected but not consistently were bursa of Fabricius, sinuses, turbinates, nasal glands, trachea, bronchi and parabronchi, proventriculus, and feather follicles. A few birds had ulcerative keratitis. Ears examined in one bird revealed severe squamous metaplasia of the mucosa of the glands of the external ear. Kidneys were not affected in any of the birds examined.

Many birds had concurrent *Candida* infection in the crop and upper digestive tract and bacterial infections in the respiratory and ocular systems. Feed and liver analyzed for vitamin A was either extremely low or was absent. Similarly sera examined for vitamin A from many birds were low or marginally low. It is probable that vitamin A either was not added to the feed or was not added in adequate levels.

LESSONS LEARNED FROM THE EUROPEAN UNION BAN ON ANTIBIOTIC FEED ADDITIVES

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INTRODUCTION

The European Union (EU) banned the use of avoparcin, a widely used antibiotic feed additive in food-producing animals in 1997. The ban was carried out against the advice of the Scientific Committee on Animal Nutrition (1, 22), a panel of experts composed of animal scientists from various EU countries. Two years later, the EU banned the use of bacitracin, spiramycin, tylosin and virginiamycin, once more the ban was carried out against the expert scientific advice of the SCAN (1, 2, 3) citing fears of antibiotic resistance spread via the food chain and invoking the precautionary principle.

On January 1, 2006 the remaining antibiotic feed additives used in food-producing animals will be banned from use in the EU (14). Since several years have passed since the EU bans on antibiotic feed additives were implemented, and since some politicians in the USA are proposing a similar ban in this country (12, 25), it would seem appropriate to conduct an assessment of the ban results to determine if such bans have had a measurable effect on the problem of antibiotic resistance in human medicine. The purpose of this manuscript will be to evaluate and discuss published scientific information in regards to the EU bans on antibiotic feed additives and conclude if they have had a positive, a negative or no effect on the health of food-producing animals and on the problem of antibiotic resistance in human medicine.

BAN RESULTS ON ANTIBIOTIC RESISTANCE

Most likely the oldest and most complete source of data regarding antibiotic use and antibiotic resistance monitoring in animals and people is the Danish database known as DANMAP; therefore, data from these reports will be used to illustrate the results and conclusions reached in this manuscript.

As anticipated, the antibiotic feed additive bans have resulted in substantially lower levels of antibiotic resistance for the corresponding antibiotic on indicator bacteria isolated from raw meat products. This should not surprise anyone, since it is a known fact that with a few exceptions, antibiotic use will create antibiotic resistance, whether in animals or people. What the DANMAP data show, however, is that the improvements seen on indicator bacteria isolated from raw meats have not translated into lower levels of antibiotic resistance in human patients (5, 10, 11, 21). There is an abundant body of published scientific information that serves to explain this lack of correlation.

The first antibiotic feed additive used in food-producing animals banned in 1997 by the EU was avoparcin. This antibiotic was banned from use in food-producing animals because it belongs to the glycopeptide class, a critically important antibiotic used in human medicine. Vancomycin also belongs to this class and studies have shown that glycopeptide-resistant enterococci will develop in animals fed avoparcin (9); also, resistant enterococci have been isolated from raw meat of animals fed avoparcin creating a concern for passage of resistant enterococci to people via the food chain (6). This is what led to the ban of avoparcin as an antibiotic feed additive in the EU.

However, when one examines the incidence of vancomycin-resistant enterococci (VRE, bacteria commonly involved in fatal infections in human hospitals), a different picture emerges. That is because VRE infections are far more prevalent in USA hospitals than in EU hospitals (1, 20), and since avoparcin has never been used as an antibiotic feed additive in food-producing animals in the USA, it must be concluded that 100% of the VRE problem has been created by vancomycin use in humans. Obviously, a ban on antibiotic feed additives in the USA would do nothing to improve the critical VRE problem in USA hospitals. Although studies in Europe have shown that VRE can be isolated from healthy human and animal feces, the relatively low prevalence of VRE in hospitalized EU patients suggests that without substantial use of vancomycin in human medicine, the VRE problem would be very limited (20,21).

Another antibiotic feed additive used in food-producing animals banned by the EU in 1999 is virginiamycin. This antibiotic belongs to the streptogramin class and as in the case of avoparcin, concerns over cross-resistance with a new human antibiotic in the same class, Synercid, developed for treatment of vancomycin-resistant *E. faecium* (VREF) infections prompted EU regulators to call for its ban as an antibiotic feed additive in food-producing animals.

However, a very extensive sensitivity survey conducted in American and Canadian medical clinics before Synercid use began in North America found that out of more than 1,000 clinical isolates of *E. faecium* tested, 99.8% were sensitive to the new human antibiotic (15). Therefore, this study showed that after nearly three decades of continuous use of virginiamycin in food-producing animals in the USA and Canada, there was virtually no evidence of streptogramin-resistant *E. faecium* (SREF) in the human population. These results are not surprising since meat is cooked prior to its consumption, and the high temperatures achieved during cooking kill any bacteria that might have contaminated it, and dead bacteria cannot transmit antibiotic resistance. The importance of proper food hygiene and cooking has been pointed out by others as the most effective way of preventing not only transmission of antibiotic-resistance bacteria but also of preventing food poisoning in people (21).

Another study published in 2001 in *The New England Journal of Medicine* (19), which was specifically designed to prove the transfer of SREF from foods of animal origin to people, failed to do so. Between July 1998 and June 1999, the authors cultured 407 raw chickens obtained from 26 grocery stores in four states, and isolated SREF from 58.2% of them. Resistance was defined as a minimum inhibitory concentration (MIC) of at least four ppm. The authors attributed the high level of resistance to the use of virginiamycin. During the same period the authors also cultured 334 stool samples from outpatients at various medical clinics in the same four states. In contrast to the significant level of resistance found in the raw chickens, only two stool samples, or 0.6% of the total yielded SREF. It is worth noting that both samples had an MIC of four ppm reported by the authors as a "low level" resistance.

In spite of these results, the authors concluded that although "the low prevalence and low level of resistance in human stool specimens suggest that the use of virginiamycin in animals has not yet had a substantial influence, food borne dissemination of resistance may increase." They concluded by saying that "the Food and Drug Administration (FDA) was in the process of conducting a risk assessment for virginiamycin and that if such assessment demonstrated a role for food borne transmission in the emergence of SREF in humans, restrictions on the continued use of virginiamycin in food animals should be considered."

Since that manuscript was published, two risk analyses have been conducted. A quantitative risk analysis showed that the risk of the continued use of virginiamycin as an antibiotic feed additive in food-producing animals – assuming that transmission of resistance from animal foods to people occurs (an unproven assumption in this case) – the risk would be less than one statistical life saved for the entire USA population over a 15 year period and rapidly decreasing by the increased use of newer antibiotics as alternatives to Synercid (7)**.** FDA also completed its own risk assessment and also concluded that the risk from the continued use of virginiamycin in food-producing animals is very small (26). The FDA risk assessment concludes that with a food pathway attribution assumption of 10% the average risk to a random hospitalized member of the US population, the most relevant "at risk" population, of having SREF attributable to animal uses of virginiamycin and that may result in impaired Synercid therapy, ranges from six chances in 100 million to 1.2 chances in one million in one year, and that with a food pathway attribution assumption of 100% the chances would increase 10-fold. To present a comparative perspective on risk the following example is provided from an article on risk assessment of fluoroquinolone use in beef cattle (2), a study had estimated approximately a one-in-250 million chance that a person could die from a case of *Campylobacter jejuni* infection that is resistant to fluoroquinolone antibiotics, which the person might have caught by eating contaminated ground beef. In comparison to this risk, in any given year a person is 567 times more likely to be killed in a plane crash and 14,284 times more likely to be killed in a car crash.

It is because of all of this and the thorough examination of many other published research reports, that a panel of experts concluded that "there is little or no evidence that resistant enterococci from animals are a risk to human health, and that a ban of growth promoting antibiotics was not justified on this basis, and will have no impact on the prevalence of VRE in human infections" (21).

BAN RESULTS ON ANIMAL HEALTH AND PRODUCTIVITY

A manuscript by researchers from the National Veterinary Institute of Oslo, Norway (17) reported in 2001 severely impaired production performance in broiler flocks with high incidence of *Clostridium perfringens*-associated hepatitis (CPAH). The authors analyzed production performance data collected from a large processing plant in Norway, with the objective of comparing production performance data from broiler flocks with high levels of CPAH to flocks with low levels of CPAH. The study was conducted for the first 2.5 years following the ban of avoparcin, the first antibiotic feed additive to be banned by the EU. This study showed that flocks with high levels of CPAH had 25 to 43% lower profitability than those with low levels. The authors cited impaired feed conversion and

reduced weight at slaughter as the major causes for the losses. Researchers from the same Institute had reported earlier that the main effects of experimentally-induced subclinical necrotic enteritis were increased feed conversion and retarded growth rate (16). So it has become increasingly clear following the EU bans that the antibiotic feed additives, like avoparcin and virginiamycin, were preventing clinical and subclinical necrotic enteritis in poultry, even when used at inclusion rates labeled for "growth promotion"; this is in agreement with the observations made by others (21).

In another manuscript (5), the authors examined data three years after the bans were implemented and concluded that the only measurable benefit in humans was a reduction in acquired resistance in enterococci isolated from human fecal carriers; however, the authors stated that despite the growth promoter ban and the reduction of carriage of resistant enterococci in animals and humans, there had been no reduction in the prevalence of resistant enterococcal infection in humans. On the other hand, the authors also stated that the antibiotic feed additives had an important prophylactic activity previously unrecognized and that their withdrawal was now associated with a deterioration in animal health evidenced by an increased incidence of diarrhea, weight loss and mortality in post-weaning pigs, and necrotic enteritis in broiler chickens. The authors closed by saying that "the theoretical and political benefit of the widespread ban of growth promoters needs to be more carefully weighed against the increasingly apparent adverse consequences."

BAN RESULTS ON HUMAN HEALTH

An unintended consequence of the EU ban on the prophylactic use of antibiotic feed additives in food-producing animals may have an even greater adverse effect on public health. In a manuscript published in December, 2004 (14) the authors indicate that following the EU bans, the incidence of *C. perfringens*-associated disease in poultry and its detection in poultry meat has increased substantially and is emerging as a real threat to public health. According to the authors, toxins formed by *C. perfringens* type A and type C present in poultry meat can cause food poisoning and necrotic enteritis in people, respectively. Since *C. perfringens* food poisoning is not a reportable disease, its incidence is in all probability greatly underestimated. Nevertheless, *C. perfringens* was recognized in Norway as the most common cause of food poisoning during the decade of the 1990s (3). According to F.V. Immersel, *et al*., 2004, with the ban of the remaining three antibiotic feed additives (avilamycin, monensin and salinomycin) with activity against *C. perfringens*, the public threat of *C. perfringens*-induced food poisoning is expected to increase even more. Time will tell the magnitude of the consequences of the bans on antibiotic feed additives as related to food poisoning in humans.

According to the latest available report by DANMAP (10), "the use of antibiotics in humans and animals and the occurrence of resistant bacteria continued to increase through 2004." In the mean time, antibiotic use for therapeutic purposes in food-producing animals has increased every year since the first bans, from 48,000 kilograms the year after the bans to 112,500 kilograms in 2004.

An interesting theory has recently been proposed on how antibiotic use in food-producing animals may actually reduce consumer risk (13): a professor of veterinary medicine provided various ways by which antibiotic feed additive use in food-producing animals may actually lower the risk of food poisoning in people. This seems to be in agreement with a recently published manuscript that indicated for example, that the use of virginiamycin in turkey feeds significantly reduced the incidence of *Salmonella* spp. (8), since virginiamycin has no direct activity on *Salmonella* spp., we must assume that the changes produced in the intestinal microflora were less favorable to its growth. Likewise, the use of antibiotics, whether added to the feed to prevent disease or in the drinking water to treat diseases like airsacculitis of poultry, may also aid in reducing the risk of food poisoning to consumers. In a series of studies conducted to determine the effect of airsacculitis (an infection of the air sacs) of broiler chickens on the overall quality of the carcass (24), the researcher found that airsacculitis-positive flocks had lower body weights, more fecal contamination, more processing errors, and higher levels of *Campylobacter* spp. The author concluded that broiler chicken companies should emphasize control of airsacculitis in the flocks as a means of preventing subsequent food borne bacterial infection.

Finally, it has recently been reported that concentrations of various antibiotic feed additives and ionophore anticoccidials similar to those normally used in poultry rations had an inhibitory effect on the transfer of a multiresistance-conferring plasmid in *E. coli* in an *in vitro* test system (18). The authors concluded that based on the results of these tests, feed additive antibiotics and ionophore anticoccidials may actually inhibit resistance transfer mechanisms within poultry and livestock.

LESSONS LEARNED

There is little to no evidence to support the claim that the use of antibiotic feed additives in animal feeds has contributed to the problem of antibiotic resistance

in human medicine. This conclusion is further supported by the fact that of the 20 most serious bacterial infections exhibiting problems with antibiotic resistance in human medicine, 12 are in no possible way related to antibiotic use in food-producing animals, as these bacteria cannot be acquired via the food chain. Of the remaining eight, assuming that transfer of bacterial resistance from animals to people occurs (an unproven assumption in most cases), the calculated percent contribution to antibiotic resistance in all cases is 1% or less, in most cases less than 0.5% (4).

The EU banned the use of various antibiotic feed additives at levels labeled for growth promotion. Almost immediately a surge of enteric disease problems in food-producing animals followed.

The surge in enteric diseases of food-producing animals was followed by a surge in antibiotic use in food-producing animals for therapeutic purposes. The antibiotics used to treat food-producing animals belong to the various classes of antibiotics most frequently used in human medicine. This might have actually had a more adverse effect on the creation of antibiotic resistance in people than the use of the antibiotic feed additives.

The surge in use of antibiotics for therapeutic purposes in food-producing animals has clearly proven that the prior use of antibiotic feed additives had a health promotional and disease prevention effect in food-producing animals even when used at concentrations labeled for "growth promotion."

Although the antibiotic feed additive bans implemented by the EU achieved the objective of reducing the incidence of resistance on indicator bacteria in raw food products of animal origin, this has not resulted in any measurable improvement on the problem related to antibiotic resistance in human patients or human hospitals. This may be explained by the fact that monitoring of antibiotic resistance in raw meat products is not representative of the bacteria that may actually reach the consumer. Proper cooking of foods of animal origin destroys any bacteria that might have contaminated them, and dead bacteria cannot transmit antibiotic resistance to people.

While the incidence of food borne diseases in the USA population has continued to decline, in the EU it has continued to increase – at least for certain bacteria like *Salmonella*, *Campylobacter*, and *C. perfringens*. Therefore, it is becoming increasingly apparent that the bans on antibiotic feed additives have not resulted in a safer food supply.

The USA should learn from the EU experience and proceed with caution and only make decisions supported by science and quantitative risk analysis rather than implementing bans that may actually have effects opposite to their intended ones.

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COMPARISON OF PRODUCTION AND MORTALITY IN LAYERS FED BIO-MOS VERSUS TREATMENT WITH TYLAN AND TETRACYCLINES

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INTRODUCTION

The lay ranch had a history of unexplained production drops in the 40-50 week of age time period. The problem had been repeated over multiple flocks. No mortality or clinical disease was associated with the decreased production. The most common laboratory isolates were *Escherichia coli* and *Gallibacterium* spp.

The ranch was *Mycoplasma gallisepticum* (MG) positive and flocks were started on Tylan at 50g/ton and maintained at that level until seroconversion – usually around 40 to 50 weeks. The producer felt that tetracycline had some benefit in increasing production as well. Continuous inclusion of Bio-mos in the lay ration was evaluated as a cost-effective alternative to long term Tylan and periodic tetracycline administration.

MATERIALS AND METHODS

The study included a flock brooded together and placed on the lay ranch in seven houses. All birds received two pounds/ton of Bio-mos for the first two weeks on the pullet ranch and one pound/ton for weeks 3 – 17.

The Bio-mos treatment group (houses D, E, F, & G) received one pound/ton of Bio-mos through 60 weeks. They received no antibiotics. The control group (houses A, B, C) received no Bio-mos. This flock was grown continuously on Tylan with periodic treatment with 200 mg/ton of tetracycline.

The group was comprised of two strains: Hyline W-36 (Houses D & E) and Bovan Whites (Houses A&B and F&G). House C was a mixed house with an unknown ratio of strains and was not included in any of the strain-specific analysis. The W-36 flocks lacked a control group and were not evaluated by strain. Only the Bovans were present in sufficient numbers to evaluate the effect of the treatments within one strain of birds.

Mortality, hen-day production, eggs per hen housed and average feed consumption were examined at 60 weeks. Due to small sample number of flocks, no statistics were performed.

RESULTS

Houses fed Bio-mos had a cumulative mortality of 6.05% for week 19-60. The control flocks had a cumulative mortality of 9.72% for the same time period. Treated Bovans had a mortality of 10.1% while the Controls were 11.7%. There was excessive mortality in weeks 23-25 due to a viral challenge. All flocks were subject to field exposure.

The Bio-mos treated group produced 235.6 eggs per hen housed. The controls produced 227.1 eggs per hen housed for a difference of 8.5 eggs at 60 weeks. Strain impacted these values as well. The treated Bovans produced 232.7, while the controls produced 222.9. The difference was 9.8 eggs per hen housed.

When all strains were evaluated, the treated group had average of 21.3 pounds of feed consumed per hundred chickens for the study period. The control group averaged 21.5 pounds per hundred. When the Bovans were evaluated, the treated group consumed 22.1 pounds per hundred and the controls consumed 21.6 pounds per hundred.

DISCUSSION

Bio-mos was successful in ameliorating the production drop of unknown origin at this ranch. Additionally, continuous feeding of Bio-mos had a greater benefit than continuous feeding of Tylan and periodic treatment with tetracycline.

Although mortality was not included as a problem in this ranch's history, the treated groups had a lower cumulative mortality than the controls. During this trial, all houses were exposed to a respiratory virus around 23 weeks of age. There was a dramatic difference in the mortality spikes of the treatment groups at that time. It is unclear what role the Bio-mos played. With a reduction of subclinical disease, the birds may have had greater reserves to respond to the viral infection or Bio-mos may have played an immunostimulatory role.

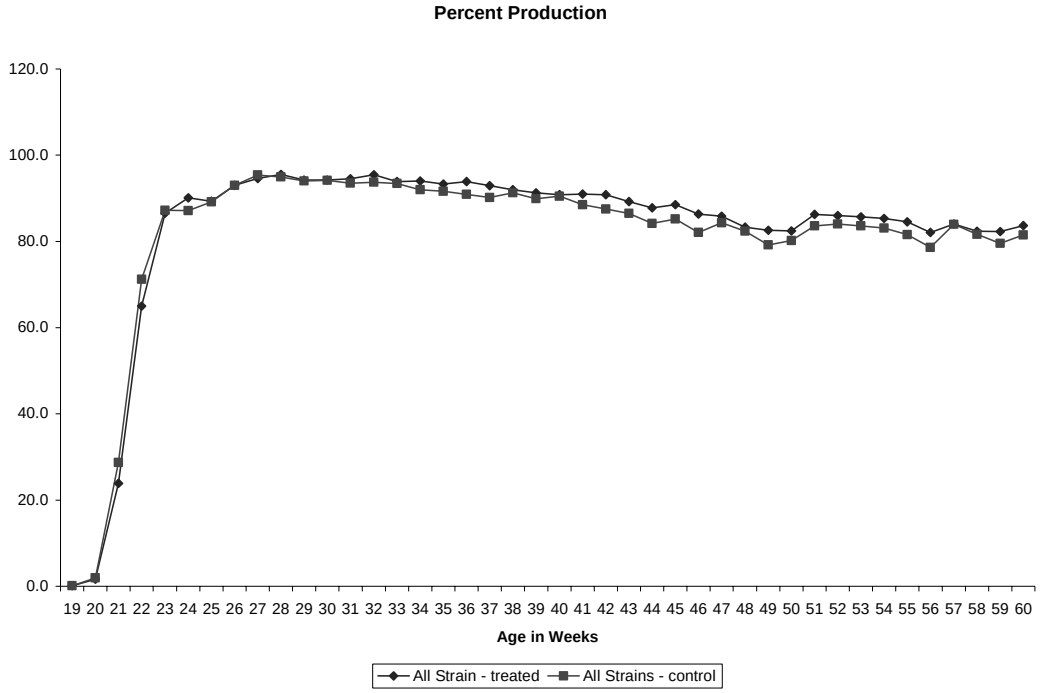
Evaluation of the production curves indicates that flocks treated with Bio-mos had a greater persistency of lay. Although on a week by week basis, Bio-mos treated flocks had a slightly lower percent while coming into production, they maintained peak longer and decreased in percent production more slowly. It is unclear whether the differences early in the cycle are artifact from the viral challenge, or a treatment effect resulting from a delayed response to Bio-mos treatment.

The greatest measure of product impact for this ranch is the difference in eggs per hen housed, since this value takes into account both production and mortality. All treated housed had higher eggs per hen housed at 60 weeks than the un-treated controls. Although statistics were not performed, the 8-10 egg difference is of great economic significance.

The role of Bio-mos on feed consumption is mixed in this study. While pure cost would argue for the lowest feed consumption possible, preservation of higher consumption can lead to higher production, more eggs of optimal size, and lower mortality.

The impacts of strain on production and mortality are well documented. The study yielded information on the interplay of strain and products designed to boost production and decrease mortality. Further studies with appropriate controls for all strains being evaluated could provide even greater information.

Figure 1. Percent egg production.



EVALUATION OF OLIGOSACCHARIDES FOR THE POST-ANTIBIOTIC GROWTH PROMOTER ERA

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INTRODUCTION

The avian gastrointestinal tract constitutes a complex microbial ecosystem comprised of several hundred different species of bacteria. The hindgut, in particular, is densely populated with in excess of 1011 bacteria per gram of contents. These organisms and their metabolic activities are not inert to the avian host and can have positive and negative impacts on health. The balance of this ecosystem is dynamic and may be adversely altered by stress, diet, medication and a host of environmental factors. The maintenance of a community of bacteria that contains a predominance of beneficial species and minimal putrefactive (protein degrading) or potentially pathogenic species is believed to be important for maintaining intestinal health.

A number of dietary supplementation approaches have been proposed with regard to maintaining a eubiotic microbial ecosystem. The first is the oral administration of live, beneficial microbes, also known as probiotics, with the most interest being shown in the lactic acid bacteria and *Bifidobacterium* spp. There are a number of advantages and disadvantages with probiotics, which are beyond the scope of this paper. However, since these aforementioned bacteria are dominant genera present in the hindgut of healthy birds, a second strategy is to increase their numbers by supplying those already present in the intestine with a selective carbon and energy source that provides them with a competitive advantage over other bacteria in the ecosystem. In 1995, Gibson and Roberfroid (1) defined these dietary "prebiotic" components as "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, that can improve the host health." The third strategy is to supplement specific carbohydrates, which play multiple roles in gut health, such as immunomodulation, an anti-adhesive effect and enhancement of intestinal tissue recovery. The best known and well characterized are composed of

polymers of mannose, such as Bio-Mos® (Alltech Inc., Nicholasville, KY).

PROPOSED MECHANISMS OF PREBIOTIC ACTION TO IMPROVE AVIAN HEALTH

From the definition of Gibson and Roberfroid (1) it can be surmised that any fermentable dietary component that arrives undigested in the colon has the potential to act as a prebiotic. To date, all prebiotics used in animal and poultry feed have been carbohydrates, ranging from small sugar alcohols and disaccharides, to oligosaccharides, and large polysaccharides, all with a variety of sugar compositions and glycosidic linkages. *Bifidobacteria* are unique in that they use these diverse chemical structures as either an energy or carbon source, as they have an unusual array of glucosidic enzymes not found in the majority of gut bacteria. The exact mechanisms by which such a chemically diverse range of carbohydrates preferentially stimulates one particular genus in a population with many saccharolytic species is unclear. However, the result of providing a selective fermentable carbohydrate to a beneficial microbial population (e.g. *Bifidobacteria* spp.) can have a number of direct and indirect effects on the metabolic activity of the microbial ecosystem including, inhibition of putrefactive and pathogenic organisms; provide colonization resistance; increased production of SCFA; and reduce intestinal pH, thereby increasing mineral solubility and improving mineral absorption.

The "prebiotic" substrates that are currently being investigated as dietary aids in poultry nutrition include the mannaoligosaccharides (MOS) (1, 2), fructoligosaccharides (FOS) (4, 5), inulin (6), and the newer isomaltoligosaccharides (7). There are many others with potential that need to be considered in the future such as galacto-oligosaccharides, soybean oligosaccharides, xylo-oligosaccharides, palatinose polycondensates, resistant starch, β-oligo-saccharides.

Mannanoligosaccharides. Yeast cell wall mannoproteins are highly glycosylated polypeptides, often 50-95% carbohydrate by weight, that form radially extending fimbriae at the outside of the cell wall (8, 9). Many mannoproteins carry N-linked glycans with a core structure of Man10-14GlcNAc2-Asn structures very similar to mammalian high mannose N-glycan chains. "Outer chains" present on N-glycans consist of 50-200 additional α-linked mannose units, with a long α-1,6-linked backbone decorated with short α-1,2 and α-1,3-linked side chains. These complex structures determine the cell surface properties (9) which are believed to be the basis of the three primary modes of action of MOS observed in animal and poultry studies: 1) adsorption (agglutination) of pathogenic bacteria containing Type 1 fimbriae, 2) modulation of the host immune response, and 3) enhancement of intestinal morphology (3, 10).

The most studied and well understood mode of action of MOS involves the competitive blocking of bacterial lectins. Adhesion of pathogens to the epithelium surface of the gut (colonization) is the first critical stage leading to infection. Mannose-specific lectins (Type 1 fimbriae) on the bacterial surface recognize glycoproteins (rich in mannose) on the host cell surface. The control of bacterial mediated attachment has been proposed as a possible means of reducing enteric infection. Oyofo and coworkers (11) tested the effect of different sugars on the adherence of *Salmonella typhimurium* to epithelial cells from one-day-old chicks *in vitro* and found that mannose and methyl-α-D-mannoside were the most efficient in inhibiting adherence. They reported that mannose addition decreased the number of adherent bacterial cells to a defined intestinal surface by more than 90% when compared to a control with no carbohydrate added. In three follow-up *in vivo* studies, Oyofo and coworkers (13) observed a significant protective effect from supplementing mannose (2.5% w/v) in the drinking water of chicks for 10 days; *Salmonella*-challenged control chickens were 78, 82, and 93% colonized whereas *Salmonella*-challenged mannose treated chickens were only 28, 21, and 43% colonized. In other studies, addition of Bio-Mos at significantly lower dietary inclusion levels (0.4% w/w) to the mannose concentrations (2.5 % w/v) used by Oyofo and coworkers (11) resulted in the successful reduction of *Salmonella* and *E. coli* in the ceca of young broiler chicks (3). This confirmed earlier in vitro studies that indicated differences exist in the ability of different mannose-based sugars to block pathogen attachment (13). Firon and coworkers (13) demonstrated that compounds containing both α-1,3 and α-1,6 branched mannan (as found in the outer cell wall of *S. cerevisiae*) had approximately 37.5 times the binding capacity for *E. coli* as D-mannose. In another interesting study, Fernandez and coworkers (14) demonstrated a reduction in colonization of *Salmonella enterica* serovar *enteritidis* (PT4) in the ceca of young broiler chicks receiving the cecal contents from hens fed Bio-Mos (2.5% w/w) through the diet. When the chicks diets were supplemented with the same Bio-Mos as given to the hens, an even greater protection was observed, as demonstrated by fewer *Salmonella*-positive birds observed, 11/24 (46%) for Bio-Mos treatment compared with those fed mash alone (17/24 (79%). The ability of Bio-Mos to interfere with the attachment of pathogenic bacteria in the gut raises the possibility that Bio-Mos could also inhibit the binding between bacteria that is required for plasmid transfer via conjugation (15). Lou (16) demonstrated that dietary Bio-Mos supplementation decreased the

proportion of specific groups of gram-negative antibiotic resistant fecal bacteria in swine. Work continues in this area to confirm these earlier findings.

Numerous studies have investigated the effect of Bio-Mos on humoral and cell immunity. While the exact mechanisms have not been completely elaborated, significant evidence has been accumulated to propose Bio-Mos plays a multi-purpose role in immune modulation. Dietary inclusion of Bio-Mos has been shown to affect humoral immunity in turkeys by enhancing plasma IgG and bile IgA antibody levels (17). In another study, with sows receiving Bio-Mos 14 days pre-farrowing and throughout lactation, higher concentrations of colostrum IgG and IgM were observed compared to the untreated sows (18). This increase in colostrum Ig's correlated well with the piglets from supplemented sows being significantly heavier at weaning. Non-specific cellular immunity has also been positively influenced in studies investigating macrophage activity. The stimulation of phagocytosis by Bio-Mos has been demonstrated to be dose dependent in vitro (19). This may be due to the presence of a mannose receptor (MR), which is involved in microbe recognition and phagocytosis in the absence of specific opsonization and acts like a true lectin in the lectin phagocytosis of microorganisms (20). MR is expressed on tissue macrophages, dendritic cells (mostly on Langerhans cells), endothelium, and rat microglia. In addition to acting as a scavenger of mannose-containing glycoconjugates on the surface of a wide spectrum of microorganisms such as *E. coli*, *Klebsiella pneumoniae*, and *Salmonella*, MR mediates their ingestion by macrophages (21). MR is the main molecule involved in antigen recognition and the binding process in antigen-presenting cells. Therefore, activation of immune cells by yeast-associated mannan may facilitate antigen processing and serve to stimulate the initial stages of the immune response. Recently, there has been some evidence to suggest that Bio-Mos may suppress the pro-inflammatory immune response. Ferket and co-workers (2) induced an acute immune response in turkey poults by intraperitoneal injection of LPS from *Salmonella typhimurium* and measured fever response. Poults fed a diet containing Bio-Mos showed little fever response compared with the control (no additive) group, which experienced an increase of $+0.4^{o}C$ in body temperature. Greater control of the immune response, particularly the fever response, can be beneficial to the host in terms of energy savings, maintaining feed intake, and reducing stress. Further studies are necessary to understand the highly complex and diverse effects the yeast cell wall mannanoligosaccharides have on the immune system of the host.

There is increasing evidence that Bio-Mos modifies the morphology and structure of the intestinal mucosa, although whether this is a direct or indirect (pathogen control) effect remains unclear. Early studies at Oregon State University demonstrated a reduction in crypt depth of turkey poults fed diets containing 0.1 % Bio-Mos through eight weeks of age in three sections of the intestine comprising the distal half of the duodenal loop, Meckel's diverticulum, and at the junction of the jejunum and cecum (22). These changes in crypt depth were correlated to a statistically significant increase in growth rate through eight weeks of age, suggesting an inverse correlation between the parameters measured. Santin and co-workers (23) showed that inclusion of yeast cell wall at 0.2% in broiler diets aided in intestinal development with an increase in villus height during the first seven days of life, and could be positively correlated with an improved body weight gain over the entire production period. Another detailed study evaluated the response of the intestinal mucosa of broiler chickens to Bio-Mos included in sorghum/lupin-based diets at 0.0, 1.0, 3.0 or 5.0g kg-1 diet (24). Supplementation with the highest level of Bio-Mos resulted in longer ($P<0.01$) jejunal villi. The RNA content of the ileal mucosal homogenate was significantly greater ($P<0.05$) in chicks receiving 3.0 and 5.0 g Bio-Mos kg-1 diet than in other groups. The protein/RNA and RNA/DNA ratios in ileal homogenates were significantly ($P<0.01$) influenced by the presence of Bio-Mos in the diet. This was not translated into increased mucosal growth or differences in digestive enzyme activities in the ileum. However, with Bio-Mos inclusion in the diet, there were significantly greater specific activities of maltase ($P<0.01$), leucine aminopeptidase ($P<0.05$), and alkaline phosphatase ($P<0.001$) in the jejunum. Improvements in the intestinal mucosa with dietary supplementation of Bio-Mos have been linked to a reduction in morbidity and mortality attributable to necrotic enteritis (25). The bottom line of these observed changes brought about by mannan-oligosaccharides, specifically Bio-Mos, is reflected in comparable growth performance and improved livability to that seen with antibiotic growth promoters in both broilers and turkeys (26, 27).

SUMMARY

Carbohydrates have long been known to be an important dietary component, although traditionally have been seen as energy yielding molecules and structural components. Recently, studies have demonstrated that non-digestible carbohydrates play an important role in animal production and human health. Moreover, there is a growing recognition that non-digestible carbohydrates are more than an energy source for the colonic microflora but play a vital role in cellular metabolism, protein structure and function,

cell-to-cell communication and host immunity. The functional properties of mannanoligosaccharides make them attractive for use in poultry diets.

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FACTORS THAT MAY CONTRIBUTE TO OUTBREAKS OF HISTOMONIASIS IN COMMERCIAL TURKEY FARMS IN CENTRAL CALIFORNIA: A DESCRIPTIVE EPIDEMIOLOGY STUDY

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This study investigates the prevalence and the extent of histomoniasis in commercial meat turkey ranches in Central California in the past 10 years (1995-2005) based on the cases of histomoniasis (blackhead) submitted to the California Animal Health and Food Safety (CAHFS) Laboratory Fresno and Turlock branches. Most of the cases came from the major meat turkey producing premises. There has been an increase in positive cases for histomoniasis since 1995. The rise in the number of *Histomonas* cases coincided with the ban by the FDA on dimetridazol and ipropran, nitroimidazole compounds commonly used to treat blackhead in the 1990s. Twenty-eight *Histomonas* positive cases were submitted to the laboratory in 2001-2005 for a total of 172 birds; 142 (82%) had typical hepatitis and enterotyphlitis lesions with the histomonads observed on histopathology. When compared to 1995-2000, only nine *Histomonas* positive cases were submitted with a total of 42 birds and 24 (57%) had typical lesions as mentioned. This shows a three-fold rise in our blackhead cases in the past 10 years. Mortalities of 10-50% were not uncommon.

Cases submitted to the laboratory typify those reported around the country with regards to its baffling epidemiology. No cecal worms or larvae were observed in the intestines of the affected birds on post-mortem and histopathology from any of the cases submitted. Majority of cases were submitted between April and August; *Histomonas* was not detected in the month of January in the past 10 years. Of the five major meat turkey companies in central California, only three submitted blackhead cases in 2001-2005. This research looks into where the protozoan comes from and how the birds gain access to it. Risk factors including location in central California, distances from the nearest turkey or meat ranch, type of soil, litter used in the house, problems with darkling beetles or rodents, sharing of employees and equipment, method of treatment, and other biosecurity measures required will be discussed.

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

TURKEY *EIMERIA*: INCIDENCE, PATHOLOGY, AND CONTROL

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Several trials were conducted to determine the efficacy of Clinacox against *Eimeria* isolates collected from turkey farms that had used different methods of control for coccidia; these included drugs, vaccine, or a rotation between drugs and vaccine. Groups of young poults were inoculated with oocysts coming from each of the sources were provided with feed fortified with or without Clinacox at 1.0 ppm. Growth was depressed an average of 37% for the infected unmedicated control groups (IUC) vs. the uninfected unmedicated groups (UUC). The average gross lesion score of IUC birds was 2.0 and a microscopic parasite burden score of 3.27 was assessed, compared to no lesions and zero parasite burden in the UUC birds. A higher index score indicates better efficacy. The average index for the isolates obtained from sources that had used vaccination were 38 and 91 for the IUC and Clinacox groups, respectively. The average scores for the samples from sources not using vaccination were 35 and 60 for IUC and Clinacox medicated birds, respectively.

Coccidiosis in turkeys has not been recognized as a major disease of importance, and the impairment in performance is underestimated or ignored, although coccidiosis in turkeys has been described in the US as long as 109 years ago. Seven species of *Eimeria* are described as parasites for turkeys, but four are generally considered important *(E. adenoeides*, *E. gallopavonis*, *E. meleagrimitis*, and *E. dispersa)*. There is also a general belief that there are no gross lesions or poor clinical signs of coccidiosis in turkeys. It is possible that these agents may be misdiagnosed because of the lack of clinical signs. Based on data from the 1960s (Edgar's files) showed that the predominant species were *E. adenoeides, E. gallopavonis*, and *E. meleagrimitis*, and *E. dispersa* were the least prevalent. In a second survey 1982 to 1983 (Edgar) the prevalent species were *E. meleagrimitis*, *E. adenoeides*, and *E. gallopavonis*; *E. dispersa* was the least prevalent. Fitz-Coy, 1990 found the most common species were *E. meleagrimitis, E. adenoeides*, and *E. dispersa*, and *E. gallopavonis* was the least prevalent.

Several trials were conducted to determine the efficacy of some commonly used anticoccidial against isolates collected from a range of companies using different practices to control coccidia. Some of the *Eimeria* were isolated from farms that had used only anticoccidial drugs to control the disease. Some of the isolates were from farms that had used a combination of drugs and biologics for the control of coccidiosis. There were also samples collected from farms in which only vaccines were used. The initial litter samples were collected from farms and sent to a designated laboratory for propagation. Coccidia were propagated by feeding young coccidia-free poults the material. Following harvesting and cleaning, the predominant species were determined. The findings were that *E. meleagrimitis* and *E. adenoeides* were the most commonly encountered species, followed by *E. dispersa* and *E. meleagridis*, and the least prevalent was *E. gallopavonis*. The levels of weight suppression for the IUC groups for each isolate tested were approximately 35% compared to that of the UUC groups.

Index scores were determined using growth, gross lesions, microscopic parasitism, and livability. Growth of coccidia naïve poults was depressed by an average of 37% and mortality may occur. The average gross lesion score was 2.0 and a microscopic parasite burden score of 3.27 for the control birds compared to no lesions and zero parasite burdens in the UUC birds. Under the conditions of this study, the infectivity of the isolates was severe and similar on the IUC birds regardless of source. The average score for the samples collected from locations where Clinacox was used, but no Coccivac-T, were 35 and 60 for IUC and Clinacox, respectively. The average score for the samples collected from those farms where Clinacox and Coccivac-T used for control measures were 35 and 86 for IUC and Clinacox, respectively. The average score for the samples collected from those farms where Coccivac-T was the only method for control methods were 35 and 86 for IUC and Clinacox, respectively.

Pathological changes caused by mild to moderate infection of *Eimeria* species in young turkey poults, varied from petechiae, hyperemia and white spots in the lower small intestines, ceca, and rectum. Moderate to severe infections caused whitish cheesy plugs in the ceca including the cecal tonsils. Severe infections in young poults caused by *E. adenoeides*, *E. meleagrimitis*, and *E. gallopavonis* may lead to death.

Under the conditions of this study, the infectivity of the isolates was severe and similar on the IUC birds regardless of source. On the other hand, the response of the isolates to medication with Clinacox was different. Birds challenged with isolates from sources where Coccivac-T live vaccine had been used showed better

response to medication than birds challenged with isolates from where Clinacox had been used but no vaccination was applied. Using Coccivac-T live vaccine in rotation with Clinacox appeared to provide a sparing effect on the efficacy of the drug.

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CHARACTERIZATION OF *EIMERIA* SPECIES FIELD ISOLATES WITH NOVEL PHYSICAL AND GENETIC PROPERTIES

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SUMMARY

It is possible to differentiate all seven accepted species of *Eimeria* which infect chickens within a single PCR reaction (1, 2). The technique involves amplification of the first or second internal transcribed spacer (ITS-1 or ITS-2) regions, using genus-conserved primers. Resolution is by denaturing polyacrylamide gel electrophoresis (DPAGE). Banding patterns generated by this method have been found to be conserved within a species, across all strains analysed so far, except for minor band variations between some strains in *E. mitis* and *E. praecox*. The banding pattern of each species contains at least one band that does not overlap with any of the other species, allowing each species to be distinguished within a sample containing multiple-species. A benefit of this technique over species-specific methods is that it has the potential to detect organisms with divergent DNA sequences, such as divergent strains or novel species.

In the course of analyzing oocysts from Australian field outbreaks, several novel banding patterns were identified in mixed-species infections in flocks from several different regions. Novel banding patterns were detected in at least three flocks from different geographical regions of Australia. The *Eimeria* oocysts responsible for two of the banding patterns were isolated using single oocyst culture techniques and hyper immunization. The isolates were amplified and their physical and genotype properties were investigated further.

The PCR banding pattern of the first isolate (designated EPL-X001) was of similar size to, but distinct from *E. maxima*. The second isolate (strain designation EPL-X002) gave a more distinct banding pattern. Oocysts of this type were also isolated from the same farm as EPL-X001. The species represented by the unknown bands possessed ovoid to spherical oocysts.

Both strains were characterized by histology (preferred asexual and sexual replication sites), oocyst morphology, prepatent period (PPP), and genetic sequencing of the 18S and 28S rRNA regions.

RESULTS

The physical characteristics of both isolates do not comply with published details (Table 1). Oocysts of strain EPL-X001 had a length of ~23-33 µm (mean 30 µm) and a width of ~20-28 µm (mean 23 µm). Oocyst shape was ovoid to sub spherical, with a mean length/width (L/W) index of 1.29. ITS-1 and ITS-2 DPAGE analysis compared EPL-X001 to five different strains of *E. maxima* (including two non-Australian strains of European and North American origin), and three strains of *E. brunetti* (all of Australian origin). All eight strains produced a conserved banding pattern for their species. This was to the exclusion of EPL-X001. The minimum prepatent period (PPP) was ~130 hours, which is nearly 10 hours longer than *E. maxima* and *E. brunetti*, and eight hours less than *E. necatrix* (138 h). Histology sections collected at patency indicated a preference for the upper intestine (small intestine and duodenal loop). Again, this does not entirely match *E. maxima* and *E. brunetti*, and appears closer to *E. acervulina* or *E. praecox*. Sequencing of the nuclear small subunit "18S"rRNA region, and subsequent phylogenetic analysis, revealed that EPL-X001 groups with *E. maxima* and *E. brunetti*. However the sequence analysis was not clear enough to conclusively prove whether it was closely related to either. Sequencing of

the nuclear large subunit "28S"rRNA region, and subsequent phylogenetic analysis, conclusively revealed that EPL-X001 groups with *E. maxima* rather than *E. brunetti*. EPL-X001 was found to exhibit some sequence variation to the other five strains of *E. maxima* sequenced. Preliminary results from cross protection studies indicate EPL-X001 could be antigenically distinct.

Oocysts of EPL-X002 had both a length and width of ~18-23 µm (mean length ~21 µm, mean width ~ 19 µm). Oocyst shape was ovoid to spherical, with a mean length/width (L/W) index of 1.1. The shape was most similar to *E. mitis*, however the oocysts were closer to *E. tenella* in dimension. The PPP was > 160 h, which matches no described species. To exclude the possibility that the oocysts were second-generation daughter oocysts, fecal material oocyst flotation failed to detect oocysts at any time prior. Histology results indicate a preference for the upper intestine (small intestine and duodenal loop) and most closely matched *E. praecox*. Sequencing of the nuclear small subunit "18S"rRNA region, and subsequent phylogenetic analysis, suggests that EPL-X002 is closest to *E. mitis*. A paucity of *E. mitis* 18S sequences on the databases means that it is difficult to assess the significance of variation between the sequences, and hence how close EPL-X002 is to E. mitis. ITS-1 and ITS-2 DPAGE analysis compared EPL-X002 to four different strains of *E. mitis*, all of Australian origin. All strains produced a conserved banding pattern for their species (with minor variation in band intensity),

The significance of these two new isolates is subject to further research. Information on the relative pathogenicity to known field isolates, and the distribution of these isolates within the Australian poultry industry are being obtained. It remains inconclusive as to whether the two isolates reported in this paper represent new species or are highly divergent strains of known species.

Vaccine manufacturers will need to monitor the significance of these isolates and make commercial decisions as to their significance. If these isolate reduce live bird performance, it seems highly probable that, regardless of species, live coccidiosis vaccine manufacturers will need to review vaccine formulations.

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Table 1. Summary of characteristics for *Eimeria* strains EPL-X001 and EPL-X002, and comparison with data for other species. Data were taken from (3).

	EPL-X001	EPL-X002	*E. acervulina*	*E. brunetti*	*E. maxima*	*E. mitis*	*E. necatrix*	*E. raecox*	*E. tenella*
Preferred site of infection	Duodenum -SI	Duodenum – upper SI	Duodenum – upper SI	Lower SI – LI	Mid-lower SI	Lower Si - LI	Mid-SI & caeca	Duodenum & upper SI	Caeca
Length (µm)	23.0 – 33.3 mean: 29.9	17.9 – 23.0 mean: 21.1	17.7 - 20.2 mean: 18.3	20.7 - 30.3 mean:24.6	21.5 - 42.5 mean: 30.5	11.7 - 18.7 mean: 15.6	13.2 - 22.7 mean: 20.4	19.8 - 24.7 mean: 21.3	19.5 - 26.0 mean: 22.0
Width (µm)	20.5 – 28.2 mean: 23.2	17.9 – 23.0 mean: 19.2	13.7 - 16.3 mean: 14.6	18.1 - 24.2 mean: 18.8	16.5 - 29.8 mean:20.7	11.0 - 18.0 mean: 14.2	11.3 - 18.3 mean: 17.2	15.7 - 19.8 mean: 17.1	16.5 - 22.8 mean: 19.0
Shape, L/W index	ovoid to subspherical 1.29	ovoid to spherical 1.10	ovoid 1.25	ovoid 1.31	ovoid 1.47	subspherical 1.09	oblong ovoid 1.19	ovoidal 1.24	ovoid 1.16
Min PPP (h)	130	> 160 h	97	120	121	93	138	83	115*

*Australian *E. tenella* strains have been reported with longer PPP.

ORGANIC TRACE MINERAL COMPLEX SUPPLEMENTATION IMPROVES THE IMMUNE RESPONSE, GUT STRENGTH, AND PERFORMANCE IN COCCIDIOSIS VACCINATED AND CHALLENGED BROILERS

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SUMMARY

The objective of this study was to compare zinc sources in coccidiosis vaccinated and challenged broilers fed a zinc deficient basal diet. Zinc supplementation resulted in significant improvements in performance, livability and specific antibody response in coccidiosis vaccinated birds. Birds fed organic trace mineral complex (Mintrex® Zn) had significantly greater tibia zinc and jejunum strength after challenge compared to all other sources.

INTRODUCTION

After invasion of the host intestine, *Eimeria* elicits both nonspecific and specific immune responses which involve many facets of cellular and humoral immunity (2, 7, 8, 9). Antigen-specific immunity is mediated by antibodies, lymphocytes, and cytokines (10) Response to a coccidial infection is exacerbated by zinc deficiency (1, 14). Poor performance of coccidiosis-infected birds may result from a zinc deficiency brought about by decreased absorption of zinc from the intestine (15, 16). The patterns of response of serum zinc (Zn), copper (Cu), and iron have been described as similar to what is seen following an acute phase response (12). There are no reports, however, describing the effect of zinc deficiency and supplementation on coccidial vaccination and later challenge of immunized birds. Organic zinc has been demonstrated to have immune benefits in broilers (4, 5, 6). This study was designed to examine the vaccination and challenge effects in birds fed a Zn deficient diet (35 ppm Zn from ingredients) supplemented with inorganic (zinc sulfate, ZnS) or organic (zinc methionine, ZnMet or organic trace mineral complex, Mintrex Zn) sources of zinc fed at equal supplementation levels. Mintrex Zn is an organic zinc source using 2-hydroxy-4-methylthio butanoic acid (HMTBA) as the organic ligand.

MATERIALS AND METHODS

Day old Cobb x Cobb broilers were assigned to four treatment (trt) groups: a low zinc basal diet (trt 1), the same basal diet supplemented with inorganic zinc sulfate (trt2), ZnMet (trt 3), or Mintrex Zn (trt 4) all fed at 40 ppm zinc. The diet was a corn soy starter designed to meet NRC (11) recommendations. There were six replicate pens with 10 birds per pen. The study was conducted in an environmentally controlled battery room. On day 0, all birds were vaccinated using ADVENT® Coccidiosis Control.

A mixed coccidiosis challenge with three species of *Eimeria* (*acervulina*, *maxima*, and *tenella*) was administered on day 24. Birds were lesion scored for *E. acervulina* on day 28, *E. tenella* on day 29, and *E. maxima* on day 30. Serum samples were obtained on day 20. Tissues were collected on days 20, 28, 29, and 30 for morphology, gut strength (using an Imada MV-110 digital forced tester), and zinc analysis (by ICP).

Analysis of variance (13) was used to compare treatments with the pen as the replicate for performance, lesion scores, and tissue analysis. Performance index was calculated as follows: ((cumulative livability*(body weight / day of study))/Cumulative dead bird corrected feed to gain).

RESULTS AND DISCUSSION

Performance results indicated that the basal diet was deficient and birds responded to zinc supplementation from all sources. Under the conditions used in this study there were no significant differences between zinc sources at this early time point (day 21), but only Mintrex Zn gave feed to gain significantly better than the basal (Basal 1.982a, zinc sulfate 1.889ab, ZnMet. 1.863ab, and Mintrex Zn 1.848b).

Table 1 shows the antibody response to the coccidiosis vaccination (ELISA analysis performed by H. S. Lillehoj, USDA, Beltsville, using the method described by Ding et al, 2005). The two antigens used in this study are identified by specific antibodies generated against the coccidial vaccine. The anti-

microneme antibody MIC2 is directed against an organelle essential for parasite invasion into gut cells. The second antibody anti-3-1E is directed to the surface of the invading sporozoite. Clearly the birds fed Mintrex Zn had a higher specific antibody response than zinc deficient birds or birds on other zinc sources. Single degree of freedom contrasts between Mintrex Zn and all other treatments were significant, as is indicated in Table 1.

On day 24, all birds were challenged with three species of *Eimeria*. The vaccine proved efficacious and all birds were protected, showing low lesion scores (typically 0 or 1, data not shown). However, the parasite does invade the gut epithelium to some extent, and morphometry and gut strength measures were performed to assess the status of the jejunum and ileum of the post-challenged birds. Table 2 shows the effect on the jejunum of these birds. This is the part of the gut parasitized by *E. acervulina* and *E. maxima*. It also shows all birds given zinc supplementation had better gut status, with longer villi and deeper crypts than the deficient birds. For crypt depth, an indication of epithelial growth rate, birds fed Mintrex Zn were significantly higher than birds on the other zinc treatments. This improved gut status is reflected in the higher gut breaking strength. Coccidiosis damages the wall, resulting in a greater risk of breakage. Table 2 indicates that both organic zinc sources were better able to protect the integrity of the gut compared to the zinc deficient or zinc sulfate treatments. The distal ileum, on the other hand, is not directly parasitized by the coccidial species used here, but nevertheless, the ileum mucosa had a significant response to zinc supplementation, with Mintrex showing the best ileum development in the 28 day-old birds.

The higher antibody response predicts that birds fed Mintrex Zn should be able to resist the parasite more effectively than birds on other sources. Here performance index, a combination of livability, feed conversion, and gain during the immediate post-challenge period, shows that Mintrex Zn birds did have the best overall performance index (Basal 215.6b, Zn sulfate 259.3ab, ZnMet. 265ab, and Mintrex Zn 281a). The efficacy of Mintrex Zn as a zinc source is further demonstrated in the post-challenge tibia zinc levels. Tibia zinc is the most reliable indicator of whole body zinc status, which tends to be depleted during an immune challenge. Mintrex Zn birds were able to fight the infection yet maintain significantly greater tibia zinc levels (Basal 115.2c, Zn sulfate 214.7b, ZnMet. 214.7b, and Mintrex Zn 246a).

CONCLUSIONS

Broiler performance was improved with supplemental zinc. Mintrex Zn-fed birds produced significantly more anti-coccidia antibodies following coccidiosis vaccination. Zinc supplemented birds maintained gut integrity during the coccidiosis challenge but Mintrex Zn had superior jejunum crypt depth and ileum mucosa thickness than any other treatment. The superior immune response to coccidial challenge was reflected in post-challenge performance and tibia zinc levels. Mintrex Zn fed birds were able to control the coccidial challenge with less impact on growth and whole body zinc status than any other treatment.

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Table 1. Mintrex Zn significantly improved antibody response to vaccination.

Antibody response O.D. background (1:16 dilution)		
	Anti-microneme antibody MIC2	Antibody anti-3-1E
Basal	0.036b	0.001b
Zn Sulfate	0.050b	0.081ab
ZnMet.	0.052b	0.058ab
Mintrex Zn	0.146a	0.125a

Table 2. Effect of Mintrex Zn on post challenge gut health.

	Jejunum villus length (microns)	Jejunum crypt depth (microns)	Jejunum breaking strength (force, g)	Ileum mucosa thickness (microns)
Basal	886b	619b	0.225b	910bc
Zn Sulfate	994ab	790b	0.265b	850c
ZnMet.	1048a	775b	0.334a	928b
Mintrex Zn	1113a	950a	0.370a	1051a

UNIFORMITY OF BROILER CHICKEN INFECTION AFTER COCCIDIOSIS VACCINE DELIVERY *IN OVO*

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ABSTRACT

Broiler *in ovo* vaccination is widely practiced today, particularly in the United States, where hatcheries have routinely incorporated this procedure for Marek's vaccination. Inovocox™ is a live oocyst coccidiosis vaccine administered *in ovo* as an aid in the prevention of coccidiosis in broilers. The vaccine contains *Eimeria acervulina, Eimeria maxima* (two strains), and *Eimeria tenella.* Previously presented data have demonstrated that administration of Inovocox through the Inovoject® automated *in ovo* injection system results in uniform, consistent delivery to each egg and a high level of vaccine infection in individual animals. The present study investigated whether broilers vaccinated *in ovo* with Inovocox and reared in floor pens were protected from challenge and whether vaccinated birds exhibited similar performance parameters as birds fed the anticoccidial salinomycin. Results indicated protection from homologous challenge by three weeks as measured by lesion score reduction. Body weight and feed conversion ratios (FCR) were determined from eight replicate pens per treatment group at days 21, 35, 42, and 49. Broilers vaccinated with Inovocox did not differ significantly in body weight or FCR from broilers grown with salinomycin.

INTRODUCTION

Coccidiosis vaccination provides an alternative to in-feed anticoccidial drugs for commercial broiler production. Automated *in ovo* delivery of coccidiosis vaccine can provide convenient and uniform dosing to every chick in the flock (1). Inovocox is a live oocyst coccidiosis vaccine under development by Embrex specifically for *in ovo* administration. Safety and feasibility of achieving immunity against coccidiosis via live oocyst administration *in ovo* has been previously demonstrated by either manual or automated injection (2, 3, 4, 5). Further studies support the conclusion that *in ovo* coccidiosis vaccination does not interfere with Marek's vaccine efficacy (6) or with Bursaplex® vaccine efficacy (7). More recent studies have shown Inovocox delivery through the Inovoject is not only uniform in terms of numbers of oocysts delivered, but also results in a high rate of vaccine infection early in the life of the birds (8).

The objectives of the present experiment were to 1) demonstrate that uniform oocyst delivery through the Inovoject results in expected efficacy against homologous challenge and 2) evaluate performance data in birds vaccinated with Inovocox compared to salinomycin treated birds during a 49 day growout.

MATERIALS AND METHODS

Vaccination and hatch. The test formulation of Inovocox vaccine was comprised of a 1X dose of *E. acervulina, E. tenella,* and two strains of *E. maxima* in 50 uL Marek's diluent and delivered by the Inovoject automated egg injection system. The non-vaccinated group (NV) received Marek's diluent as a control. A total of 800 candled embryos at 18 days of embryonation were injected per treatment and percent hatch was greater than 97% for both vaccinated and sham vaccinated treatments. Birds were vent sexed at hatch at Embrex Avian Research Center and transported to Southern Poultry Research for placement.

Efficacy. Two pens of 62 birds per pen were placed on fresh wood shavings for each treatment prior to challenge at day 20 or 21. Birds in the efficacy portion of the experiment did not receive salinomycin in the feed. For each challenge strain, 10 birds from each pen were used. An additional 10 birds from each of the NV pens (for a total of 20 birds) served as non-challenged controls. At six days post challenge, (five for *E. tenella*), lesion scores for each challenge species were compared between the (V/C) treatment to the non-vaccinated challenged treatment (NV/C).

Inovocox performance. Eight pens of 62 mixed sex birds (equivalent numbers of males and females) per pen were placed on fresh wood shavings for each treatment. On days 21, 35, 42 and 49 feed conversion and average live weights were compared between V and NV birds fed salinomycin (60gm/ton through D35).

RESULTS AND CONCLUSIONS

Vaccine efficacy was assessed by lesion score reduction. All vaccinated groups had significantly lower lesion scores post single species challenge than the non-vaccinated challenged controls. For bird performance, no significant treatment effects were seen for feed conversion on days 0-21, 0-35, 0-42 or 0-49 between vaccinated birds and salinomycin fed controls. No significant treatment effects were seen for average live weight based on pen weights for days 21, 35, 42 or 49 between vaccinated birds and salinomycin fed control birds. No significant treatment effects were seen for average live weight males V and NV or female V and NV at D49.

Based on the data collected in this study, performance parameters of broilers vaccinated with a 1X live coccidial vaccine Inovocox were equivalent to nonvaccinated, salinomycin-fed controls. Overall, Inovocox was shown to be efficacious when measured by the potency test and had no negative affects on bird performance parameters when injected at E-18 using the Inovoject. Taken together with previously presented data, the current study provides further verification that the convenient automated vaccination of broiler embryos with Inovocox results in reliable efficacy and performance parameters similar to that of the in-feed anticoccidial salinomycin.

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EVALUATION OF INCINERATION FOR DISPOSAL OF POULTRY MORTALITIES

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SUMMARY

There have been notable improvements in incinerator design and it was of interest to evaluate for a one-year period the efficiency and operational costs of three commercially available units for the disposal of poultry carcasses. A total of six flocks were evaluated for each of two broiler producers and a period of eleven months or the equivalent of a complete breeder flock cycle was evaluated for a broiler breeder producer. The broiler-breeder farm had an average mortality weight of 5.82 lbs (2.64 kg) over the four-quarter test, and averaged 19.83 lbs (8.79 kg) of mortality per gallon of fuel. Broiler #1 had an average mortality weight of 2.08 lbs (0.94 kg) over the six-flock test period, and averaged 24.98 lbs of mortality per gallon (2.99 kg/L). Broiler #2 had an average mortality weight of 0.93 lbs (0.42 kg) over the test period, and averaged 49.89 lbs of mortality per gallon (5.98 kg/L). Operational costs were calculated based on $0.85/gallon ($0.22/L) for propane and $0.98/gallon ($0.26/L) of diesel. The broiler breeder farm averaged 4.26 cents/lb (9.39 cents/kg) with a range of 3.55 to 4.72 cents/lb (7.83 to10.41 cents/kg), Broiler #1 averaged 3.59 cents/lb (7.92 cents/kg) with a range of 2.69 to 4.01 cents/lb (5.93 to 8.84 cents/kg), and Broiler #2 averaged 1.99 cents/lb (4.39 cents/kg) with a range of 1.83 to 2.07 cents/lb (4.04 to 4.56 cents/kg). Microbiological samples of residual materials remaining after incineration were examined and were found to be virtually devoid of detectable levels of bacteria. Incineration is shown to be a very cost effective environmentally friendly method of disposal.

INTRODUCTION

Recognized as one of the most biologically safe methods of disposal, incineration curtails the spread of disease and does not contribute to nor create water quality problems. The comparatively small amount of waste by-product does not attract insects or scavengers and can be disposed of easily in an environmentally friendly manner. The main environmental concern is the emission of particulate that may be generated during the process. Recent advances in refractory materials and better engineering have contributed greatly to improvements in incinerator efficiency. This study was designed to measure the efficiency and operational costs of several incinerators in poultry farm settings. Information obtained from this project will assist the poultry grower in making a sound economic and environmental decision concerning incineration options.

MATERIALS AND METHODS

There have been major improvements in commercially available incinerators during the past few years. Manufacturers claim recent, major improvements in efficiency and longevity of equipment - important concerns for implementing producer best management practices. However, some manufacturers are interested in obtaining *non-biased data* pertaining to the use and appropriateness of these units for disposal of poultry carcasses. Three manufacturers donated commercial incinerators that were installed on a broiler breeder and two broiler farms where they were subjected to a one-year evaluation process. Units were located on a concrete slab, with two units being placed under shelter. Each participating grower was furnished with a hanging scale to facilitate daily recording of mortality numbers and corresponding weight. In addition, fuel or propane usage rates were obtained to determine burn rate. Data were accumulated for a total of six flocks for each of the two broiler producers, and a period of eleven months or the equivalent of a complete breeder flock cycle was evaluated for the broiler breeder producer. Descriptions of the incinerators are as follows:

Broiler breeder:
Farm Type: Broiler Breeder, 2 Houses
Incinerator Type: Destructor Junior, National Incinerator, P. O. Box 266, Boaz, AL 35957
Capacity: 250 lbs Fuel Type: Propane

Broiler #1:
Farm Type: Broiler, 4 Houses, 7 lb average bird
Incinerator Type: Model A-15, Shenandoah Manufacturing Co., P. O. Box 839, Harrisonburg,

VA 22801
Capacity: 500 lbs. Fuel Type: Diesel
Broiler #2:
Farm Type: Broiler, 4 Houses, 7 lb average bird
Incinerator Type: Burn Easy Model 30 with thermocouple controlled burner. R & K Incinerator Co., 6125 West 100 South, Decatur, IN 46733
Capacity: 500 lbs. Fuel Type: Diesel

RESULTS AND DISCUSSION

Location and installation. There is no question that the placement of an incinerator on a concrete pad and under a covered shelter will extend the life of the unit. Exposure to the environment not only allows deterioration of the unit's exterior materials, but also contributes to rainwater entering the smoke stack, wetting the ash and creating reactive compounds that will result in the deterioration of the internal components of the unit. The use of a shelter results in up to two times greater life expectancy and reduces the need for grate replacement, or complete refurbishment or replacement. Once installation under a shelter is complete, it is essential that where the stack exits the roof an appropriate sealant be utilized to prevent the seepage of rainwater down the exterior surface of the stack to the level of the incinerator. Poorly sealed stacks will result in unexpected deterioration of the unit. Finally, a properly fitted cap will also reduce or eliminate the seepage of rainwater into the combustion chamber of the incinerator.

Economic evaluation. Summary of inputs, fuel usage and fuel costs appear in Table 1. For the broiler breeder farm a total of 4,692 mortalities were incurred during a 50-week production cycle. Initial flock size was 17,600 birds and the total mortality expressed on a monthly basis was 2.2%. Broiler breeders are large size birds that probably require the greatest amount of fuel for complete cremation. As a result, costs will be greater for the disposal of broiler breeder carcasses, which averaged 5.82 lbs (2.64 kg). It is interesting to note that actual cost of disposal tended to decrease with increased flock age. Older birds have a tendency to accumulate more body fat and this contributes to the combustion process. Disposal costs for broiler breeder carcasses averaged 4.26 cents/lb (9.39 cents/kg) with a range of 3.55-4.72 cents/lb (7.83 –10.41 cents/kg).

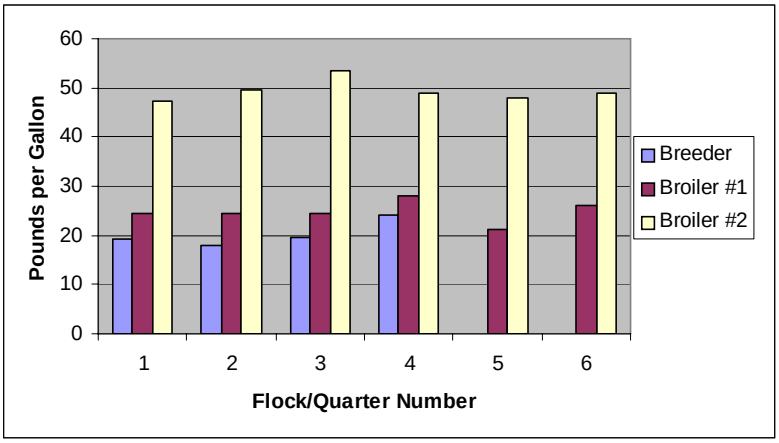
For the broiler farms, there was a significant difference in fuel costs between the two incinerators tested. The average cost of disposal for Broiler #1 vs. Broiler #2 was 3.59 vs. 1.99 cents/lb (7.92 vs. 4.39 cents/kg), respectively. The costs incurred by Broiler #1 were 80% greater as compared to Broiler #2. Average bird size for Broiler #1 was 2.08 lbs (0.94 kg), while that for Broiler #2 was 0.93 lb (0.42 kg). A major contributor to this difference was observed to be in cull management within the first week of growout. However, it is unlikely that the size of the bird accounts for the greatest proportion of this difference in incinerator efficiency. Differences in efficiency and cost are more likely to be related to the variability in model design and operation. It is interesting to note that the Burn Easy incinerator has a thermocouple that controls the burner. This device cycles the burner off at an internal temperature of 1300^{O} F (704^{O} C) and cycles the burner back on at lower temperatures to insure complete combustion. This type of system has a greater reliance on the presence of combustible products (i.e. fat) in the carcass for its contribution to the incineration process.

Another method of data expression is to determine the amount of pounds incinerated per gallon of fuel (Figure 1). Average fuel costs used in this analysis were 0.85 and 0.98 dollars/gallon ($0.22 and $0.26/L) for propane and diesel, respectively. The broiler breeder incinerator was propane dependent, while the broiler incinerators used diesel fuel. Results indicate that the Broiler #2 incinerator was most efficient in that it incinerated nearly 50 lbs/gallon (6 kg/L) used, while the Broiler #1 and broiler-breeder incinerator combusted about 25 and 20 lbs/gallon (3.0 and 2.4 kg/L) of fuel, respectively. It appears that the Broiler #2 incinerator has the capability to incinerate twice the carcass mass for the same amount of fuel, or at the same cost.

Table 1. Summary of incinerator inputs, fuel usage, and fuel cost.

Category	Broiler-Breeder	Broiler #1	Broiler #2
Number of mortalities	4,692	24,495	35,282
Pounds (kilograms) of mortalities	27,285 (12,374)	50,844 (23,059)	32,677 (14,820)
Average mortality weight (lbs/kg)	5.82/2.64	2.08/0.94	0.93/0.42
Gallons (liters) of fuel used	1,376 (5,208)	2,035 (7,703)	655 (2,479)
Pounds per gallon (kg/L)	19.83 (2.38)	24.98 (2.99)	49.89 (5.98)
Cents per pound (kilogram)	4.26 (9.39)	3.59 (7.92)	1.99 (4.39)
Cost range (cents/lb)	3.55-4.72	2.69-4.01	1.83-2.07

Figure 1. Amount of mortality incinerated per gallon of fuel.



AVIAN INFLUENZA IN ITALY: SEROLOGICAL AND VIROLOGICAL FINDINGS IN WILD DUCKS

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SUMMARY

Three groups of mallards were monitored during 2004-2005 for *Orthomyxovirus* A, kept as decoys for shooting. Two of them were housed in an aviary directly on lake water; the third bird group was on ground floor. Over the same period we collected samples from 15 wild mallards shot in the northern part of Italy. Influenza viruses, including subtypes H5 and H7, were identified – especially in the birds living on water.

INTRODUCTION

In Italy, as in many other European countries, in recent years there has been a sudden increase in avian influenza both in commercial poultry and in wild birds. The first influenza virus in wild birds was isolated from the tern (*Sterna hirundo)* in South Africa as described by Becker (1), but epidemiologic surveys in the 1970s indicated that the *Anseriformes* were widely infected (2), particularly the mallard (*Anas platyrhyncos*). This species seeks food on the surface of ponds, filtering the water through its beak, so it gets infected more frequently than birds feeding on the pond bottom (diving ducks and Limicolae), as *Orthomyxovirus* have a lipid envelope which allows them to float.

MATERIAL AND METHODS

Three groups of ten mallards each, kept as decoys, were tested from three different places. The first two groups lived in cages on the lake shore, in contact with the wild birds; the third group was kept in a garden, and carried to the lake only for shooting parties. The birds were four- and ten-year-olds. Pool of feces from each group and blood samples from every single bird (a total of 120 samples) were collected in March, July, and November 2004, and March 2005. Samples of feces from 15 mallards that had been shot were checked in October 2005.

RESULTS AND DISCUSSION

Table 1 shows the different serological pattern for the ducks kept in a garden, which were all negative at two tests, whereas most of the ducks in the other two groups showed seroconversion over the whole year, probably as a result of contact with infected wild birds living in the same lake.

The positive specimens were typed to establish the virological subtypes.

In lake A where a good proportion of birds were positive in all four seasons, three (n. 1, 6 and 8) were positive for H5 a year later and one (n. 6) had double the HI value. We found the subtype H7 in one duck

from the lake B (n. 5). We also found the subtypes H2, H9 and H11. Ducks had no clinical signs according with previous reports (4, 5).

In the 15 mallards shot, virological tests were negative.

The subtypes H5 and H7 were found in decoy ducks, with some changes in the antibody titer over the year, probably due to repeated contact with infected birds of the same lake.

We concluded that wild ducks, though a natural reservoir for potentially harmful influenza viruses, are not an immediate source of danger to farmed birds in areas with a low density of commercial birds, as in the situation considered. It would continue this research in lakeside areas with higher concentrations of commercial poultry.

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Table 1. Positive findings for Orthomyxovirus A in three groups of decoy mallards over 12 months*.

PLACE		MARCH 2004	JULY 2004	NOV. 2004	MARCH 2005
LAKE A	1	+ (H5)	+	+	+ (H5)
	2	-	+	-	-
	3	+	-	+	+ (H11)
	4	+	-	+	nn
	5	+	-	-	-
	6	+ (H5)	+ (H5)	+	+ (H5)
	7	+	-	+	+ (H5)
	8	+ (H5)	+	+	+ (H5)
	9	+	+ (H5)	+	+ (H5)
	10	+	-	-	-
	Positive	9	5	7	6
LAKE B	1	-	+	-	-
	2	+	+	-	nn
	3	+	+	+	+
	4	+	+	+	+ (H9)
	5	-	-	+	+ (H7)
	6	+	+	+	+
	7	-	+	+	+ (H9)
	8	+	+	+	+ (H2)
	9	-	-	-	+(H9)
	10	-	-	+	+
	Positive	5	7	7	8
GARDEN	1	-	+	-	+
	2	-	-	-	-
	3	-	+	-	-
	4	-	+	-	-
	5	-	nn	-	-
	6	-	-	-	-
	7	-	-	-	-
	8	-	-	-	-
	9	-	-	-	-
	10	-	-	-	-
	Positive	0	3	0	1
Total positive		14	15	14	15

* between brackets the subtype, when established nn = not known

HIGHLY VIRULENT EXOTIC NEWCASTLE DISEASE VIRUS STIMULATES DIFFERENTIAL CYTOKINE GENE EXPRESSION IN CHICKEN SPLENOCYTES FROM LOW VIRULENT LASOTA STRAIN

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The immune system can be divided into two functional components, the innate and adaptive, that differ in their mechanism of pathogen recognition. The innate immune response is responsible for detecting invading microorganisms during the initial stages of infection, which is a crucial determinant of disease resistance or susceptibility. Toll-like receptor (TLR) family members are responsible for recognizing the presence of invading microorganisms through pathogen-associated molecular patterns and initiation of immune responses. Recently, several chicken TLR's have been identified, including TLR7, which is reported to be involved with recognition of ribonucleic acid components characteristic of viral genomes (e.g. single-stranded (ss) RNA).

Newcastle disease virus (NDV) is classified as member in the Avulavirus genus, within the *Paramyxoviridae* family. The virus is enveloped and contains a negative-sense, ssRNA genome. NDV isolates have been classified as lentogenic (low), mesogenic (intermediate) or velogenic (highly virulent) depending on the severity of disease produced by the isolate in chickens. During May 2002, highly virulent exotic NDV (ENDV) was isolated from ring neck pheasants in northern California, which preceded isolation from commercial poultry in December 2002. More than 19,000 premises would be quarantined in five states, including California, Nevada, Arizona, Texas, and New Mexico, before the last positive isolation from commercial poultry was made on March 26th, 2003. In this study, the innate immune response induced by highly virulent ENDV from the 2002-03 California outbreak was compared to the low virulent NDV LaSota vaccine strain following *in vitro* infection of chicken splenocytes.

Using real-time RT-PCR, both pathotypes of NDV induced cytokine gene expression, however, ENDV induced higher levels of gene expression of IL-1β, IL-6, interferon (IFN)-α and IFN-γ. The addition of chloroquine abrogated cytokine induction in ENDV-infected splenocytes indicating endosomal maturation is important for downstream cytokine pathway signaling. Using a LaSota virosome model, increased cytokine gene expression was determined to be dependent on the presence of viral RNA since virosome preparations did not induce increased cytokine expression. These findings support the role of avian TLR7 in recognition of ssRNA and suggest differences in innate immune response between different pathotypes of NDV.

IDENTIFICATION OF INFECTIOUS BURSAL DISEASE VIRUS BINDING PROTEINS USING VIRUS OVERLAY PROTEIN BINDING ASSAYS AND MASS SPECTROMETRY

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ABSTRACT

Infectious bursal disease virus (IBDV) infects B-lymphocytes in the bursa of Fabricius, resulting in immunosuppression. The cellular receptors of IBDV, which are responsible for viral attachment and tropism, remain unknown. In this study, membrane proteins of chicken tissues were analyzed using virus overlay protein-binding assay (VOPBA) with an attenuated strain GZ911. The detected IBDV-binding proteins, which are potentially receptors or co-receptors of IBDV, were identified using mass spectrometry. The VOPBA analysis identified four bands of bursal

membrane proteins that bind to IBDV viral particles specifically, with molecular weights of about 43, 36, 34, and 29 kDa respectively. The identities of the proteins in these IBDV-binding bands were characterized by LC QSTAR® Pulsar quadruple time-of-flight mass spectrometer and the spectra obtained were analyzed against the NCBI nonredundant protein database. Totally 152 proteins were identified as significant hits with a significant threshold of 5%, while 26 of them are unique membrane proteins of various species, including chB6, annexin II and MHC class II of *Gallus gallus*. Although the functions of these IBDV-binding proteins in IBDV entry warrant further investigations, our results provide the insight on the putative identity of cellular receptor(s) of IBDV.

INTRODUCTION

Infectious bursal disease virus (IBDV) causes a highly contagious immunosuppressive disease in young chickens, resulting in great economic loss in poultry industries worldwide. IBDV is a member of the genus Avibirnavirus of the family *Birnaviridae*, whose genome consists of two segments of double-stranded RNA. Replication of IBDV causes depletion of B-lymphocytes by apoptosis, which results in morbidity, mortality and severe immunosuppression. Surface immunoglobulin M (sIgM)-bearing B lymphocytes were thought to be the target cells for IBDV infection.

Identification of viral receptors is critical for studying the viral pathogenesis and vaccine development. Viral receptors were conventionally identified by receptor-destroying enzymes that destroy components on the cell surface required for virus attachment. Competition or interference assay was also used for studying shared usage of a receptor by more than one virus. Virus overlay protein-binding assay (VOPBA) can be used to determine the size and number of virus binding proteins. Methods for protein identification have changed dramatically with the rapid development of mass spectrometry (MS). A protein or a mixture of proteins is digested with a proteolytic enzyme and the peptides are analyzed using MS techniques. Among different MS techniques, MS/MS has been used in the identification of complicated protein mixtures and several software tools for database search have been developed.

Previous experiments with proteases showed that IBDV receptor was composed of proteins. Furthermore, VOPBA has identified the cell membrane proteins that bound to a highly virulent IBDV strain OKYM, and an attenuated IBDV strain Cu-1. Molecular weights of putative IBDV receptors were also determined. However, strain-specificity and identity of cellular receptors of IBDV have not been investigated. In this study, we characterized the IBDV binding proteins, which could be putative viral receptors, with an attenuated IBDV strain GZ911 in bursal membrane proteins using VOPBA and MS/MS analysis.

MATERIALS AND METHODS

Cell line, virus and animal. A tissue culture adapted IBDV strain GZ911 was propagated in Africa green monkey kidney cells (Vero; ATCC CCL-81). Viral particles of GZ911 were purified as described previously. Healthy three- to six-week old specific pathogen free (SPF) leghorn chickens were used to collect tissues for preparation of membrane proteins.

Biotinylation of virus. Purified virus was biotinylated using sulfosuccinimidyl-6-(biotinamido) hexanoate (Pierce Biotechnology, Rockford) as previously described. To validate biotin labeling, the biotin-labeled IBDV was resolved in SDS-PAGE and then transferred to an Immun-Blot PVDF membrane (Bio-Rad Laboratories, CA, USA). Biotin-labeled virus was detected by streptavidin-alkaline phosphatase (AP) (Invitrogen, CA, USA) followed by color development using 5-bromo-4-chloro-3-indolyl -phosphate/ nitro-blue tetrazolium (BCIP/NBT) (Zymed Laboratories, CA, USA) as substrate. The reaction was stopped by repeated washes with distilled water.

Virus overlay protein-bBinding assay (VOPBA). Membrane proteins of chicken bursa, kidney, spleen, thymus, and leg muscle were prepared from three-week old leghorn chickens. The VOPBA was conducted as described in the following section. The membrane proteins were resolved in SDS-PAGE and transferred to an Immun-Blot PVDF membrane. Membrane was incubated overnight in blocking buffer [1% (w/v) BSA in PBS] at room temperature. After three washes in PBS, the membrane was incubated with biotin-labeled GZ911 in blocking buffer for one hour at room temperature. The membrane was then washed three times with PBS and binding of virus was detected with streptavidin-AP followed by color development using BCIP/NBT. To check for binding between membrane proteins and streptavidin-AP, the experiment was repeated without adding biotin-labeled GZ911. To confirm specificity of the binding assay, inhibition of biotin-labeled viral particle binding to bursal membrane proteins was tested. Before the biotin-labeled GZ911 was added, the purified GZ911 was incubated with the transferred membrane for one hour and then the membrane was washed three times in PBS.

Mass spectrometry and protein identification. To identify these IBDV binding proteins, the four bands of corresponding molecular sizes were excised, digested with trypsin and subjected to MS analysis. The trypsin in-gel digestion of proteins was carried out

as descried elsewhere. For analysis of trypsinized peptides, Electrospray Ionization - Quadruple- Time Of Flight (ESI-QUAD-TOF) was performed using Ultimate capillary LC system (LC Packings, CA, USA) coupled to a QSTAR® Pulsar quadruple time-of-flight mass spectrometer (Applied Biosystem/ MDS Sciex QSTAR® Pulsar I). Using Analyst QS® software, the MS/MS ion was searched against the NCBI nonredundant protein (NCBInr) database in Mascot. The same search parameters were used for all searches: trypsin cleavage; *eukaryota*; allow up to one missed cleavage; monoisotopic mass values; no restriction on protein mass; peptide mass tolerance ± 0.3 Da; fragment mass tolerance ± 0.2 Da; variable modification: carbamidomethyl (C), deamidation (NQ), oxidation (M), propionamide (C). Peptide matches were grouped into protein matches. According to the significance threshold, which had a default setting of 5%, protein hits with significant scores were listed as significant hits. Since peptides less than 10 amino acids matches are expected to be found merely by chance, annotations were carried out on peptides longer than 10 amino acids only. The subcellular locations of these proteins were either found according to Genbank search or prediction using Psort II program.

RESULTS

Biotinylation of virus. In these experiments, two bands were detected with molecular weights about 48 and 32 kDa, which were identical to the reference viral proteins, VP2 and VP3. These results indicated that biotin has been successfully linked to the IBDV proteins, where VP2 and VP3 are the major components.

Identification of IBDV-binding proteins. Four IBDV-binding bands, designated B1, B2, B3, and B4, were observed in membrane proteins extracted from bursa. Molecular weights of these protein bands were approximately 43, 36, 34, and 29 kD, which were estimated by comparing with protein molecular weight marker (MBI Fermentas, Lithuania) using Kodak 1-D image analysis software (Kodak NEN, NY, USA). In the negative control, no band was detected in the bursa membranes proteins, demonstrating the bands (B1-4) were not from non-specific binding between streptavidin-AP and the bursa membranes proteins. In the case of kidney and muscle, a number of bands were detected in VOPBA. However, similar bands were also observed in the negative control, indicating that these proteins bound to streptavidin-AP non-specifically. No detectable band was observed in membrane proteins from spleen and thymus. In consistent with the increased protein quantities as shown in SDS-PAGE, signals for IBDV-binding bands increased in a dose-dependent manner in VOPBA. The specificity of virus binding was further confirmed by a competitive binding assay. In this case, 40 μg bursal membrane proteins were pre-incubated with purified non-biotin-labeled GZ911 viral particles. The biotin-labeled virus binding was blocked and the four virus-bound bands could not be observed.

Mass spectrometry analysis. Totally, 189, 238, 140 and 130 spectra were identified in protein bands B1, B2, B3 and B4 respectively. Of these, four spectra of B2, six spectra of B3 did not find matched peptides. This may be caused by differences in database, unsuspected posttranslational modification, nonspecific cleavage, poor quality of spectrum and/or nonproteinaceous compounds. To find corresponding proteins, identified peptides were annotated with a significance threshold of 5%. In other words, protein matches with a probability of less than 5% to be a random event are listed as "significant hits". Band B1, B2, B3 and B4 had 39, 30, 33 and 50 significant hits respectively. Twenty-six unique membrane proteins were identified according to Genbank search or prediction using Psort II program. Due to the incompleteness of chicken protein database, most of the membrane protein matches were identified from other organisms, only chB6 (gi 1184241), annexin II (gi 113949) and MHC class II (gi 104768) were of chicken origin.

CONCLUSION

We have identified four protein bands that specifically bound with biotin-labeled IBDV, GZ911, in bursal membrane preparations by VOPBA analysis, and the identities of these IBDV-binding proteins were characterized by MS/MS techniques. Our results provided the first report on the putative identity of IBDV receptor(s) in chicken and raised the possibility of multiple receptors involved in IBDV entry.

COMPARISON OF MEDIA FOR THE RECOVERY OF *CLOSTRIDIUM PERFRINGENS* FROM POULTRY LITTER

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SUMMARY

In times of disease outbreak it is important to get an accurate count of litter bacterial numbers. One of the more important pathogenic bacteria found in litter is *Clostridium perfringens*. Several different media are commercially available for the isolation and cultivation of *C. perfringens*. To date no research has been performed to determine which medium is best for the recovery of this significant pathogen in poultry litter samples. In this experiment five differential and two selective media were examined for their ability to recover *C. perfringens* from several litter samples. Additionally, the ability of this media to recover *C. perfringens* from pure cultures was determined. One of the differential media, TSC, had shown to be most effective in getting accurate *C. perfringens* counts from both litter and pure culture.

INTRODUCTION

Clostridium perfringens is widely distributed in the environment, occurring naturally in soil, dust, and in the intestine as part of the normal microflora in warm blooded animals. Under the right circumstances *C. perfringens* can induce either necrotic enteritis or gangrenous dermatitis in poultry. Besides being in poultry's gut, this potential pathogen is often found in the litter, occasionally at high levels. Enumeration of *C. perfringens* from litter is often performed using media that have been adapted from typical food safety *C. perfringens* determination. These media are effective for determining *C. perfringens* numbers in food; however, their effectiveness in determining litter *C. perfringens* numbers has never been determined. The purpose of this study was to determine the best selective or differential medium for cultivating *C. perfringens* from litter. In order to determine the best media, two trials were performed using two selective media and five differential media.

MATERIAL AND METHODS

Trial 1

Media. Eight different media were used in this study. One, the reduced blood agar (RBA), was the unselective media that, for the known samples, would give the baseline counts. Two selective media: Clostrisel and reinforced clostridial agar (RCM) and five differential media: McClung-Toabe agar (MT), oleandomycin polymyxin sulphadiazine perfringens agar (OPSP), Shahidi-Ferguson perfringens agar (SFP), sulfite polymyxin sulfadiazine agar (SPS), and tryptose sulfite cycloserine agar (TSC) were used. All of these media were bought as premixed powder and were made according to the manufacturers' directions. There was a modification in SPS and TSC; these two media were made without the addition of egg yolk. Not using egg yolk is often performed and has no adverse effect on recovery of *C. perfringens.*

***C. perfringens* isolates**. In this trial five known *C. perfringens* isolates were utilized. Three (K1-K3) of the five isolates were taken from clinical cases of necrotic enteritis, one (K4) from a clinical case of gangrenous dermatitis, and one (K5) was ATCC culture 43402. All five isolates were removed from a -80^{o}C freezer and grown on RBA overnight at 37^{o}C under anaerobic conditions. A single colony that displayed typical *C. perfringens* double zone hemolysis on RBA was then taken and used to inoculate 10mL of reduced brain heart infusion broth. This broth was grown anaerobically at 37^{o}C; after 24 hours each isolate was serially diluted in sterile saline (0.85% NaCl). Each sample had 0.1 mL spread plated to the following media in duplicate: Clostrisel, MT, RCM, OPSP, SFP, SPS, TSC, and RBA. After incubating for 24 hours at 37^{o}C under anaerobic conditions the plates were counted.

Litter microbiology. Pine shaving litter that had at least two subsequent flocks on it was selected for sampling. Six samples were collected using the grab sample technique described previously (1). Briefly, samples were collected from three areas within the pen using a clean glove. These three areas were under the feeder, under the watering line, and from the middle of the pen. They were then combined in a sterile bag and thoroughly mixed and transported to the laboratory. In the lab, pooled samples were diluted 1:10 in sterile filter bags using sterile saline and thoroughly mixed in a stomacher for 90 seconds. After being stomached, the 1:10 dilution would then be serially diluted with sterile saline. From these dilutions 0.1mL would be plated onto the following media in duplicate: Clostrisel, MT,

RCM, OPSP, SFP, SPS, and TSC. These plates were incubated anaerobically at 37°C for 24 hours, after which all suspect *C. perfringens* colonies were counted from each plate. From each plate five suspect positive colonies were streaked onto RBA and then incubated anaerobically at 37°C overnight. A positive *C. perfringens* is one that exhibits double zone hemolysis. From these results a ratio was created that would be used to adjust the final suspect *C. perfringens* count to give the final overall count.

Trial 2

The same procedures were followed as in Trial 1 except that the six litter samples came from different pens.

Statistical analysis. Data collected from both trials were converted to log10, combined and then analyzed using SPSS ver 12.0. A GLM was performed with the $P<0.05$, if there was any significant difference between the media, the means would be separated out using Tukeys Multiple Comparison Test.

RESULTS

Medium MT for both tables was removed due to consistently poor results, which left RBA, Clostrisel, RCM, OPSP, SFP, SPS, and TSC. Overall, the results presented in Table 1 show that all the media produced similar results compared to the unselective media (RBA). The only significant differences involved OPSP. This medium recovered 1.7 and 0.6 $\log_{10}$ lower *C. perfringens* amounts than RBA for K2 and K3 respectively. The other medium that produced lower bacterial counts is Clostrisel with sample K3. Clostrisel recovered 1.3 $\log_{10}$ less bacteria than RBA for sample K3.

The dirty litter produced *C. perfringens* numbers that ranged from over 0 to over 7.0 $\log_{10}$. These differences are not surprising, given that the litter was unseeded and the variable nature of *C. perfringens* in the litter. As can be seen in Table 2 the two non-differential media, Clostrisel and RCM, consistently had higher counts. This is not unexpected since these two media are selective only for *Clostridium* and do not differentiate species of that particular genus. Between these two media, RCM was consistently overwhelmed with *Clostridium* at the tested dilutions used. Clostrisel recovered 0.5-2.8 $\log_{10}$ less *Clostridium* colonies then RCM. The four differential media (OPSP, SFP, SPS, and TSC) produced comparable results to each other. TSC gave either the highest or close to the highest number of positive colonies for *C. perfringens* on 10 of the 12 samples tested. SFP produced high numbers on seven of the 12 samples, with SPS and OPSP producing high numbers on six of the 12 samples. The medium that produced the lowest overall counts was SPS, which produced the lowest number five out of 12 times. OPSP and SFP produced the low counts four out of 12 times. TSC had low counts only one out of 12 times. The differences in the amount of *C perfringens* recovered from these four media were as extreme as 3.5 $\log_{10}$ in sample L12 to a close as 0.05 $\log_{10}$ for sample L4.

DISCUSSION

The results presented herein show that the pure cultures of *C. perfringens* were most readily recovered using RCM, SFP, SPS, and TSC when compared to the number of colonies that grew on RBA. Clostrisel and OPSP were inhibitory with strain K3, which is a clinical *C. perfringens* isolate from a chicken that had necrotic enteritis. Strain K2, when plated on OPSP, also was not fully recoverable. The results concerning OPSP were not unexpected, since several authors (2, 3) have reported that this media can suppress growth of *C. perfringens*. The similarity in the counts for K1-K5 when plated on SFP, SPS, and TSC are not surprising, since these three media only differ in the type of antibiotic(s) used (3). RCM and Clostrisel are selective for *Clostridium* spp. and not for *C. perfringens* only. These two should have good recovery of *C. perfringens* when compared to RBA. Overall this statement held true, especially with RCM; however with isolate K3, Clostrisel had a significantly lower recovery when compared to RCM or RBA. The main inhibitory ingredient with Clostrisel is sodium azide. Since this isolate was tested two separate times in duplicate, it can be inferred that this isolate of *C. perfringens* is susceptible to sodium azide.

Litter samples, which contained an unknown number of *C. perfringens*, were best recovered with TSC. This medium was followed by SFP, OPSP, and SPS. Clostrisel was able to isolate a fair number of suspect *Clostridium* spp bacteria, while RCM was overgrown at the dilutions tested. From these results RCM is not selective enough for determining the *Clostridium* that may be present in litter. Colonies formed on Clostrisel were only tested for the presence of *C. perfringens* and not other *Clostridium* spp that may have grown. For recovery of overall *Clostridium* counts Clostrisel is a good medium of choice. The differential media that had the lowest recovery were OPSP, SFP, and SPS. Perhaps the failure of these three media to culture *C. perfringens* is due to their composition. As mentioned above, OPSP is known to suppress some strains of *C. perfringens*. A problem that both OPSP and SFP share is that they both tend to allow sulfite reducing facultative anaerobic bacteria to grow. This requires that these two media require additional testing to confirm the presence of *C. perfringens* and that this testing may incorrectly skew the results to the low side. SPS occasionally fails to

produce black colonies (4), which would produce incorrectly reported low counts.

Our results show that TSC is the best medium for isolating *C. perfringens* from poultry litter. Other investigators have shown that TSC is the preferred medium for isolating *C. perfringens* from ground beef (3), shellfish (5), and lean meats (4, 5). The only problem experienced with TSC is that it would occasionally give false positives, though at a lower rate than OPSP and SFP.

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Table 1. Combined $\log_{10}$ transformed results from *C. perfringens* isolates K1-K5.

Isolate ID	RBA	Clostrisel	RCM	OPSP	SFP	SPS	TSC
K1	9.76	8.74	9.05	8.91	9.75	9.45	8.23
K2	$11.04^{a,b}$	$10.99^{a,b}$	$11.00^{a,b}$	9.26^{b}	11.22^{a}	$11.05^{a,b}$	$10.18^{a,b}$
K3	10.90^{a}	9.57^{c}	10.86^{a}	10.27^{b}	$10.66^{a,b}$	$10.65^{a,b}$	10.83^{a}
K4	11.14	10.43	11.32	10.94	10.83	10.66	10.87
K5	11.56	10.93	10.97	11.27	11.07	10.84	11.30

^{a,b}Letter differences in a row indicate a statistically significant difference at $P<0.05$.

Table 2. Amount of *C. perfringens,* expressed in $\log_{10}$, that was recovered from twelve litter samples.

Litter Sample ID	Clostrisel	RCM	OPSP	SFP	SPS	TSC
L1	5.48^{b}	$>7.00^{a}$	3.24^{e}	3.57^{d}	$3.50^{d,e}$	4.51^{c}
L2	$4.18^{b,c}$	$>7.00^{a}$	4.27^{b}	3.93^{c}	4.23^{b}	$4.13^{b,c}$
L3	5.47^{b}	$>7.00^{a}$	4.95^{c}	4.71^{d}	5.48^{b}	5.14^{c}
L4	6.43^{b}	$>7.00^{a}$	6.24^{b}	6.19^{b}	6.19^{b}	6.24^{b}
L5	5.53^{b}	$>7.00^{a}$	3.89^{d}	5.22^{b}	4.71^{c}	$5.07^{b,c}$
L6	5.14^{b}	$>7.00^{a}$	4.53^{d}	$4.72^{c,d}$	4.50^{d}	$4.99^{b,c}$
L7	6.00^{b}	$>7.00^{a}$	$3.18^{c,d}$	$2.79^{c,d}$	2.69^{d}	3.24^{c}
L8	5.86^{b}	$>6.99^{a}$	3.38^{c}	3.09^{d}	3.39^{c}	3.38^{c}
L9	5.99^{b}	$>7.00^{a}$	2.76^{d}	$3.39^{c,d}$	3.60^{c}	2.84^{d}
L10	6.55^{a}	$>7.00^{a}$	3.18^{b}	2.70^{b}	1.00^{c}	2.74^{b}
L11	5.55^{a}	$>7.00^{a}$	0^{b}	1.00^{b}	1.00^{b}	0^{b}
L12	$5.70^{a,b}$	$>7.00^{a}$	3.01^{b}	$5.92^{a,b}$	2.48^{b}	$3.18^{a,b}$

$^{a-e}$Letter differences in a row signify a statistically significant difference at $P<0.05$.

SURVEYING THE POULTRY HEALTH KNOWLEDGE OF BACKYARD AND SMALL POULTRY FLOCK OWNERS

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SUMMARY

There has been concern that poultry kept in backyard or small flock situations can serve as reservoirs of disease agents for commercial poultry enterprises. Moreover, there is also concern that backyard and small poultry flocks can serve to disseminate exotic diseases like avian influenza and exotic Newcastle disease to commercial operations. Although there are opportunities for commercial poultry producers to attend continuing education programs, such programs may be lacking for backyard and small flock owners. Education of these poultry flock owners is of utmost importance to improve the health status of these flocks. Before educational programs can be developed, it is necessary to know the baseline level of knowledge these flock owners possess. Hence, the purpose of our study was to survey backyard and small poultry flock owners on basic poultry health concepts. Flock owners were surveyed at extension educational programs and county fairs. Preliminary results indicated that disease knowledge of the owners varied with the flock types (exhibition, youth groups, organic producers, and backyard pet chickens). For example, organic producers were more knowledgeable about antimicrobial resistance and medication procedures when compared to other groups of flock owners.

DEEPLY MULTIPLEXED RT-PCR ASSAYS FOR HIGH-THROUGHPUT SURVEILLANCE OF ANIMAL DISEASES

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In the era of bioterrorism, bird flu, SARS, and West Nile virus, it is more imperative than ever to build an infrastructure for testing of environmental and clinical samples that is robust, efficient, and sensitive. At the core of such an infrastructure lay the assays that are placed in the hands of the public health community. These assays must be sufficiently specific, sensitive and robust to give public health officials the confidence to initiate a national or regional response should a positive reading occur. Researchers at Lawrence Livermore National Laboratory (LLNL) have developed deeply multiplexed assays for use by the Laboratory Response Network and the National Animal Health Laboratory Network, among others.

A critical challenge facing US Agriculture in detecting and responding to outbreaks of animal disease is the availability of rapid, validated diagnostic assays for the detection of multiple diseases or multiple strains of the same disease in a single assay. Laboratory methods currently used to detect animal diseases are generally single agent and can be time-consuming, labor-intensive, and difficult to scale up to meet diagnostic demands in the event of an outbreak.

The Bioassays and Signatures Group (BSG) of the Chemical and Biological National Security Program (CBNP) at the Lawrence Livermore National Laboratory (LLNL) has developed rapid, reliable and sensitive assays geared towards enhancing the National Security of the United States. As such, LLNL is involved in a number of projects with multiple collaborators developing TaqMan, Multiplex, Microarray, and other assays for the early detection of Infectious Disease Threats. For example, a TaqMan assay developed at LLNL in collaboration with the California Animal Health and Food Safety Laboratory (CAHFS) at UC Davis was used to combat the 2002 exotic Newcastle disease outbreak in California.

One of our most promising current areas of research is the development of deeply multiplexed nucleic acid assays. These assays can detect multiple

genome regions of many pathogens in a single tube assay simultaneously and with great sensitivity & selectivity. We have developed two panels of such assays, one targeting human pathogens, and another targeting mammalian agricultural pathogens. We have demonstrated the ability to simultaneously extract and amplify both DNA and RNA targets with a high degree of efficiency. With the help of our many collaborators, our assays are being implemented into the National Animal Health Laboratory Network (NAHLN), and the Laboratory Response Network (LRN).

The current assay can detect 17 distinct genomic regions from a panel of agricultural foreign and domestic viruses that are clinically indistinguishable from Foot and Mouth Disease virus in cattle, sheep and pigs. The 21-plex assay that we have developed is run in a single tube and in addition to the 17 genomic signatures, includes four internal controls. This assay can be run in a 96-well format using standard laboratory instrumentation which is commercially available. We can use the capabilities of the BSG to develop any number of different multiplex panels with a theoretical "plex ceiling" of 100.

Our hope is that with the help of new collaborators with expertise in avian diseases, we can extend the multiplex approach to the development of new assays. We hope to develop both assays for the diagnosis of multiple avian diseases in a single tube (ILTV, *Pasteurella multocida*, FPV, APV, duck viral enteritis, HEV, and APV) as well as assays for the detection and characterization of infectious pathogens whose genetic diversity is beyond the current capabilities of single TaqMan assay detection. Such genetically diverse agents could include but are not limited to infectious bronchitus virus (IBR), avian leukosis virus (ALV), and avian influenza (AI).

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FOOD/AGRICULTURE INFRAGARD: NATIONAL SECURITY AND THE VETERINARY PROFESSIONAL

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Vulnerabilities to the food distribution and agricultural production systems have been identified by the Federal Bureau of Investigation (FBI) and the intelligence community. Adversaries, including both international and domestic terrorist groups, are motivated to conduct attacks so as to cause damage the economy, foment public unrest, and cause death and disease.

"Food/Agriculture InfraGard" is a new national security program designed to better protect the United States agricultural production system and food supply. The program, which is sponsored by the Federal Bureau of Investigation (FBI) is designated as a "Special Interest Group" (SIG). The SIG provides a nexus for cooperation between industry, academia and the FBI. Veterinarians enrolled in the program will play a significant role by serving as subject matter experts for animal health and production issues. The program has a critical need for many kinds of professionals, including veterinarians, who have training and experience in the areas of foreign animal diseases (FADs) and/or weapons of mass destruction.

Veterinarians enrolled in the program will be considered both customers and subject matter experts for use in the intelligence cycle. As customers, they will inform the FBI as to their informational needs to better serve their specific commodity or clientele. As subject matter experts, veterinarians may also be called upon to assist the FBI in detecting, deterring, assessing, and preventing malicious attacks (criminal and terrorist origin) on the agricultural production and food processing sectors.

Information flow. Informational flow is a critical element of the Food/Agriculture SIG. The FBI's intelligence cycle begins by defining the information requirements. In this first stage ("Requirements") of the cycle, veterinarians can help define specific system needs from the perspective of both the science (e.g. what FADs are most pertinent to the U.S. economy; what type of reports are most useful for serving the veterinary clientele, etc.) and professional agribusiness interconnectivities (e.g. how an incident affects a given commodity, as well as other commodities; and what type of informational flow is most useful during an disease incident, etc.).

The second stage of the cycle is entitled "Planning and Direction." Although, private and industry veterinarians would not generally have a great deal of input at this stage, those professionals embedded within the military and intelligence communities would assume significant roles in setting intelligence priorities and establishing assessment

methodologies. This stage of the process is affected by other intelligence being gathered throughout the world. For example, if an adversarial nation were known to be conducting research on an animal disease, that if introduced into the United States, would have a significant economic impact, intelligence on that disease or the exact nature of the foreign research and its exploitability potential, would become a priority.

The third stage of the intelligence cycle is "Collection." Although, the term might conjure images of spies and spy craft, in fact it is here that veterinarians, both inside industry and those in private practice, can serve a vital role. "Intelligence" is another name for information. Intelligence data about animal production and disease occurrence are inextricably tied up with the techniques and products of epidemiology. Veterinarians serve as additional eyes and ears of surveillance in the field. Although government agencies, such as the United States Department of Agriculture, continually monitor disease patterns throughout the U.S., the scope of the surveillance is limited by budgetary, personnel, and facility constraints. With the insertion of private and industry veterinarians into the epidemiology and intelligence collection systems, the amount of data being collected can increase exponentially, thereby increasing the likelihood that a disease or significant mortality event (natural and bioterrorism related) is observed early and therefore contained more quickly.

The fourth stage of the intelligence cycle is called "Processing and Exploitation." It is here that the complex and incomprehensible reams of information collected through various means are turned into products that facilitate their interpretation. This stage is solely the responsibility of specialists within the intelligence community and would not be in the purview of veterinarians (either inside or outside of government), who would lack the technical expertise to perform the mission.

The fifth stage of the intelligence cycle is called "Analysis and Production." It is here that private and company veterinarians can serve another vital role. Compiled and processed data, without verification and interpretation by subject matter experts, is of little or no value. The Food/Agriculture InfraGard SIG is designed to provide a mechanism by which information can be safely shared between government and industry. Once shared, it can then be independently verified or interpreted by subject matter experts both inside and outside the government. Sometimes called "full spectrum" analysis, the incorporation of multiple views and perspectives better ensures a correct interpretation. Veterinarians within the SIG will be expected to help the government interpret and give meaning to the vast array of information being collected both by industry and the intelligence community.

The sixth and final stage of the intelligence cycle is perhaps its most important. Information which is "stovepiped" or compartmentalized and not shared back to all customers loses much of its value. Food/Agriculture InfraGard provides the conduit by which both general information and that which is of a more immediate concern or "actionable" quality can be efficiently and rapidly shared with the customers, including agribusiness and the veterinary community. The importance of a functioning system where information can be shared in a timely and efficient manner cannot be overemphasized. As the business community has long realized, "information is power and power is profit."

Once the intelligence and information cycle has been completed, to continue to be of value, the system must begin the cycle again. Working properly the system will be in perpetual motion, always collecting, always evaluating the needs of the customers and always seeking to increase the value and the utility of the data. Functioning properly, intelligence data returned to the customers will generate new ideas, new questions and therefore cause the identification of new needs and the synthesis of new priorities and methodologies to achieve the goal. To do this efficiently, a robust system must be built and constraints to information flow must be minimized. To do anything less is to create a system that will soon be of little interest to the business community.

Security of data. A great deal of industry concern for data sharing revolves around the issue of Freedom of Information Act (FOIA) mandated access. Companies rightly worry that sensitive and confidential data, once shared, may fall into the wrong hands. Once released, information can never be recalled and the consequences of its liberation could likely be economically devastating. FBI managers of the Food/Agriculture InfraGard program recognize the sensitivity of the issues and mechanisms are being built into the system to protect individuals, companies, and industries that participate. Information collected by the FBI during open cases (those being actively investigated) is never released to the public nor is it subject to FOIA release. For example, if a poultry company were being threatened in some manner, the FBI could not be forced to release confidential information related to the case, which had been shared by the company. The same would be true for information shared by practicing veterinarians, who may possess sensitive information pertinent to open cases being conducted by the FBI. If information concerning sector or commodity vulnerabilities or credible threats is shared by private citizens or industry through the Food/Agriculture InfraGard conduit, it too would be protected from FOIA suits and not released. If particularly sensitive, the information might in fact

not be shared with the entire SIG, but would remain a private and confidential matter between the submitting individual or commodity and the FBI. Anyone within the government releasing such information would be subject to criminal prosecution and severe punitive action.

Application process. Veterinary professionals interested in participating in the Food/Agriculture InfraGard SIG are strongly encouraged to join. To become a member of the SIG one must first join the InfraGard Program. The program is limited to U.S. Citizens. Applicants are vetted by the FBI and, if approved, receive security software which allows them to receive InfraGard restricted information. Specific requirements and application information is available on the public InfraGard Website, which is located at: www.infragard.net. Once a member of InfraGard, members can go to the secure Infragard Website and make application to Food/Agriculture InfraGard SIG. Subject matter experts in the Food/Agriculture InfraGard SIG are encouraged, but not required to submit original articles, which can then be viewed by other members of the SIG. One of the main benefits for participating is the development of professional contacts and identification of specific subject matter experts contained within the SIG.

SUMMARY

Food/Agriculture InfraGard SIG is a new group being developed within the FBI's InfraGard Program. Its main purpose is to provide a conduit for information/intelligence flow between government, academia, and industry. A critical need of the program is the incorporation of veterinary professionals, both inside and outside of industry. These individuals can serve both as subject matter experts and as intelligence customers. Veterinarians must clearly define their needs for information and articulate those needs to the SIG leadership within the FBI. Only then can the program evolve into an effective tool, which can serve industry, help protect commercial agriculture and the food supply, and thereby strengthen national security.

REPLICATION AND ENVIRONMENTAL SHED OF A NOVEL AVIAN HERPESVIRUS FROM EXPERIMENTALLY INFECTED CHICKENS, TURKEYS, AND QUAIL

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ABSTRACT

A Novel Avian Herpesvirus (NAHV) chimeric serotype 1 and serotype 3 virus has been developed by Schering-Plough Animal Health as a vaccine against Marek's disease (MD) and as a backbone construct for carrying other poultry disease antigens. This report describes the replication and shed of the vaccine virus following overdose inoculation in chickens, turkeys and quail. These results show that the chimeric virus replicates similarly to other MD viruses in each host species. However, shed of the virus from the feather follicle epithelium was limited or absent and no infection of contact chickens occurred.

INTRODUCTION

Marek's disease of chickens is caused by a cell-associated lymphotropic alphaherpesvirus. Clinical signs include immunosuppression, polyneuritis, and tumor formation. Although vaccination has been used to control MD since the 1970s, the disease continues to be a serious threat to the health and welfare of poultry. HVT is the most commonly used vaccine against MD, but may not protect against disease caused by Serotype 1 virus or variant virulent strains of MD often implicated in field outbreaks of MD.

A novel avian herpesvirus (NAHV) was constructed as a chimeric virus containing the unique long (UL) regions of the Herpes Virus of Turkeys (HVT, Serotype 3) and the unique short (US) regions of the Marek's Disease virus (MD, serotype 1) by cosmid reconstruction recombinant DNA technology. NAHV provides protection against Marek's disease in chickens and will be used as a vector for expression of other poultry disease antigens. A minimum protective dose for NAHV has been determined using the USDA challenge model. The virus has also been tested for safety at a 10X dose, and for reversion to virulence following backpassage in chickens. The results of these studies have confirmed the efficacy and safety of this novel vaccine.

In these studies, the dissemination of the NAHV in the host species (chicken) and the ability of the virus to replicate in non-target species, turkey and quail were

investigated. The shed of the virus to contact chickens and into the environment were also of interest due to the genetically modified organism (GMO) classification of the vaccine virus.

MATERIALS AND METHODS

Chickens, turkeys, and quail were vaccinated subcutaneously with 0.2 mL of diluted NAHV containing 1.5X, 4X or 5X the minimum release titer for the product measured in plaque forming units. A group of unvaccinated contact sentinels was housed with the chickens. Negative control groups were placed for all species. The sampling schedule and tissues sampled for each group are as follows. Chickens were vaccinated at day of age. Three chickens from the vaccinated, contact control, and negative control were sampled at 0, 7, 14, 21, and 28 days of age. The contact sentinel chickens were also sampled on day 42. Tracheal swabs, cloacal swabs, feather, spleen, bursa, blood, and litter samples were collected. Samples were pooled by group. Turkeys were considered maternal antibody positive and were vaccinated at three weeks of age to allow for the degradation of maternal antibody. Spleens were collected from three turkeys, from the vaccinated and negative control group on days 0, 7, 14, 21, and 28 post-vaccination. Feather and litter were collected on days 21 and 28 post-vaccination. Quail were vaccinated at day of age. Vaccinated quail were sampled on days 7, 14, 28, and 56. Samples collected were a visceral organ pool (liver, kidney), lymphoid organ pool (spleen, bursa), digestive organ pool (duodenum, proventriculus), respiratory organ pool (trachea, lung), and feather. Gross examination for tumors was conducted at every necropsy. Samples were prepared for both virus isolation and polymerase chain reaction (PCR) assay using primers specific to NAHV. Only the results of the PCR testing will be presented here.

RESULTS

Chickens. There was no clinical illness or mortality due to the vaccine during the study. No tumors developed when examined at 28 days post-inoculation. PCR analysis demonstrated that the recombinant virus disseminated into the spleen, bursa, and feather follicle epithelium of vaccinated chickens. Spleens were positive by day 7 post-inoculation and remained positive through the 28 day sampling. Bursae were positive at day 7 post-inoculation only. Feather follicle epithelium was positive at 21 days post-inoculation and negative at day 28. There was no virus detected in litter samples collected throughout the course of the study. Sentinel birds exhibited no evidence of infection by the NAHV vaccine in any tissues.

Turkeys. There was no illness or mortality in any of the vaccinated turkeys. No tumors were identified when examined at 28 days post-inoculation. The NAHV was detected in the spleen on days 14, 21, and 28 post-vaccination and on days 21 and 28 post-vaccination in the feather follicle epithelium. No virus was identified from the negative control group. The virus was not detected in highly sensitive but limited environmental sampling, suggesting the shed of cell-free virus is not extensive.

Quail. There was no clinical illness or mortality due to the vaccine in any of the vaccinated quail. No tumors developed when examined at 56 days post-inoculation. PCR results identified NAHV in samples from the visceral lymphoid, digestive, and respiratory tissue pools. The lymphoid pool (bursa, spleen, thymus) tested positive on day 7 and throughout the 56 day study. The respiratory pool was positive on day 7 and 14 but negative on days 28 and 56. This probably represents viral replication in the lung tissue. The digestive organ pool (duodenum, proventriculus) was positive for virus on day 56 and in visceral (kidney, liver) organs on day 7, 28, and 56. Since the duodenum and proventriculus as well as kidney and liver are common target organs for tumor formation in quail, this represents a normal dissemination pattern for MD infections in quail. No virus was detected in feather follicle epithelium.

DISCUSSION

The sites and timing of positive virus identification for NAHV was consistent with the published literature for Marek's viruses in chickens and was not different than reports for commercially available vaccine strains. The NAHV replicated in the spleen, bursa, and feather follicle epithelium following ubcutaneous injection. The absence of NAHV virus in tracheal and cloacal swabs suggests that there was no shedding by either oral or fecal route. The absence of shedding by the NAHV vaccine to sentinel chickens indicates that the virus presents a low risk for environmental spread or for genetic modification of the virus through serial passage. In turkeys, the NAHV reached a detectable limit in the spleen by day 14 post-inoculation and remained positive at 21 and 28 days. Detection by 14 days post-inoculation is later than expected based on the published pathogenesis of HVT in turkeys. The feather follicle epithelium became positive one week after the spleens (day 21) which is the normal pattern described for Marek's viruses in the published literature. The absence of virus in litter samples in the turkey study suggests that shedding

from the FFE to the litter was minimal and supports environmental safety of the vaccine.

In quail, the dissemination pattern of the NAHV virus was consistent with published references on the pathogenesis of MD in this species. Over the course of the study the NAHV was detected in the visceral, lymphoid, digestive, and respiratory organ pools of vaccinated quail, but not in the feather follicle epithelium.

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EPIDEMIOLOGY OF INFECTIOUS BURSAL DISEASE IN BOLIVIA

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Infectious bursal disease (IBD) continues to be an important disease throughout Latin America. Different outbreaks for very virulent IBD (vvIBD) have been reported. The immunosuppression resulting from an IBD virus infection could be associated to the many cases of respiratory disease in chickens that affect this region.

In order to gain a better understanding of the disease we employ various diagnostic tools: the bursameter in the field, imaging processing (IP), and the reverse transcriptase/polymerase chain reaction-restriction fragment length polymorphism (RT-PCR/RFLP).

Computer-imaging analysis was used in order to measure bursal integrity. Using this tool we are able to "see" the changes that occur in the bursal tissue as a result of field infections or vaccine take. It is a very sensitive technique that enables us to establish timing, severity, and recovery of the field challenge.

We use Jackwood's PCR method, in which a 743 base pair sequence of the variable region of VP2 is amplified, then reacted with two restriction enzymes BstN1 and Mbo1. With this tool we are able to see what kind of virus we have in the field; to detect all IBDV strains, classify IBDV strains in molecular groups, and detect multiple IBDV strains in a single sample.

This diagnostic tool provides information that will enable us to better understand the different aspects of the IBD situation, such as time of challenge, percentage of bursal damage, and regeneration as changes in the field virus. According to IP most of the field challenge occurs between the third and fourth weeks of age. We have 30 positive results by PCR and have found 17 viruses for Molecular Group 1, seven for Group 3, one for Group 4, two for Group 6, and three that have mixed virus.

These results are not conclusive, and we still need to continue these studies in order to understand the behavior of the virus. It is important to continually monitor flocks for changes in the wild type IBDV populations.

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EXPRESSION OF VP2, CASPASE 3 AND CASPASE 8 GENES IN IBDV INFECTED CHICKS

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SUMMARY

Apoptosis is a type of individual and active cellular death characterized by nuclear and cellular fragmentation into apoptotic bodies (1). In contrast of the necrosis, there is no release of the cellular contents into the interstitium and, consequently, inflammation does not occur in neighboring of the apoptotic cells (2). Little has been published on apoptosis in the lymphoid system of birds. However, it is well accepted that the involution of thymus and bursa of Fabricius are mediated by apoptosis. The infectious bursal disease (IBD) is an acute and highly contagious viral infection of young birds, which affect particularly the B-lymphocytes of the bursa of fabricius (BF). Infectious bursal disease virus (IBDV) cause intense immunosuppression (3, 4). The immunosuppressive effect of the IBDV is related to the morbidity and mortality of birds (6) and susceptibility to other infections (6). Such immunosuppressive effect causes great economic losses for the poultry industry. Immunosuppression, at least in part, is due to increased apoptosis in BF (7, 8). However, the genetic mechanism and the metabolic pathway on how apoptosis is triggered by IBDV are still unknown.

Thirty one-day-old SPF chicks were inoculated with IBDV to evaluate apoptosis. Birds were distributed in five experimental groups of six animals each: Group 1, control - non infected birds euthanized at the beginning of the experiment; Groups 2 to 5, infected birds euthanized at different post inoculation periods: 24 h for Group 2, 48 h for Group 3, 72 h for Group 4, and 96 h for Group 5. Before euthanasia, birds were weighed and evaluated. After decapitation they were submitted to autopsy. Fragments of the BF were harvested for histological processing and for the extraction of RNA. Sections 5 µm thick were stained with hematoxilin and eosin (HE) and TUNEL (Terminal deoxinucleotidil transferase Uracil Nick End Labeling) for morphometrical evaluation of apoptosis, obtaining an apoptotic index. The analysis of the apoptotic indices at the different post inoculation periods (24, 48, 72, and 96 hours) suggested that IBDV induces hypotrophy of the Bursa of Fabricius by the progressive activation of apoptosis.

For the extraction of total RNA, the technique described by Chomczynski & Sacchi was used (5). Two hundred mg of each sample were homogenized in 0.5 mL of solution D (4 M guanidine isothiocyanate of, 25 Mm of sodium citrate pH 7.0, 0.5% sarcosil, 0.1 M 2-mercaptoetanol) and transferred to two microfuge tubes of 1 mL. To the homogenate was added 0.1 mL of sodium acetate 2M pH 4, 0.5 mL of phenol (water-saturated), 0.5 mL of chloroform, isoamilic alcohol (49:1). The suspension was agitated per 10 seconds, cooled in ice per 15 minutes and centrifuged per 20 minutes 10000G/4ºC. The watery phase was transferred to new microfuge tubes. Then 0.5 mL of isopropanol was added and the tubes were stored at -20ºC/1h, followed by centrifugation at 10000G/20 min/4ºC. Pellet of RNA was dissolved in 0.3 mL of solution D, transferred to a microfuge tubes of 1.5 mL and precipitated with one volume of isopropanol at -20º C.

The precipitate was centrifuged at 14000G/4ºC/10 minutes and the pellets of RNA ressuspended in ethanol 75% and centrifuged again. Pellets were vacuum dried 15 minutes and diluted in 100 µL DEPC water (0.5 mL of pirocarbonate dissolved in extreme-pure water - q.s.p. 1 liter - autoclaved solution). Extracted RNA's obtained from the samples of BF were quantified and used for the study of the expression of genes VP2 of the IBDV, caspase 3 and caspase 8 through the reverse transcription and of the technique of real time PCR. Primers were selected based on the analysis of the DNA sequences of caspases 3 and 8 and also of VP2 in the GeneBank, using Blast software (http://www.ncbi.nlm.nih.gov/blast/blast.cgi). The reverse transcription reaction was carried through using 2.0 µg of aliquoted total RNA and with the volume completed with water for PCR FS of 8.0 µL. Then 1.0 µL (1 pmol/µL) of primer reverse VP2, 5.0 µL of oligo

dT (50 pmol/µL) was added, and the mixture was incubated 37°C/10 minutes. After incubation, the samples were placed in two sequentially ice and added of buffer reaction 10x, 2.0 µL of dNTP mix (1.25 mM/µL), 2.0 µL of DTT 0.1M, 2.0 µL (20 U/µL) of reverse transcriptase (RT). The samples were incubated at 41°C/60 minutes, placed in ice, and later stored at -20ºC.

The PCR was based on the method described by Mullis & Faloona (9). Amplicons were dissolved in 10x buffer (20% of Ficoll 400; 0.1 M Na2EDTA, pH 8; 0.1% of SDS; 0.25% of blue of bromophenol). To obtain a pure and specific PCR fragment of the VP2 for quantification and use as standard in Real Time PCR, 40 µL of amplicon obtained in conventional PCR were purificated in gel of agarose 2%. Electrophoresis was carried out with 85 volts for approximately 90 min. in 1x TBE buffer. With the aid of a blade, the referring band of the amplicon was cutout of the gel. The band was placed in a bag of dialysis of 1.5 x 4.0 cm, with 1.0 mL of 0.5x TBE buffer and submitted the separation of the gel for electroelution during 60 min. at 100 volts. The fragment of the gel was purificated using: isoamílic alcohol (1:1 added, v/v) and the inferior phase was transferred to another microfuge tube. After that, acetate of sodium and isopropanol were added 1:1. The pellet of precipitated DNA was obtained by centrifugation at 14,000 rpm/4ºC/15 minutes. The supernatant was discarded, pellet was washed with 500 µL of 75% etanol, and centrifuged again for five minutes. Pellets were dried out in vacuum and rehydrated in 300 µL of water mili-Q SF. The amount of purificated DNA was quantified in a GeneQuant spectrophotometer. After the purification, a gel of poliacrilamide with 2.0 µL (200 ng) of 50 pb DNA marker to ladder and 15 µL (300 ng for VP2) of the purificated fragment was set for confirmation of the integrity and pureness of the fragment. For the reaction of Real Time PCR a SYBR GREEN PCR kit was used (No. Cat. 4304886; Warrington, UK - Core Reagents of the PE Biosystems). A basic protocol was used keeping the usual concentrations of reagent in a final volume of reaction of 20 µL. In short, the reaction was consisted of: 6.65 µL of water for PCR (SF); 1.5 µL of buffer (10x SYBR Green PCR buffer); 1.2 µL of dNTP mix (200 µM each); 1.5 µL of MgCl2 (25 mM); 3.0 µL of PNA to primer mix (1.5 pmol of PNA1 and 1.5 pmol PNA3) or 3.0 µL of GAPDH to primer mix (sense and antisense – 1.5 pmol each); 0.15 µL (5 U/µL) of enzyme AmpliTaq GoldTM and 1.0 µL of DNA of the reverse transcription reaction. The negative controls for VP2, caspase 3 and caspase 8 were set substituting the samples for the same volume of water in the reaction. The reaction in real time was carried through in device ABI Prism 7000 SDS.

As positive control and for posterior quantification of the other results, a standard amplicon curve of purificated VP2 was constructed from serial dilutions. For confirmation of the size of the other fragments amplified by Real Time PCR a dissociation curve were carried through in ABI Prism 7000 SDS. Also, the fragments amplified later were visualized through electrophoresis in poliacrilamide gel stained with silver nitrate. Results of the real time PCR demonstrated that the IBDV presented a peak of expression 24 hours after its inoculation, together with caspase 3. On the other hand, caspase 8 presented a discrete increase of expression at 24, 48, and 72 hours, decreasing at 96 hours post inoculation. These results suggest that the IBDV induce apoptosis in BF, through the expression of caspases 3 and 8 genes.

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GLOBAL EXPRESSION AND PATHOGENESIS ANALYSES IN AVIAN INFLUENZA VIRUS-INFECTED CHICKENS

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Avian influenza A virus (AIV) is a member of the *Orthomyxoviridae* family and contains a segmented and negative-stranded RNA genome. Highly pathogenic strains of AIV such as the H5N1 virus cause high mortality, while lowly pathogenic strains produce milder or asymptomatic infection in birds. To further understand the pathogenesis and the underlying molecular mechanisms associated with the infections of both high-path and low-path AIV strains, global expression pattern analysis was performed using Affymetrix chicken cDNA microarray chips on RNA prepared from infected lungs. Several classes of proinflammatory genes, activated lymphocyte and macrophage genes, apoptotic genes and stress-induced genes were up- or down-regulated significantly. Interestingly, marked down-regulation of a set of immune response genes were observed in chickens infected with a low-path AIV strain, which may emphasize the importance of immune responses in AIV-induced pathogenicity. How the AIV infection alters the expressions of these genes and what the roles the altered expressions play in the infection and disease course are under further investigation.