PROCEEDINGS OF THE SIXTY-SECOND WESTERN POULTRY DISEASE CONFERENCE

March 25-27, 2013 Sacramento, CA

THE 62nd WESTERN POULTRY DISEASE CONFERENCE DEDICATION

VICTOR MANUEL MIRELES MIRANDA

Dr. Víctor Mireles' partnership with the WPDC started in 1975, when Dr. Arnold Rosenwald (Rosy) decided that the many Spanish-speaking attendants deserved English-Spanish translation. Rosy arranged for the required equipment, for Víctor, and his assistants. This service was provided free of charge to WPDC for many years, sometimes with the assistance of "amateur" translators that gave Víctor brief breaks.

Víctor's career as translator really goes back to the 1968 Olympic Games in Mexico City when he volunteered Italian-Spanish-Italian translation. In 1969 he started as a veterinary student at the Universidad Nacional Autónoma de México. Eventually he worked as a volunteer at the Department of Avian Diseases, and upon graduation, he worked for Mexican divisions of American Cyanamid and Salsbury Laboratories. All this time Víctor offered translation services at conferences that he attended. In 1985 he decided to start his own translation company, serving the needs of the poultry and pharmaceutical industries with translations from and to Spanish, English, Italian, Portuguese, and German, among others. He continued his close association with WPDC until 1998. In 1999 the logistics and costs associated with English-French-Spanish translation in Vancouver, dictated that simultaneous translation would not be offered. From then on only WPDC/ANECA meetings were translated, the last one in Puerto Vallarta, 2008.

Víctor is remembered by his many colleagues and friends first and foremost as a gentleman, excellent translator, interpreter, and professional that served the poultry industry worldwide. But very few knew him for his love of opera, classical music, art in general, and birds. Those who worked with Víctor remember him fondly for his dedication, care, generosity, and ability to multitask: listening to a discussion while typing a translation and asking his secretary to give him the next piece of work.

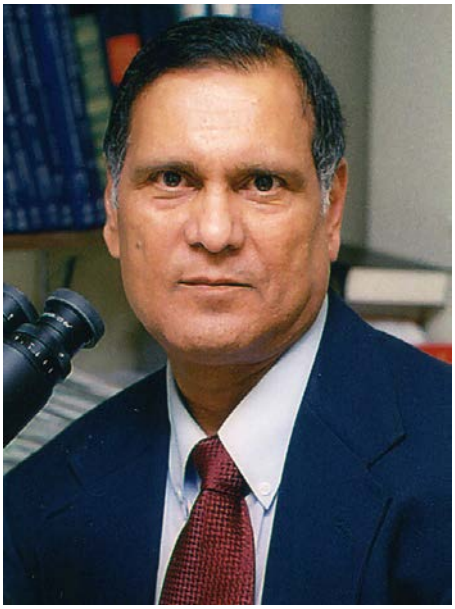
In addition to his services as translator, Víctor made three presentations at WPDC, two on Marek's disease and one on the "use and abuse of shortened forms of expressions and acronyms in poultry science."

A couple of days before his death Víctor sent a message gloating about the good life, having achieved his dream: "I settled in my new home in Acapulco, a glorious place in front of the sea, with the most gorgeous view, and the sound of the ocean. The unheated swimming pool gets sun all day, and is warm by 11 AM, even though it is winter, as if such thing existed here. After a dip, surrounded by jungle, with no more neighbors than raccoons, ant eaters, iguanas and parakeets, one can sip a beer or a tequilita, before attacking some fresh shrimp."

The poultry industry lost Dr. Víctor Mireles, an exceptional translator and interpreter, but his memory will remain in all of those who knew him, worked with him, or received his services.

62ND WPDC SPECIAL RECOGNITION AWARD

A. SINGH DHILLON



Dr. Dhillon was born and raised in India and attained his DVM degree from Punjab University (India). After graduation he was selected from a pool of 2,000 veterinarians to serve as a commissioned officer with the Indian Army for six years. Dr. Dhillon moved to the USA to advance his education. In 1972 he completed his MS, and went on to graduate with a PhD degree from the University of Wisconsin in 1975. Dr. Dhillon then worked for five years as an avian pathologist at the Animal Disease Diagnostic Laboratory in West Lafayette, IN. In 1981, he and his family moved to Washington. He served as a Professor for Washington State University and the Director for the Avian Health and Food Safety Laboratory (WADDL) for 28 years.

During his entire professional career, Dr. Dhillon has worked closely with commercial poultry farmers and other bird owners. He has always demonstrated a keen interest in poultry microbiology and has numerous publications in refereed journals. In 1993 he served as program chair for the WPDC, and the following year became their president. In 1997 Dr. Dhillon took the lead in significantly expanding the role of the laboratory. It changed from being exclusively a poultry diagnostic facility to one which has taken on additional public health service roles. These include microbiologic testing for pathogens and sanitation evaluation for the food-related industries.

Dr. Dhillon is not only an extraordinary poultry specialist and microbiologist, but is also a devoted family man and a dedicated member of his community. He and his wife Premendra (Pam) live in Puyallup, WA. Dr. Dhillon has three children and five grandchildren. He has lived in Puyallup over 30 years, and during this time has served on many public boards, including the Senior Center Advisory Board for the Puyallup School System, becoming a Chair Person in 2006. Dr. Dhillon has also been a member of the Toastmasters International Organization for 30 years, leading several groups and starting two others in the Puyallup area, including the WSU-Cougar club.

Currently, Dr. Dhillon is an emeritus professor for Washington State University and continues to help out with diagnostic work at the Avian Health and Food Safety Laboratory. He volunteers at the local food bank and with the Puyallup River Bank Beautification Project. He is an avid wine maker, and has won several prizes at the local fairs. He and Pam enjoy time together, with their family, and with the community.

62nd WPDC CONTRIBUTORS LIST

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SPECIAL ACKNOWLEDGMENTS

The 62nd 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 once again given substantial financial support. Many companies and organizations, including some that also contribute financially, send speakers at no expense to the Conference. We thank all these people, and acknowledge their support and contribution.

The WPDC is extremely honored to give a special acknowledgement to **Zoetis Global Poultry** and **CEVA Animal Health** who contributed at the Super Sponsors level. In addition, WPDC is pleased to acknowledge our Benefactor contributors, **American Association of Avian Pathologists, Inc.**, and **Merck Animal Health.** Once again, the WPDC is forever grateful to our distinguished Patrons, Donors, Sustaining Members, and Friends of the Conference who are just as important in making the conference a success. All our contributors and supporters are listed on this and previous pages. We greatly appreciate their generosity and sincerely thank them and their representatives for supporting the WPDC.

We thank Dr. Portia Cortes for graciously accepting the position as Program Chair for the 62nd WPDC.

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

We thank Dr. David Frame for editing and producing another outstanding Proceedings of this meeting. Dr. Frame is indebted to Mr. Dana Frame for his meticulous proofreading and formatting of the Proceedings in preparation for publication. Special thanks once again go to Zoetis Global Poultry for sponsoring the flash drives of our proceedings. We again acknowledge and thank *Omnipress* (Madison, WI) for the handling and printing of this year's Proceedings. Once again, we acknowledge Bruce Patrick (Graphic Communications, Brigham Young University) for the cover design of the printed proceedings.

Last but not least, we express our sincere gratitude to all authors who submitted manuscripts. Without their willing participation, there would be no conference.

62nd WESTERN POULTRY DISEASE CONFERENCE OFFICERS

PRESIDENT
Dr. Vern Christensen
PO Box 126
Mona, UT 84645

PROGRAM CHAIR
Dr. Portia Cortes
USDA APHIS VS
10365 Old Placerville Rd, Ste.210
Sacramento, CA 95827

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Dr. David D. Frame
Utah State University
Cooperative Extension
325 West 100 North
Ephraim, UT 84627

SECRETARY-TREASURER
Dr. Richard P. Chin
California Animal Health & Food Safety
Laboratory System - Tulare
18830 Road 112
Tulare, CA 93274-9042
rpchin@ucdavis.edu

62nd WPDC PROCEEDINGS

The Proceedings of the 62nd Western Poultry Disease Conference are <u>not</u> refereed, but are presented as a service and a source of information to those attending the conference and to others who wish to gain some insight as to the information presented. Copies of the Proceedings are available in either book or electronic formats.

Copies of these Proceedings are available from: Dr. R. P. Chin
CAHFS-Tulare
University of California, Davis
18830 Road 112
Tulare, CA 93274-9042
rpchin@ucdavis.edu

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Still available…

Proceedings of the WPDC 5-year compilations (2002–2006; 2007-2011). The electronic versions of the 55th WPDC and the 60th WPDC contain the printed proceedings of the 51st through the 55th, and the 56th through the 60th Western Poultry Disease Conferences, respectively. Copies can be purchased from the WPDC Secretary-Treasurer.

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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 4th Poultry Health Sym. (PHS)	D. C. Young	W. J. Mathey	1st combined WPDC & PHS	1st listing of distinguished members
20th WPDC – 1971 5th PHS	W. J. Mathey	Ramsay Burdett		
21st WPDC – 1972 6th PHS	R. Burdett	Marion Hammarlund		
22nd WPDC – 1973 7th PHS	M. Hammarlund	G. W. Peterson		
23rd WPDC – 1974 8th PHS	G. W. Peterson	Craig Riddell		
24th WPDC – 1975 9th PHS	C. Riddell	Ralph Cooper		
25th WPDC – 1976 10th PHS	R. Cooper	Gabriel Galvan		
26th WPDC – 1977 11th PHS	G. Galvan	Don H. Helfer	Hector Bravo	
27th WPDC – 1978 12 PHS	D. H. Helfer	Art Bickford		
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30th WPDC – 1981 15th PHS	G. Y. Ghazikhanian	Mahesh Kumar		
31st WPDC – 1982 16th PHS	M. Kumar	Robert Schock		
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

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38th WPDC – 1989	B. Kelly	Masakazu Matsumoto		Louise Williams
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41st WPDC – 1992	R. P. Chin	Rocky J. Terry	Marcus Jensen	Henry E. Adler * *(posthumous) R. A. Bankowski C. E. Whiteman
42nd WPDC – 1993	R. J. Terry	A. S. Dhillon	W. W. Sadler	Royal A. Bagley
43rd WPDC – 1994	A. S. Dhillon	Hugo A. Medina		G. B. E. West
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 21st ANECA	D. D. Frame R. Salado C.	Mark Bland G. Tellez I.	Don Zander M. A. Marquez	Pedro Villegas 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 27 ANECA	K. Takeshita J. Carillo V.	Barbara Daft Ernesto P. Soto	Hiram Lasher	Bachoco S.A. de C.V. Productos Toledano S.A.
52nd WPDC – 2003	B. Daft	David H. Willoughby		Roland C. Hartman
53rd WPDC – 2004	D. H. Willoughby	Joan Schrader		G. Yan Ghazikhanian
54th WPDC – 2005	J. Schrader	Stewart J. Ritchie	W.D. Woodward	R. Keith McMillan
55th WPDC – 2006	S. J. Ritchie	Peter R. Woolcock		M. Hammarlund
56th WPDC – 2007	P.R. Woolcock	Bruce Charlton	R. Keith McMillan	M. Matsumoto
57th WPDC – 2008 33rd ANECA	B. Charlton M. A. Rebollo F.	Rocio Crespo Maritza Tamayo S.	A. S. Rosenwald* *(posthumous) A. S. Rosenwald*	B. Daft Ernesto Ávila G.
58th WPDC – 2009	R. Crespo	Victoria Bowes		G.L. Cooper
59th WPDC - 2010	V. Bowes	Nancy Reimers		
60th WPDC - 2011	N. Reimers	Larry Allen		John Robinson
61st WPDC - 2012	L. Allen	Vern Christensen		
62nd WPDC - 2013	V. Christensen	Portia Cortes	Víctor Manuel Mireles M.	A. Singh Dhillon
63rd WPDC – 2014 39th ANECA	P. Cortez Néstor Ledezma M.	Ernesto Soto Priante Ernesto Soto Priante		

MINUTES OF THE 61ST WPDC ANNUAL BUSINESS MEETING

President Allen called the meeting to order on Monday, April 2, 2012, at 5:10 PM, at the DoubleTree Resort by Hilton, Paradise Valley, Scottsdale, AZ. There were 19 people in attendance.

APPROVAL OF 60th WPDC BUSINESS MEETING MINUTES

The minutes from the 60th WPDC business meeting were reviewed and a motion was carried to approve them as printed in the Proceedings of the 61st WPDC.

ANNOUNCEMENTS

Dr. Allen acknowledged all the contributors; in particular, Pfizer Poultry Health Division which contributed at the Super Sponsor level by sponsoring the flash drives of the proceedings. In addition, Dr. Allen acknowledged those at the Benefactor level, which included the American Association of Avian Pathologists and Merck Animal Health. He also thanked all the contributors for their generous donations. The efforts of the current WPDC officers were acknowledged for their work and participation in the organization of this year's meeting. Dr. Allen acknowledged the contributions of Dr. Victor Mireles and Dr. Al DaMassa to the WPDC, both of whom passed away this past year.

REPORT OF THE SECRETARY-TREASURER

Dr. R. Chin presented the Secretary-Treasurer report. As estimated, there was an overall gain for the 60th WPDC due to the generous contributions ($38,370) and lower costs at the Sacramento Holiday Inn. WPDC had a total gain of approximately $26,326. WPDC currently has approximately $77,000 in reserves.

Contributions for the 61st WPDC are once again outstanding. Currently, we have $36,200 in contributions. Unfortunately, at the beginning of the meeting, we only had 153 registrants (last year, we had 199 registrants). This will obviously affect total income dramatically. Unfortunately, Dr. Chin was unable to compile all the number prior to the meeting, though he was expecting a slight loss due to the lower number of registrants.

There were no objections to paying for WPDC officers' expenses if they needed it to attend.

REPORT OF THE PROCEEDINGS EDITOR

Dr. David Frame presented the Proceedings Editor report. There are a total of 63 papers in the proceedings. This is down from previous years. It will cost about $1200 for editing. We printed 400 books and made 400 flash drives. The flash drives were again sponsored by Pfizer Poultry Health Division. There was a brief discussion regarding whether or not to continue printing the proceedings. It was decided to continue as is for 2013, but we will probably not have a book printed for the joint meeting with ANECA in 2014. After which we will decide whether or not to stop printing in book format for good.

NEW BUSINESS

The 62nd WPDC will be in Sacramento, CA, at the Holiday Inn Capitol Plaza, March 24-27, 2013 (this is the week prior to Easter). ACPV will sponsor a workshop on Sunday, March 24, 2013.

Last year, it was agreed to hold the 63rd WPDC with ANECA in Mexico, April 2-5, 2014, at the Sheraton Buganvilias (same location as our previous joint meeting in 2008). Dr. Ernesto Soto agreed to be the program chair for both ANECA and WPDC. He will ask ANECA and WPDC members to form a committee to assist him in the program development. Dr. Allen nominated the following officers for 2012-2013:

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

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

It was voted to hold the 64th WPDC, in 2015, in Sacramento, CA, at the Holiday Inn Capitol Plaza. The location for 65th WPDC (in 2016) was discussed. Following our usual rotation, it was agreed to hold the meeting in Vancouver, BC, Canada. Dr. Chin stated that the Fairmont Hotel was very expensive and he would get proposals from other hotels in Vancouver.

Dr. Allen mentioned that CE credits will be emailed to everyone by the AAAP office. There were no additional items for discussion.

Dr. Allen turned the presidency over to Dr. Vern Christensen who adjourned the meeting at 5:30 PM.

TABLE OF CONTENTS

A CALIFORNIA FARMING FAMILY - LEADING THROUGH CHANGE

Jill Benson, senior vice president of JS West & Companies, shares their egg farming family story and how they continue to move forward in the face of uncertainty and conflict in a politically charged environment in defining the California egg industry's future.

Proposition 2 was passed by California voters in 2008. This vague and poorly written egg layer housing law has a 2015 deadline looming ahead. Voters, lawyers, judges, scientists, regulators, animal rightists, and farmers continue to chime in and voice what they think hen housing should or shouldn't be.

Jill Benson, fourth generation farmer in her family's 104 year old enterprise, will provide background and on-going journey they've taken to create and secure a future for California's egg industry. Learn how one farming family, along with the egg industry are forging ahead with solutions in spite of an uncertain future.

UNUSUAL OUTBREAKS OF INFECTIOUS AND NON-INFECTIOUS NEOPLASIA

G. Zavala and S. Cheng

Department of Population Health, University of Georgia
953 College Station Road, Athens, GA 30602

SUMMARY

A collection of recent outbreaks of neoplastic disease in the United States and selected countries is summarized. Such outbreaks have been studied clinically, microscopically, and using molecular detection methods. Unusual non-infectious neoplastic syndromes have been identified recently in meat type chickens, including myelocytomas, melanomas and lymphomas. Avian leukosis virus subgroup C (ALV-C), an infrequent ALV subtype was detected in bantam chickens. ALV-J continues to circulate in color chickens of Asia and South East Asia. ALV-J, a virus previously thought to have been eradicated from the United Sates, was recently detected in a colony of color chickens produced within the United States. Reticuloendotheliosis virus (REV) appears to circulate in wild birds and is occasionally detected in birds expressing lymphomas. REV has been a significant neoplastic condition of chickens in China in recent years. Marek's disease virus continues to induce outbreaks of neoplastic disease in focal areas of the United States and other countries of the American Continent.

Putative spontaneous non-infectious lymphoid leukosis. One of the most common pathological manifestations of infection with avian leukosis viruses (ALV) is lymphoid leukosis (LL) (4), which generally consists of B cell lymphomas derived from transformed B cells originating from the bursa of Fabricius. The most common ALVs responsible for LL include subgroups A and B (ALV-A and ALV-B, respectively), although other subgroups have been implicated as well (5, 12). LL caused by ALV-A or ALV-B has been eradicated from commercial poultry bred in the Western world (1, 13, 14). However, there have been occasional reports of LL in meat type chickens. Numerous efforts to detect exogenous virus sequences in tumors obtained from young meat type chickens with a diagnosis of LL have failed. Thus, such cases of LL tumors have been tentatively considered spontaneous tumors (18). An unusual feature of such tumors is the young age at which they have been reported (less than eight wk of age); their anatomical location, which tends to involve the area covering the thoracic inlet; and the breeds or breed crosses involved, which have been produced by primary breeding companies where exogenous ALVs have not been detected in years.

Putative spontaneous non-infectious and infectious myelocytomas. Myelocytomas are best known as the primary type of tumor induced by subgroup J ALV (ALV-J) (8, 11, 7, 9, 6, 5, 10), a virus that was first identified in the late 1980s, and first reported in the early 1990s (10). However, myelocytomas as a type of tumor were first reported in the early 20th century, long before ALV-J was ever a problem in meat type chickens. Our laboratory has documented spontaneous myelocytomas in meat type chickens; and also myelocytomas induced by ALVs

other than ALV-J. One recent case related to a multifocal myelocytomas detected in a family of bantam chickens from which ALV-C was isolated and characterized (17). In another case, myeloblastosis associated virus type 1 (MAV-1) was isolated from sarcomas affecting commercial egg layers (16). The MAV-1 virus inoculated into commercial layer embryos induced not sarcomas but myelocytomas instead (16). Thus, more than one virus can cause myelocytomas. In addition, myelocytomas were detected in various flocks of broiler breeders from breed crosses produced by two different primary breeding companies that had not detected exogenous ALV in any of their genetic lines several years. Multiple attempts to detect exogenous viruses in the tumors and blood of affected hens were unsuccessful. In all cases, similar tumors were not detected in the grandparent source flocks or in sister flocks produced by the same grandparent flocks and the genetic lines involved remain free of exogenous ALVs to this date. Thus it was concluded that myelocytomas might develop spontaneously, although it not known what circumstances may lead to the expression of non-infectious myelocytomas.

ALV-J has been eradicated from all fast growing meat type chicken lines selected in the Western world. Myelocytomas, hemangiomas, and other types of tumors associated with ALV-J infection have been reported in slow-growing colored chicken lines in Asia, particularly in China and Taiwan (2, 3). Although ALV-J was thought to be completely eradicated from any and all lines of commercial chickens in the United States, very recently (2012) ALV-J infection was confirmed in various colonies of commercially bred slow-growing colored chickens expressing myelocytomas (19).

Putative non-infectious melanomas. A low incidence of cutaneous and systemic melanomas in processed broiler chickens has been detected, documented, and reported recently (15). The frequency of carcasses with melanomas or melanosarcomas has been estimated to be seven carcasses for every 3.6 million broilers processed (0.0001944%). Although not a highly frequent problem, melanosarcomas could potentially increase in frequency and become a significant challenge for at least one primary breeding company.

Subcutaneous sarcomas. Subcutaneous sarcomas have been reported in recent years (16). Such tumors have been detected in commercial layers of various countries, including the United States, Canada, Mexico, Brazil and Japan. Virus isolation attempts plasma and tumors have rendered myeloblastosis associated viruses (MAV), particularly of subtype 1 (MAV-1), which carries an envelope gene most similar to ALV-A, but with a backbone displaying some diverging properties. MAV-1 isolates can induce myelocytomas (in addition to sarcomas) when inoculated in high titer into susceptible embryos.

SUMMARY

Avian retroviruses are mostly under control and it is clear that primary breeding companies have successfully eradicated all exogenous retroviruses capable of inducing neoplastic disease. However, the threat of recombination and emergence of novel infectious, replication-competent, transmissible avian retroviruses persists and the breeding industry must continue their successful efforts to continuously monitor their genetic lines to avoid unwanted neoplastic disease in their genetic lines and the commercial product derived from them.

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2. Cui, Z., S. Sun, and J. Wang Reduced serologic response to Newcastle disease virus in broiler chickens exposed to a Chinese field strain of subgroup J avian leukosis virus. Avian Dis 50:191-5. 2006.

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A NATURAL OUTBREAK OF LYMPHOMA IN A COMMERCIAL QUAIL FLOCK

R. Crespo[A] , I. Gimeno [B] , S. A. Dhillon [A] , and G. Zavala [C]

[A]Avian Health and Food Safety Laboratory-Washington Animal Disease Diagnostic Laboratory, Washington State University, 2607 West Pioneer, Puyallup, WA 98371

[B]Poultry Health Management, College of Veterinary Medicine, North Carolina State University, 1060 William Moore Drive, Raleigh, NC 27607

[C]Poultry Diagnostic Research Center, College of Veterinary Medicine, University of Georgia, 953 College Station Road Athens, Georgia 30602

SUMMARY

Several reports of naturally occurring lymphomas have been described in quail (2, 4). Experimental studies have demonstrated that quail are susceptible to the three major groups of avian oncogenic viruses (Marek's herpesvirus, reticuloendotheliosis virus, and avian leucosis virus) and the lesions have been well-described (2). Most of these studies have been done on Japanese quail (*Coturnix coturnix japonica*) and a few have used Bobwhite quail (*Colinus virginianus*). It has been demonstrated that these two species have different susceptibility to Marek's disease (3). There are no reports of natural outbreaks or experimental studies of oncogenic conditions in Gambel's quail (*Callipepla gambelii*).

A commercial game fowl farm reported sickness and off balance in 25% of the Gambel's quail (about 150 birds) at around four to eight months old. In the spring of that year (about six months prior), the parental flock had been diagnosed with poxvirus dermatitis. No other quail species or other game fowl on the same premises were affected. The birds were fed a commercial ration that contained 19% protein. The feed was medicated with several antibiotics, including bacitracin, sulfamethoxine, and neomycin. Three birds were submitted for necropsy. All birds had moderately enlarged spleen and dilated ureters with urate deposits. One bird had a couple of pale foci on the liver. No bacteria were isolated from the livers or spleens. No *Salmonella* was isolated from spleens or intestines. Histologically there was moderate to severe, multifocal infiltration of lymphocytes and lymphoblasts in the heart, peripheral nerves, liver, and kidney. There was also less severe infiltration of lymphocytic neoplastic

cells in the spleen, muscle of the gizzard, lung, and around the blood vessels in the brain.

Blood samples from live Gambel's quail from the same group and other quail species on the farm were collected on FTA cards and tested by real time PCR (1) for Marek's herpesvirus (MDHV). All samples were negative for MDHV. In addition, multiple paraffin-embedded tissues, from the birds submitted for necropsy, were tested for MDHV (serotype 1, Meq oncogene, pp38 gene, and Vil8 gene), avian leucosis virus (polymerase and envelope genes), reticuloendotheliosis virus (Gag gene), and lymphoproliferative disease virus of turkeys by polymerase chain reaction. All samples were negative for the presence of these genes. Because of the poor performance of the flock, the entire Gamble's quail flock was euthanized. Based on the epidemiology of this outbreak, viral etiology is still suspected, however the causative agent of the lymphoma has not been found.

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FEMORAL HEAD NECROSIS IN BROILER CHICKENS

H. L. Shivaprasad

California Animal Health and Food Safety Laboratory System - Tulare Branch, University of California, Davis, Tulare, CA 93274

SUMMARY

Leg problems are a common occurrence in broiler chickens and cause significant economic losses to the industry. There are many such conditions and one of them is femoral head necrosis (FHN), also called femoral head degeneration, brittle bone disease, bacterial chondronecrosis with osteomyelitis, femoral head separation, etc. The incidence of FHN in a flock can range from sporadic occurrence to 0.75 % or more resulting in lameness, decreased weight gain, increased morbidity, mortality, and hemorrhages due to fractures resulting in downgrading of carcasses at the processing plant. Gross lesions of FHN include discoloration and brittleness sometimes with swelling of the femoral head, condyles popping off or dislocating easily, fractures, etc. Histopathology of FHN includes osteochondrosis, chondronecrosis, disorganization of trabeculae, increased osteoclasts, periosteal and sub-periosteal thickening due to fibrosis, osteonecrosis, etc. The cause of FHN is not known; trauma, nutrition, metabolic, genetics, infection (bacteria, virus), management (heavy birds, wire floor model), and others have been proposed.

Six cases of FHN in broiler chickens with locomotor problems submitted to the laboratory were examined. These were white chickens and their ages ranged from 23 d to 52 d. The older and heavier the chickens were, the more severe the clinical signs and lesions. Clinical signs included leg weakness, down on legs – one or occasionally both legs stretched outwards, etc., and increased morbidity and mortality in the flock. The incidence of the clinical signs ranged from 0.5 to 2% in a flock. Gross lesions ranged from mild separation of condyles of the proximal femur either unilateral or bilateral, to severe and easy separation of condyles leaving a discolored and irregular subchondral bone. In severe cases, there was brownish discoloration of the bone and easy separation of the metaphysis from the epiphysis. In extreme cases upon cutting the bone, there was severe brittleness and fractures or shattering of the proximal bone dividing it in to two or more longitudinal splits. Microscopically lesions ranged from decrease in the number and degeneration of chondrocytes in the epiphysis in milder cases to necrosis of chondrocytes, increased osteoclastic activity and fibrosis in severe and chronic cases. There was no evidence of bacteria in any of the cases examined. It is concluded that FHN is a

pathological condition of unknown etiology. FHN is probably due to a metabolic condition and the term "Femoral Head Degeneration" is proposed.

ISOLATION AND PATHOLOGICAL AND MOLECULAR CHARACTERIZATION OF CHICKEN PARVOVIRUS FROM BROILER CHICKENS WITH ENTERIC DISTURBANCE IN BRAZIL

L. Núñez[A] , F. Naranjo, S. Santander-Parra[A] , H. Mettifogo[A] , E. Astolfi-Ferreira[A] , C. Serrano, A. Ferreira[A] , and J. Piantino

[A] Avian Pathology Laboratory, Department of Pathology, College of Veterinary Medicine (FMVZ), University of São Paulo (USP), Av. Orlando M. Paiva, 87, 05508-270, São Paulo, SP, Brazil

SUMMARY

Enteric diseases in birds are important due to the losses caused by failure in the bird's performance. In this paper, the isolation of chicken parvovirus from specific pathogen-free (SPF) chicken embryos and its molecular and pathological features are described. Fecal samples and intestinal fragments from chickens with enteric disease that were identified as positive for ChPV by PCR and DNA sequencing were used. These samples were inoculated into seven d old SPF chicken embryonated eggs through the yolk sac. The embryos showed dwarfism, hemorrhage and edema but were non-enrolled. Viral isolation was confirmed through PCR amplification of the NS gene. The samples were also tested for fowl adenovirus group 1, rotavirus, reovirus, chicken astrovirus, avian nephrites virus, and infectious bronchitis virus. The result confirmed the isolation of one (1) pure sample of ChPV. The amplified product of 561 bp was purified and sequenced. The comparison of nucleotides with other sequences showed high similarity with sequences identified in the United States (94.54%) and Europe (90 – 93.65%); however, there was low similarity with Brazilian sequences (77.78 – 79.37%). In the phylogenetic analyses, the isolate aligned in a unique group with sequences from the United States and Europe, separate from the Brazilian sequences. The present study showed the first isolation of ChPV in Brazil, the pathologic features of the virus in embryos and its genetic characteristics, suggesting that there are different molecular patterns of ChPV in Brazil.

Enteric diseases are an important problem in poultry around the world. Enteric diseases are characterized by diarrhea, weight loss, and increased mortality. The most well-known enteric diseases in poultry are poult enteritis complex (PEC) and runting and stunting syndrome (RSS) in chickens (1). Many infectious agents, such as bacteria, fungi and viruses, are associated with enteric diseases and syndromes (2). Viruses, such as astrovirus, coronavirus, rotavirus and reovirus, have been commonly identified in sick birds through molecular techniques (3,4); however, since 2009, PCR has been developed to detect chicken parvovirus (ChPV) (5). ChPV is a small and non-enveloped virus with a straight chain of DNA. Its genome contains anon-structural NS gene and a VP2 gene that encodes a structural protein. The NS gene is used in viral detection with PCR. The relation of this virus to the pathogenesis of enteric diseases is not well understood (6), but it has been investigated in studies focused on determining the pathogenicity of enteric viruses. The isolation of ChPV is difficult, and few strains have been isolated around the world. The aim of the present study was to isolate chicken parvovirus and characterize it at the molecular and pathological level.

MATERIALS AND METHODS

In the present work, we used chicken embryonated eggs (SPF) and samples of feces or intestines from chickens. These samples were previously identified as being positive for ChPV by PCR and DNA sequencing. Inoculation was carried out using a suspension of the samples used in the molecular detection. The eggs were hatched and inoculated at seven d old using the yolk sac route. The embryos that died after 24 h were stored at 4 C° until necropsy. The eggs that survived until the fifth d of incubation were euthanized. In the necropsy, the embryos and the support membranes were analyzed for lesion development, and embryos and yolks were selected for making the inocula for the next passages. Viral confirmation was carried out by PCR (ChPV and FAdV-1) and RT-PCR (astrovirus, avian nephritis virus, coronavirus, rotavirus, and reovirus). Embryos that were positive with PCR were considered to be samples with the isolated virus. DNA and RNA were extracted using phenol. The amplified fragments were purified and sequenced. The sequences were analyzed

in GenBank, and phylogenetic analysis was performed using neighbor joining.

RESULTS

Viral isolation was confirmed with PCR and RT-PCR. All samples were tested for chicken astrovirus, chicken parvovirus, avian nephritis virus, infectious bronchitis virus, fowl adenovirus group 1, avian rotavirus, and avian reovirus. Chicken parvovirus was isolated and propagated in seven d old chicken embryonated eggs. The embryos showed significant alterations after one passage. After euthanasia, the embryos were examined for lesions and macroscopic alterations, such as deformities, dwarfism and enrollment, and the presence of hemorrhage, edema, and the gelatin characteristic. The macroscopic lesions were qualitatively evaluated by determining the presence (positive) or absence (negative) of injuries.

Chicken parvovirus (ChPV). The embryos showed hemorrhage, edema and dwarfism but not enrollment. The embryo mortality rate was 66.7%, and mortality occurred between 48 to 72 h after inoculation. One sample of chicken parvovirus isolated in chicken embryonated eggs was sequenced (accession number JX861894). The analysis of nucleotide similarity showed that the ChPV isolate shared 94.53% similarity with a sequence from the United States but less similarity with sequences from Canada (76.84 to 88.36%). The isolate shared 90-93.65% similarity with three sequences from Europe (Poland and Hungary). The ChPV isolate showed less similarity with Brazilian sequences (77.78 to 79.37%). The phylogenetic analysis showed that the ChPV isolate was related to the isolates of the United States, Poland and Hungary, and the Brazilian isolates (USP) were distantly related.

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DEVELOPMENT OF SUSTAINABLE STRATEGIES TO MITIGATE THE USE OF ANTIBIOTICS AND MAINTAIN GUT HOMEOSTASIS IN INTESTINAL DYSBIOSIS

H. Lillehoj

Animal Parasitic Diseases Laboratory, Animal and Natural Resources Institute, USDA-Agricultural Research Service, Beltsville, MD 20705, USA

ABSTRACT

Confronting the increased demand for poultry food products are emerging field diseases, increasing regulatory bans of antimicrobial growth promoters, high-density growth conditions, and waste management. Although biotechnology offers solutions to some of these challenges, more basic studies are needed to better understand the complex interaction between the intestinal microbiome, host immunity, and the environment that affect the outcome of field diseases. In coccidiosis, host-pathogen interaction leading to protection is complex, involving many aspects of innate and adaptive immunity to intracellular parasites.

This talk will focus on emerging strategies to enhance gut immunity and to decrease economic losses due to poultry enteric diseases with special emphasis on recent developments in coccidiosis research on host immunity and immunomodulation strategies to enhance gut immunity to *Eimeria*. Such information will magnify our understanding of host-parasite biology,

mucosal immunology, and design of future immune modulation and disease intervention strategies against avian coccidiosis.

INTRODUCTION

Poultry sectors including industry, government, and academia are confronted with a new array of challenges, such as global food security, climate change, emerging infectious diseases, regulatory ban of antimicrobials, high-intensity production conditions, and waste management. With increasing availability of biotechnological research tools, agricultural animal scientists need to consider addressing these challenges using modern research tools to develop sustainable agricultural management systems which will be compatible with environmental and consumers' needs. One of the major production challenges for the global animal industry is the increasing regulatory restrictions on the use of antibiotic growth promoters (AGPs) in animal production (5). Many AGPs have already been restricted by animal farms in the European Union (EU) and some parts of Asia. Soon other countries are expected to be under increasing scrutiny as consumers' concerns about drug resistant superbugs increase. Accordingly, scientific evidence-based publications are supporting the possibility of sustaining intensive modern farming without the use of AGPs, especially in the area of disease control (9). It is now the responsibility of agricultural scientists to convince the poultry industry the relevance of these new scientific findings so that practical and effective alternative strategies can be developed which will mitigate the use of antibiotics.

IMMUNOLOGY AND COCCIDIOSIS CONTROL

Since the 1990s, host-parasite studies in coccidiosis have been focused on identifying and characterizing the chicken immune effector molecules involved in coccidial infection (6). For example, identification of the subpopulations of intestinal lymphocytes which respond to infection in the gut led to the notion that locally produced-cytokines and chemokines regulate the quality of host immune response to *Eimeria*. It became clear that in coccidia-immune hosts, parasites entered the gut early after infection but were prevented from further development, indicating that acquired immunity to coccidiosis most likely involved mechanisms that stopped the natural progression of parasite development.

In the last decade, the application of high-throughput sequencing of intestinal-expressed sequence tag (EST) cDNA libraries from *Eimeria*-infected chickens (11) quickly led to the identification of many further cytokines, such as interleukin (IL)-16 and IL-17, which were elevated in *Eimeria*-infected tissues indicating their potential role in regulating local immune responses to coccidia. Moreover, recent studies with quantitative real-time PCR techniques have identified more than 30 different cytokines and chemokines involved in coccidia infection to further illustrate the complexity of the host immune response to *Eimeria* (1).

In late 2000, the availability of a complete sequence for the poultry genome, along with various tissue-specific chicken microarrays, has recently led to large-scale functional genomic analyses of chickens. Gene expression analyses using microarrays have become powerful tools to evaluate the complexity of host-pathogen immunobiology and most specifically, genomics technologies combined with immunology (immunogenomics) have enabled in-depth analyses of complex immunological processes based on large-scale genomic approaches (3, 10). Future gene expression analyses will lead to the comprehensive identification of immune-related transcripts and their immune pathways which are modulated during infection, together with other important genes related to cellular metabolisms which are perturbed during coccidiosis.

IMMUNE MODULATION STRATEGIES

Some examples of immune modulation strategies which we have been developing to increase host protective immunity to coccidiosis and to mitigate the use of antibiotics in poultry production include molecular vaccine (2, 7), probiotics (4), passive immunization using hyperimmune IgY antibodies, and dietary immune modulation using plant-derived phytonutrients (8). One of the initial steps triggering innate immune response involves germ-line encoded, highly conserved innate immune sensing molecules of PRRs which include TLRs, nucleotide-binding oligomerization domain proteins (NODs), retinoid-inducible gene 1 (RIG-1) and C-lectin binding receptors. *Eimeria* parasites, the causative pathogens of coccidiosis, contain several components which are stimulatory for immune cells, and they activate innate immunity and inflammatory response. In 2005, our laboratory (7) showed that a conserved antigen of sporozoites of *Eimeria*, profilin, is a parasite PAMP which stimulates T-lymphocytes and induces IFN-γ production. Recently, a significant effect of the oil-based ISA 71 VG, or aqueous nanoparticle-based Montanide IMS 1313 N VG (IMS 1313) adjuvant was demonstrated in combination with the recombinant *Eimeria* profilin subunit antigen in broiler chickens (2). These latest studies have opened other doors for the development of recombinant vaccines against coccidiosis and illustrate the importance of elucidating the underlying molecular mechanism of vaccination.

Commensal bacteria on the intestinal mucosa contain many probiotics ligands (such as long surface appendages, polysaccharides and lipoteichoic acids) which can communicate with PRRs inducing downstream signaling pathways that lead eventually to probiotic (health-promoting) effects. Direct-fed microbials (DFM) and their associated ligands that can modulate host innate immune response (4). We have recently evaluated several field isolates of *B. subtilis* strains by continuous feeding of young broiler chickens with the spore-supplemented standard poultry diet to investigate the probiotic effects of *Bacillus* strains. Depending on the *B. subtilis* strain, feeding diets supplemented with *B. subtilis* spores increased various intestinal intraepithelial T cell subpopulations, cytokine mRNA levels, and macrophage function. Following an *E. maxima* challenge infection, DFM-fed chickens showed enhanced disease resistance with higher body weight gain and decreased intestinal lesions compared with uninfected control birds. Detailed immune pathways that were affected by *Bacillus* treatment were further examined using a high-throughput gene expression analysis. Various immune-related genes, especially ones associated with inflammatory response, were up-regulated in the gut of probiotic-treated chickens.

One promising new avenue to develop a drug-free disease control strategy is the use of natural foods and herbal products to reduce inflammatory effects of infections and to enhance host defense against microbial infections and tumors. Recent studies from our laboratory provided clear evidence that dietary supplements of natural phytochemicals activate innate immunity in poultry and, in particular, enhance protective immune responses against avian coccidiosis (8, 9). In several *in vivo* trials, feeding of broiler chickens with diets supplemented with extracts of mushroom, safflower, plum, and cinnamaldehyde consistently enhanced innate immunity and provided enhanced protection against live oral parasite challenge infections.

CONCLUSIONS

Chickens will continue to provide a major and increasing supply of the world's animal protein. It is hard to imagine disease control in the field without the use of anticoccidial drugs, but it is probable that the current methods of control will continue unabated. There are, however, increasingly negative political views towards in-feed medication of livestock (especially within Europe) and an overall increasing negative view on the use of prophylactic chemotherapy provides a significant spur for work on the immunological control of avian coccidiosis. Application of the recently described innovative technology in immunomodulation and vaccination may lead to the development of alternatives to prophylactic medication. With rapidly developing technologies in functional genomics and computational biology, it is anticipated that new paradigms for coccidiosis control will be formulated. It is possible that the use of genetic tools could become one way of combating parasites, in synergy with other strategies of coccidiosis control such as vaccination, nutrition, and management.

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THE ROLE OF NUTRITION IN MAINTAINING GUT HEALTH IN THE PRESENCE OF COCCIDIAL CYCLING

M. Quiroz, J. J. Dibner, F. Yan, and C. D. Knight

Novus International, Inc. 20 Research Park Drive, St. Charles, MO 63304
marco.quiroz@novusint.com

SUMMARY

The move toward drug-free poultry feeds has increased use of coccidial vaccines; however, the curtailing of feed and therapeutic antibiotic use worldwide has increased enteric diseases, including necrotic enteritis (NE). Necrotic enteritis, caused by *Clostridium perfringens* (Cp) is one of the most economically important enteric diseases of broilers. Coccidial vaccination may predispose birds to NE. The purpose of this presentation is to place necrotic enteritis within the context of digestive health and microbial ecology by testing the hypothesis that coccidial vaccination alone does not lead to Cp overgrowth. The studies were designed to test whether diet and dietary additives also play a role in Cp overgrowth. To study the role of diet in the development of dysbacteriosis, a model has been developed that does not include a Cp challenge. Rather, the model uses a high viscosity diet that has been associated with Cp dysbacteriosis. Using this model, a series of experiments was conducted to study factors that can contribute to or mitigate the effects of subclinical enteritis. Results indicated that diet played a significant role in Cp growth while coccidiosis challenge had no significant effect. This model is being used to test novel feed additives. Determining the dietary and enteric conditions that precede clinical NE are essential in the development of dietary and feed solutions for sustainable drug free agriculture. The purpose of the research described here is to examine the relationship between coccidial cycling and dysbacteriosis involving *C. perfringens* in broiler chickens with the goal of identifying nutrition guidelines and feed additives that reduce the incidence of Cp overgrowth in the distal ileum of broiler chicks.

EFFECT OF FEEDING GRAINS NATURALLY CONTAMINATED WITH *FUSARIUM* MYCOTOXINS ON PERFORMANCE AND METABOLISM OF BROILERS AND TURKEYS

T. K. Smith[A] and A.E. Sefton[B]

[A] Department of Animal and Poultry Science,
University of Guelph, Guelph, Ontario, Canada, N1G 4K3
[B] Alltech Canada, Inc., 20 Cutten Place, Guelph, Ontario, Canada, N1G 4Z7

SUMMARY

Studies were conducted to determine the effect of feeding diets naturally-contaminated with *Fusarium* mycotoxins to broilers and turkeys. Birds were fed diets containing a blend of naturally contaminated corn and wheat throughout the production cycle. The major contaminant was deoxynivalenol (DON, vomitoxin) with lesser amounts of 15-acetyl DON, zearalenone, and fusaric acid. The feeding of contaminated diets to broilers reduced growth rates and feed consumption only during the grower phase. Birds grew and fed normally in the starter phase. There was similarly no significant effect of diet on growth of turkeys in the

starter phase although birds fed the contaminated diets gained significantly less weight than controls in the grower and developer phases. It was concluded that physiological changes in the starter phase caused by the feeding of contaminated diets, including morphological changes in the small intestine, do not immediately result in reduced growth rates. It can be concluded that diets contaminated with *Fusarium* mycotoxins should be fed to poultry only with caution.

INTRODUCTION

There are four different major modes of action of feed-borne *Fusarium* mycotoxins in poultry. Numerous *Fusarium* mycotoxins are pharmacologically active. This can result in behavioral changes arising from altered brain neurochemistry. One commonly observed behavior following the consumption of contaminated feed is feed avoidance behavior. This is actually a natural protective behavior as it will minimize mycotoxin-induced tissue damage. Many mycotoxins also inhibit cellular protein synthesis. This contributes to lesions of the gastrointestinal tract and liver which reduce the efficiency of nutrient absorption and metabolism. Probably the most economically-significant consequence of feed-borne mycotoxins in poultry is immunosuppression. This increases the frequency of secondary mycotoxic diseases. Reproductive efficiency of poultry can be reduced by feed-borne mycotoxins particularly due to adverse effects on egg and eggshell formation.

Poultry have a lesser feed refusal reaction to contaminated feedstuffs than animals such as swine and this has sometimes lead to complacency amongst poultry producers regarding the feeding of contaminated diets. Experiments were conducted, therefore, to determine if there is a lag time between mycotoxin-induced metabolic damage and reduced growth rates due to reduced feed consumption in broilers and turkeys.

MATERIALS AND METHODS

Experiment 1. A total of 360 one day old male broiler chicks were randomly distributed to 12 floor pens with one of four experimental diets fed to birds in each pen for eight wk, which included a starter period (three wk), grower period (three wk), and finisher period (two wk) (1). The diets included: (i) control (ii) low level of contaminated grains (iii) high level of contaminated grains and (iv) high level of contaminated grains + 0.2% glucomannan-based mycotoxin adsorbent (GMA, Integral, Alltech Inc., Nicholasville, KY). The control diet was corn and wheat-based,and contaminated diets were formulated by substituting control corn and corn with contaminated grains. Weight gain, feed intake, and blood chemistry were monitored over the experimental period.

Experiment 2. A total of 300 one day old male turkey poults were randomly distributed into floor pens in groups of 20 (2). Five pens were randomly allotted to each of three diets with 100 poults per diet for three wk starter, grower, developer, and finisher periods. The experimental diets included: (i) control (ii) contaminated and (iii) contaminated + 0.2% GMA. The control diet was again corn and wheat-based and the contaminated diets were again formulated by substituting control corn and wheat with contaminated grains. Weight gain, blood chemistry, and small intestinal morphology were monitored after each feeding period.

RESULTS

Experment 1. The most significant mycotoxin contaminant detected in diets was deoxynivalenol (DON, vomitoxin). Approximate concentrations of DON were 0.5 ug/g in the control diet, 6.0 ug/g in the diet with the low level of contamination, and 10.0 ug/g in diets with the high level of contamination. Co-contaminants included zearalenone, 15-acetyl DON and fusaric acid. The feeding of increasing levels of contaminated grains had no effect on growth rate or feed consumption in the starter phase ($P>0.05$). In the grower phase, however, there was a linear decline in both growth rates and feed consumption as the level of contamination increased ($P<0.05$). These effects were prevented by the feeding of GMA.

Experiment 2. Lower concentrations of mycotoxins were fed to turkeys compared to broilers. Approximate concentrations of DON were 1.0 ug/g in the control diet and 2.8 ug/g in the contaminated diets. Co-contaminants again included zearalenone, 15-acetyl DON and fusaric acid. The feeding of contaminated grains had no effect on poult growth rate in the starter phase ($P>0.05$) but growth was reduced compared to controls in both the grower and developer phases ($P<0.05$). Reduced growth rates were prevented by the feeding of GMA. The feeding of contaminated grains reduced duodenal villus height compared to controls at the end of the starter period ($P<0.05$) (3). Jejunal villus height and apparent villus surface area were also reduced ($P<0.05$). At the end of the grower phase, duodenal apparent villus surface area and jejunal villus height and apparent villus surface area were also reduced compared to controls when contaminated grains were fed ($P<0.05$). Most of these changes were again prevented by the feeding of GMA. No effects of diet were seen on morphology of the ileum or in any of

the intestinal segments at the end of the developer and finisher phases.

DISCUSSION

Poultry are considered to be less sensitive than species such as swine or dogs to feed-borne *Fusarium* mycotoxins because of a lesser degree of feed avoidance behavior in young birds. It would appear from the current studies, however, that mycotoxin-induced metabolic changes slowly occur when contaminated diets are consumed by poultry resulting in reduced growth rates only in grower periods. An example of the metabolic changes is the morphological changes seen in the small intestine of turkeys in the current study. These changes were most obvious in poults and were not seen in more mature turkeys. It is also clear that broilers have a higher tolerance for diets naturally-contaminated with *Fusarium* mycotoxins. Mycotoxin-induced changes in duodenal morphology were also seen in broiler breeder pullets fed similar diets although these birds appeared to adapt to the mycotoxin challenge in the jejunum (4). It would appear that the use of a suitable mycotoxin adsorbent can be helpful in preventing many adverse effects of contaminated diets. It can be concluded that diets naturally-contaminated with *Fusarium* mycotoxins should be fed to broilers and turkeys only with caution.

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WHAT DO WE TRULY KNOW ABOUT COCCIDIOSIS?

S. Fitz-Coy

Merck Animal Health
Salisbury, MD 21801

SUMMARY

The ubiquitous coccidian parasites invade the intestines and ceca of the host. Birds of all ages are susceptible to one or more species of coccidia, unless fully immune. It has been accepted that *Eimeria* parasites have predictable behaviors or patterns. However, more recently uncharacteristic patterns have been observed such as the morphology appears to be within a species but biological behaviors are different. These organisms are classified as cryptic species. Also, organisms within a species are described but are variants within the species. The pathology and pathogenicity are general characteristic within the species; *Eimeria praecox* is relatively benign, although relatively fecund. However the most pathogenic species, *E. necatrix,* has a relatively poor fecundity. The coccidia population within the house environment is influenced by the fecundity of the species, house management practices, and the methods of coccidia control. The most pathogenic species, *E. necatrix,* is the least prevalent whereas *E. acervulina* and *E. maxima* are prevalent. Several species of coccidian, such as *E. maxima* and *E. mivati,* parasitize the entire small intestine of the chicken. On some occasions parasites were observed within epithelial cells of the gizzards and proventriculi.

INTRODUCTION

Coccidiosis is an enteric disease of poultry of high economic importance. This disease is controlled or prevented using anticoccidial drugs or vaccines. The major cost for the prevention and control is minimal when compared to the lost due to impaired performance. For years the anticoccidial drugs were highly effective against the *Eimeria* species, but more recently there is evidence of erosion in drug efficacy. These products are used extensively in shuttle and or rotational programs and might have contributed to the

erosion in drug efficacy. The concept of using a live coccidia vaccine for the control of coccidiosis was introduced in 1950's. The acceptance was fair, but the vaccine found a place in the breeder industry. There has been a renewed interest in vaccine usage for the control of coccidiosis. Due to this interest, many vaccines are becoming available globally. Currently there are in excess of 20 vaccines; both the live and killed products are available.

Sites for parasites develop. The gizzard and digestive juices aid in the rupturing of the oocysts to release the sporocysts and sporozoites. The released sporozoites enter enterocytes and begin asexual and sexual cycles within parasitophorous vacuoles. Following numerous asexual and a sexual replication, non-infective oocysts are shed in feces that become infective following sporulation. These parasites were once believed to only develop in enteric cells; however, in severe parasitism several endogenous stages, meronts, gamonts and oocysts were seen in non-intestinal epithelial cells. Parasites were found in the cells of the proventriculi and gizzards. A study demonstrated that *E. tenella* could develop in chicken embryos (3). The parasites of *E. maxima* were found in samples taken from the gizzard-duodenal junction to the ileo-cecal junction. Therefore only preparing a wet mount smear from the mid-gut region could cause the *E. maxima* parasites to be missed. *E. tenella* develops predominantly in the ceca but occasionally some isolates were found developing in the lower ileum.

Pathology and pathogenicity. The pathogenicity and pathology of coccidia is species and strain dependent. Also, the size and numbers of late generation meronts or size of gamonts impact the severity of the pathology. Those species that develop deep within the host cell and/or within the lamina propria cause severe pathological changes. *E. necatrix* is the most pathogenic of the chicken coccidia. The large meronts develop in the sub-epithelial tissue of the small intestines therefore upon maturation destroy the vascular bed and cause severe trauma to the affected area. *E. praecox* is the least pathogenic; the endogenous stages of this species develop primarily above the host cell nucleus and just beneath the brush border. Parasite fecundity may play a role in the pathology and pathogenicity; *E. mivati*, a very fecund organism, produce large numbers of small meronts and gamonts that are less than 20 microns. The extremely great numbers in parasites within the cells cause enormous tissue damage. Laboratory altered strains such as the precocious and attenuated organisms are less pathogenic than the parent strains. The reduced pathogenicity and pathology is reflective of the lower number of asexual stages. Innate host susceptibility plays a role in the pathology and pathogenicity; some commercial strains of broilers are more tolerant to coccidia that other strains (1).

CONTROL

After many years of excellent drug efficacy, there is considerable evidence on the decline in drug efficacy. Reports on vaccinating birds with drug sensitive strains of coccidia have shown to restore anticoccidial efficacy. A study demonstrated that there are benefits for using Coccivac-B® in a rotational program with Clinacox™ (diclazuril)(4). More recently, the use of a live coccidia vaccine concomitantly with an ionophore has shown promise. It is believed that the ionophore aid in the transition from a non-vaccine program to a vaccine program with a lowered occurrence of necrotic enteritis.

Variant and cryptic coccidia. All species of *Eimeria* are antigenic; however, there are reports of poor protection or variability in cross protection among strains of *E. maxima* (4). There are commercial vaccines with multiple *E. maxima* antigens to enhance the antigen capabilities of that species. Also, there are reports about coccidia within a species that behave uniquely different from other strains within that species. But are these organisms one and the same species or are other species involved? The recent data on the genetic characterization of coccidia from chickens have added information that might have revealed more taxonomic confusions about the species of chicken *Eimeria*. How prevalent and pathogenic are these cryptic organisms?

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PEN STUDY COMPARING THE PERFORMANCE OF COCCIDIA VACCINATED BROILERS TO THOSE ALSO SUPPLEMENTED WITH SALINOMYCIN OR LASALOCID

K. Cookson[A], G. Mathis[B], J. Schaeffer[A], J. Dickson[A], M. LaVorgna[A] , and D. Waldrip[A]

[A]Pfizer Animal Health—Global Poultry, Durham, NC
[B]Southern Poultry Research, Inc., Athens, GA

INTRODUCTION

Coccidia vaccines and ionophores have each traditionally been used to control coccidiosis in broilers. More recently, producers have tried combinations of the two strategies—especially during the first cycle on vaccine—to "smooth out" the transition to having no anticoccidial treatments in the starter feeds. Our objective was to measure the effect of lasalocid (Avatec®) and salinomycin (Biocox™) on immunity development and performance in Inovocox® EM1 vaccinated broilers.

MATERIALS AND METHODS

Two thousand four hundred fifty Ross 708 males were vaccinated *in ovo* with Inovocox EM1 and placed into floor pens on used litter. Groups of 350 birds were assigned 50 per pen in a random block design to one of seven treatments: 1) No anticoccidial, 2) lasalocid (LAS) in grower, 3) salinomycin (SAL) in grower, 4) LAS in finisher (F), 5) SAL in the finisher, 6) LAS in the grower and finisher (G/F) and 7) SAL in the grower and finisher (G/F). Grower and finisher diets were fed at 18-35 and 35-50 d, respectively. LAS and SAL were included at 68 and 40 g/ton, respectively. All diets contained 50 g/ton BMD®. Eighty non-vaccinated controls (same flock source) were raised in Petersime battery cages until challenge. At 36 d, five birds from each pen and 35 controls were orally gavaged with 50,000 *E. acervulina*, 10,000 *E. maxima*, and 50,000 *E. tenella* oocysts. At 42 d, all challenged birds were necropsied and lesion scores were assessed using the Johnson-Reid method. Birds and feed were weighed by pen at 18, 35, 50, and 60 d. Two-sided tests were used to establish statistical significance at a 0.05 level.

RESULTS

Both LAS and SAL supplemented EM-1 groups were significantly lower in coccidiosis lesions after challenge compared to non-vaccinated, non-medicated controls. In addition, when comparing lesion scores caused by the species that has the greatest impact on performance—*E. maxima*—there was no significant difference in any of the EM-1 treatments (Figure 1). Thirty-five d feed conversion ratios (FCR) were significantly lower (by 4-5 points, respectively) in LAS and SAL groups compared to EM-1 controls. At 50 d, the three point FCR improvements in LAS (G/F) and SAL (G/F) groups were not significant (Figure 2). At 60 d LAS (G/F) and SAL (G/F) had a significantly lower FCR (5 to 6 points, respectively) while 2-3 points lower FCR of LAS and SAL only in finisher diets were not found to be significant. Finally, when ionophore supplementation was only in the grower feed, there was no significant difference in performance at 60 d compared to EM-1 controls.

DISCUSSION

Supplementing vaccinated broilers with either lasalocid or salinomycin in the grower diet allowed for the development of coccidia immunity while at the same time improving feed efficiency at 35 d of age. When birds were raised to 60 d, grower-only supplementation showed no benefit. However, keeping these ionophores in the finisher diet (until 50 d) allowed birds to maintain a significant performance advantage. In fact, all improvements noted above were more than enough to pay for the additional outlay in feed costs (analysis not shown), with the grower/finisher treatments yielding the highest return in 60 d old broilers.

Figure 1. Performance of EM-1 treatments +/- ionophore in grower (35 d results) or grower/finisher.

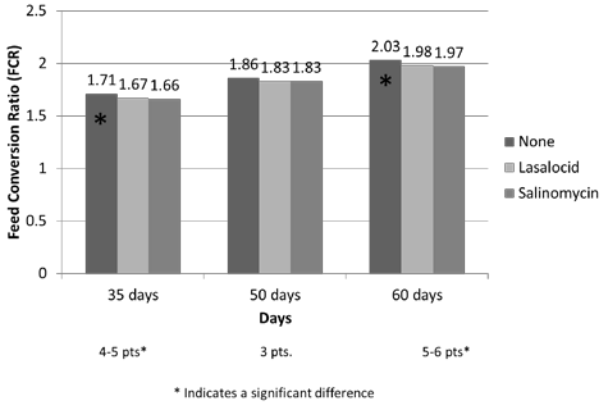
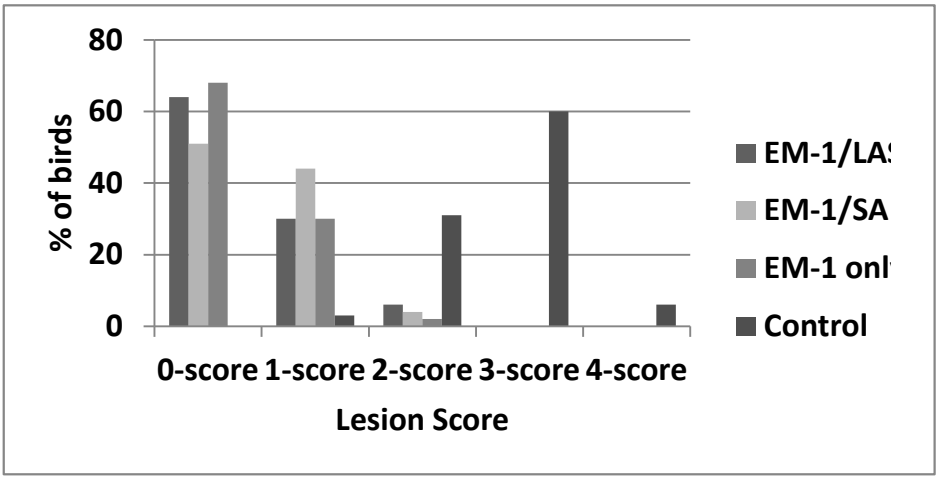


Figure 2. Distribution of *E. maxima* lesion scores after a 35 d challenge—EM-1 vaccinated vs. controls



HOW VOLUME IMPACTS COCCIDIOSIS VACCINE UPTAKE IN THE HATCHERY

J. E. McCarty

Merial Select, Inc., Gainesville, GA 30503

Successful mass application is critical to the success of any broiler coccidiosis vaccination program. All live coccidiosis vaccines require oral ingestion. A previous study done at the University of Delaware's Lasher Lab showed that the spray cabinet efficacy is approximately 88% based on oocyst shed rates five to eight d post hatch. The question remains, can we improve beyond this 88%? Using the following paper as a starting point (Caldwell *et al.* (2001) *Journal of Applied Poultry Research* 10: 107 – 111) research was implemented that clearly demonstrated increasing relative photo intensity at the time of spray application can have a positive impact on vaccine uptake. This data was presented at last year's WPDC.

Keeping with the application theme, we investigated if increasing the volume sprayed had an impact on oocyst shed. Table 1 shows the results from the initial study. Based on the findings, further research has been done to determine the optimal program needed to successfully implement a successful program in a commercial hatchery. The findings of these studies will be discussed in depth.

It was also determined that the higher shed rates have a positive impact on the shed pattern of oocyst which in turn had a direct positive impact on necrotic enteritis.

Table 1.Volume study based on oocyst per gram of feces 4-8 days post vaccination.

Volume	25 mL	30 mL
21 mL (control)	+33%	+109%

EFFECTIVE USE OF LYOPHILIZED *SALMONELLA* 9R VACCINE VIA DRINKING WATER

M. Delamer

MDLAB - CC15, 1667 Tortuguitas, Buenos Aires, Argentina.
E-mail: labdelamer@cotelcam.com.ar

SUMMARY

The vaccination by parenteral route with the live strain of *Salmonella* Gallinarum 9R (Sg) has proven to be a very effective tool in the prevention and control of fowl typhoid. The increase in labor costs, the fact that it is necessary to revaccinate every three months in order to maintain a solid immunity, and the construction of bigger productive units with hard-to-reach cages, motivate the development of a massive vaccination method that is simple, inexpensive, and effective.

INTRODUCTION

In recent decades, poultry of EDIA (Estados Desunidos de Ibero America) has been severely affected by fowl typhoid, caused by highly pathogenic strains multiresistant to antibiotics of Sg. An effective remedy to prevent and control outbreaks of this disease is the use by parenteral route of the rough 9R strain of Sg. Many producers and veterinarians have long requested the development of a method of vaccination with strain 9R. Being an enterobacteria, the only way suitable, we theorized, would be oral application.

In order to protect the viability, the 9R should arrive in large numbers to the last portion of the small intestine and the ceca, where they colonize and trigger mechanisms of cell-mediated protection and production of IgA. How to make the 9R not be killed by the very low pH of the crop and the stomachs of birds that are so sensitive to acids proved to be a problem. When you have heartburn, the intake recommended is mild alkali solutions of sodium carbonate, sodium bicarbonate, fruit salts, etc.

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MATERIALS AND METHODS

In MDLAB we investigated the action of alkalis in various concentrations on the survival of strain 9R in vitro.We added 10% of a fresh culture of 9R Sg to each of the various solutions, which were maintained for two h at 26 ° C and titrated in brain heart agar concluded this period. The initial count was 3.2×10^7 cfu/mL.

Results after two hours of exposure: 0.5% sodium carbonate 1.8×10^3; 1.0% sodium carbonate 4.5×10^2; 2.0% sodium carbonate 1.2×10^2; 0.01% sodium hydroxide 7.4×10^2; sodium hydroxide 0.1% 6.2×10^1; sodium bicarbonate 1% 3.3×10^7; and sodium bicarbonate 2% 4×10^5.

Trials with live birds. Two hundred forty male chicks were used. They belonged to a brown egg laying breed very sensitive to typhus and were bred on floor. They were divided into four pens with 60 birds in each. At 28 d of age they received one of the following treatments:

Lot 1. Injected with 1×10^8 cfu/bird of 9R lyophilized vaccine.
Lot 2. 1×10^9 cfu/bird of 9R lyophilized vaccine via drinking water consumed during two h.
Lot 3. 1×10^9 cfu/bird of 9R lyophilized vaccine in the drinking water with 1% sodium bicarbonate according to the instructions as per Appendix 1.
Lot 4. Unvaccinated controls.

Four weeks later the four groups were homogenized by feathering and body weight, leaving

50 birds in each pen. They were individually discharged by esophageal probe, first with five mL of water containing two percent baking soda, and 20 min later with 1 mL of water containing one percent sodium bicarbonate and 1 x 10^8 cfu of a fresh culture of a pathogenic strain of Sg.

Since all birds killed by Sg make septicemia, direct seed of plates with bone marrow in MC agar allows isolation in pure cultures. For the next four wk, to every dead bird, one leg was severed at the hock joint level and cultivation was performed of the tarsal bone marrow by direct seeding on MacConkey agar. All cultures were confirmed to be Sg by rapid plate agglutination against specific antiserum.

RESULTS

All dead birds after discharge were positive for Sg isolation.

CONCLUSIONS

The lyophilized vaccine 9R Sg applied in drinking water properly alkalinized, in doses of 1 x 10^9 cfu/bird, provided the same level of protection as the same vaccine given parenterally in doses of 1 x 10^8 cfu/bird .Ongoing work will seek to define the minimum dose necessary to protect effectively by applying Sg 9R vaccine in the alkalinized drinking water.

The author has not found bibliographic references to this technique and belive that this effective mass vaccination method may be extended to other similar live salmonella immunogens for both birds and other animal species, including man.

APPENDIX 1

Procedure for the application of SG 9R vaccine by oral route:

1. For every 1000 adults birds, 400 g. of sodium bicarbonate in 40 L of water with 400 grs of skimmed milk should be dissolved in an auxiliary tank.

2. Do not bring birds to any water fast (water cut). Simply change the normal supply of drinking water to the alkalinized water.

3. After the birds have drunk half this alkalinized water and the gastrointestinal tract is neutralized, add, mix and homogenize the 9R vaccine in the remaining alkalinized water.

4. Once you have finished using the water with the vaccine, return to running water immediately.

Caution: do not give any antibiotic by any route one wk before or two wk after vaccination.

Remember that solid protection provided by 9R vaccine and similar ones lasts only three months regardless of their route of application, so in order to maintain a permanent solid immunity, birds should be revaccinated every three months.

Table 1. Dead birds on the total discharged and percentages of protection.

Lot	Dead/Total	% Protection
1	7/50	86
2	42/50	16
3	8/50	84
4	43/50	14

EFFECT OF EXPERIMENTAL NEWCASTLE DISEASE CHALLENGE OF LAYING HENS RECEIVING VACCINATION PROGRAMS THAT INCLUDES RECOMBINANT HVT NDV

R. Merino

Departamento de Medicina y Zootecnia de Aves, FMVZ, UNAM. Mexico, DF, 04510, Mexico

ABSTRACT

Newcastle disease effects on poultry can be reduced by vaccination. We measured the performance of vaccinated White Leghorn type laying hens challenged with the Chimalhuacan ND strain at 28 wk old and observed for five wk post challenge (wpc). Four groups of laying hens were vaccinated at one d old as follows:

White-Marek's disease
Red- Marek's disease + Newcastle (killed virus)
Green- recombinant HVT-ND
Yellow- recombinant HVT-NDV Vectormune®

Vaccination programs included five live NDV-IBV vaccines. Red group also received twice a NDV-AIV killed vaccine. All vaccination programs protected against mortality. The mean egg production in the five wpc was lower in White group than in all others but shell-less eggs were higher. Three doses of killed and five of live ND virus avoided the drop in egg production and quality. Recombinant HVT-NDV Vectormune plus live ND virus fully protected against clinical symptoms.

INTRODUCTION

Newcastle disease (ND) threatens the poultry industry in several areas around the world, since it causes high morbidity and mortality in susceptible flocks. In order to control and prevent the occurrence of ND, good biosecurity practices need to be established and rigorously accomplished. One of these biosecurity practices is the preventive vaccination of flocks. Nowadays most vaccination programs include the use of lentogenic strains both, live or inactivated (killed) in order to provide high protective immunity and minimal adverse effects on the flock performance; however, the need for safer and more efficacious ND vaccines has lead to the development of new products, including the recombinant ones, as HVT-ND and ND-AIV.

The aim of this study was to evaluate ND vaccination programs commonly used in commercial pullets including recombinant HVT ND vaccines commercially available and the protection against the challenge with a Mexican Chimalhuacan ND strain at 28 wk of age.

MATERIALS AND METHODS

Four groups of 27 wk old laying hens were used, coming from commercial farms, identified as Yellow, White, Red and Green, with 24 hens/group. At the farm they received the Newcastle disease vaccination programs shown in Table 1.

Hens were housed in cages and received water *ad libitum*, feed according to standard procedures, and 15 hours of light per day. Ten birds per group were bled prior to challenge and every wk after that. Serum samples were tested by ELISA test (AffiniTech LTD, Arkansas, USA). All hens were challenged at 28 wk old with the Mexican standard challenge strain Chimalhuacan by the ocular route with 10^6 EID_{50}/0.2mL, then observed by five wk post challenge (wpc) for clinical signs, mortality, egg production, and egg quality (weight, broken, and shell-less).

Birds showing clinical signs and bad quality eggs were compared among groups by test of proportions. Percent of laid eggs per wk were compared by ANOVA, transforming the percent to proportion and then transforming to the arcsine-root. The mean egg weight (per week) and ELISA antibody titers were compared by ANOVA; alpha value was established in 0.05.

RESULTS

When the laying hens were received, all groups were around 90% egg production; however, three d later a mite infestation was detected; apparently the Red group was the source, since it was the most affected. All hens were treated prior to the challenge. The transport and housing stress, plus the infestation severity and treatment, caused a reduction in egg production, with variable intensity.

Egg production is shown in Figure 1. Yellow group had 54.17% production at challenge and three wpc reached 95.83%, remaining above 90% until the

end of the trial. White group had 75% production at challenge but didn't reach 80% as mean per wk trough the whole experiment. The Red group showed the lowest egg production at challenge (8.7%) but increased to 95.65% as soon as two wpc, remaining above 90% the following weeks. The Green group had 25% production at challenge, increased to 85.12% at three wpc but didn´t reached 87% the remaining weeks. Egg production during the five wpc was higher ($P< 0.01$) in Yellow and Red groups (85 and 81.49%, respectively) than White group (71.25%); egg production in Green group (76.19%) was not different from all other groups. There was no mortality after challenge. Feed consumption was not affected. Only one hen (4.16%) from Green group started with nervous signs (torticollis and incoordination) at 18 dpc, which increased and last for two wk, then started recovering at 32 dpc. There was no statistical difference ($P>0.05$) in hens showing clinical symptoms.

There was no statistical difference among groups in the amount of broken eggs in the five wk period. The mean egg weight in Green group (54.91 g) was lower ($P< 0.05$) than in all other groups (Yellow= 56 g, White= 55.86 g, and Red= 56.15 g). The White group produced more shell-less eggs, 4.3% ($P< 0.05$), than the others (Yellow 0.56%; Red 0% and Green 0.31%).

Humoral immune response to vaccination programs and after challenge is shown in Figure 1. ELISA antibody titer prior to challenge was higher ($P< 0.01$) in Red group than in the others; sero-conversion was seen in all groups except Red group. Antibodies titer remained almost the same until the fourth wpc without difference among groups ($P>0.05$). A significant reduction ($P< 0.05$) was seen in White group at five wpc.

DISCUSSION

Egg production prior challenge was variable in all groups because of the above mentioned factors; however, recovering of lay was seen soon after challenge but was largely dependent of the antibody titer at challenge. Red group had the lowest production at challenge, but the highest ELISA titer. This high titer was expected, since the hens received three doses of killed virus oil emulsion plus five live virus doses. White group had a live-ND virus only vaccination program, antibody titer at challenge was lower than Red group, but similar to Green and Yellow groups; however, egg production decayed from 75% to 60% two wpc and couldn´t reach 80% in the following weeks, so, the challenge virus didn´t kill the hens, but affected both the production and egg quality, as previously reported (1). Immunity in Yellow group that received the recombinant Vectormune HVT-NDV vaccine was not as high as the Red group, but allowed the egg production increase from 54.17% at challenge to around 95% three to five wpc, which was a little higher than Green group (25%) but couldn´t reach 87% after challenge, even when the antibody titer at challenge was almost the same.

As seen in this study, vaccination with recombinant HVT-ND and boosting with live virus vaccines induces a long lasting immunity which protects against mortality and affords clinical protection against strong challenge with velogenic NDV, as previously reported (2), and also can reduce egg-production drop caused by the infection. Each vaccination program must be established according to the immunity level needed based on the challenge in the field, as well as the costs involved.

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

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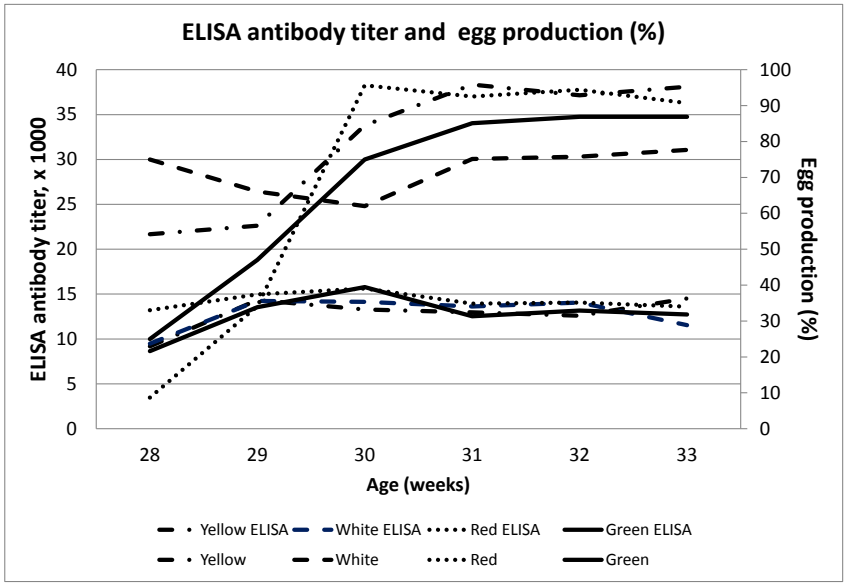
Table 1. Vaccination program against ND in commercial pullet farms.

	Age (days)							
Group	**1**	**9**	**23**	**24**	**40**	**76**	**101**	**108**
Yellow	Vectormune® HVT NDV	ND+IB live virus, ocular	ND+IB live virus, ocular		ND+IB live virus, ocular	ND+IB live virus, drinking water		ND+IB live virus, ocular
White	Marek's disease							
Red	Marek's disease + ND KV			ND+AI KV, SQ			ND+AI KV, SQ	
Green	HVT-ND							

Figure 1. Egg production and ELISA antibody titer during five weeks after challenge in laying hens inoculated with the Chimalhuacan strain of Newcastle disease virus at 28 wk of age.



FIELD SAFETY AND EFFICACY OF A VECTOR MAREK'S/NEWCASTLE DISEASE VACCINE (*R*HVT – NDV) AS ASSESSED BY CLINICAL AND PRODUCTIVE PERFORMANCE IN A LARGE POPULATION OF COMMERCIAL BROILERS

L. Sesti [A], C. Kneipp [A], R. Paranhos [A], P. Paulet [B], and C. Cazaban [B]

Ceva Animal Health
[A] Brazil, [B] France

INTRODUCTION

Newcastle disease (ND), along with avian influenza, are the most serious health threat to the modern poultry industry around the world due to their induced severe clinical (mortality and morbidity) and political consequences (export of poultry products). Therefore, it's imperative for the industry to have available effective, and predominantly safer, immunological tools to prevent and/or control ND, even in countries whose production systems are free of velogenic ND viruses (2,4).

The objective of this work was to investigate the safety and efficacy of a vector *r*HVT-NDV vaccine in

comparison with different ND vaccination programs with conventional live vaccines in a large field trial involving six companies and over nine million broilers in the northeast region of Brazil (states of Pernambuco and Ceará).

MATERIALS AND METHODS

Testing vaccine. The new ND commercial vaccine tested was a turkey herpesvirus-based recombinant vaccine (*r*HVT) expressing a key protective antigen (F glycoprotein) of the ND virus (Vectormune® ND, from Ceva Animal Health, Lenexa, Kansas, USA).

Experimental groups. The data to be presented are from three companies (integrated production systems) which had different conventional ND vaccination programs.

The respective ND vaccination programs compared in each company and volumes of broilers tested in each were as follow:

Company 1- *r*HVT-NDV d one SQ (437,000 broilers) vs. apathogenic Phy.LMV. 42 strain d one spray + lentogenic La Sota 18 d DW (555,000 broilers)

Company 2- *r*HVT-NDV d one SQ (761,000 broilers) vs. apathogenic Phy.LMV.42 strain d one spray (754,000 broilers)

Company 3 -*r*HVT-NDV d one SQ (1,080,000 broilers) vs. lentogenic C2 strain d one spray (1,210,000 broilers).

In all companies the populations of broilers used in each group were always contemporaneous (one wk vaccination with each treatment group) and had absolutely the same vaccination program, management procedures, nutrition levels, type of houses and disease challenges in general.

Sampling for laboratory analysis. The sampling schedule in ten flocks of each group in each company was as follows: 20 blood samples for HI (8 HU) and IDEXX ELISA serology were taken at 1, 14, 21, 35, 42 and 49 d of age; five tracheas for histopathology at 14, 21, and 28 d of age; and pool of five wing feathers from each of five birds for *r*HVT polymerase chain reaction (PCR) at 21 d of age.

Productivity data. Key productivity parameters (daily weight gain, slaughter weight, feed conversion, final mortality and European productivity index) were collected and statistically analyzed for the entire broiler population used in each group in each company. In addition, a detailed financial output from each experimental group of broilers was calculated taking into account all main broiler production costs and income involved (unit cost of DOC, feed, mortality, feed conversion, vaccination program, sales price per kg of live slaughter age broiler).

Statistical analysis. All productivity, clinical and laboratory results were statistically analyzed by completely random analysis of variance and means compared by Tukey HSD All-Pairwise test at $p<0.05$ level. (Statistix 9.0 software, www.statistix.com)

RESULTS

Serology. HI seroconversion from *r*HVT-NDV vaccinated broilers was almost 100% negative in all companies with only a few individuals presenting titers slight above the positive threshold limit (3 $\log_2$) after 42 d of age while the on ELISA serology, a low but clear seroconversion could be observed in all flocks at 42 and 49 d. Most interesting was the significant uniformity (very low CVs) of titers in *r*HVT-NDV vaccinated broilers after 35 d of age.

Histopathology of tracheas. At almost all ages tested (except for Company 1 at 14 d and Company 3 at 28 d), all broilers *r*HVT-NDV vaccinated presented significantly lower lesion scores (congestion, deciliation, mononuclear inflammatory infiltrate and epithelial hyperplasia) on sections from upper, mid, and lower regions of the trachea when compared to conventional live vaccines vaccinated broilers (Table 1).

***r*HVT PCR.** All ten flocks sampled at three wk of age in the *r*HVT-NDV vaccine group in each company were found to be positive for the molecular detection of the *r*HVT indicating an excellent vaccination take.

Productivity and financial data. The main productivity and financial data sets from both experimental groups are depicted on Table 2.

It is evident the very strong trend, and sometimes significant differences, for better results for the *r*HVT-NDV vaccinated broiler group when compared with the conventional live vaccines group. This is clearly demonstrated by the numerically and/or significantly higher values of the European Productivity Index which takes into consideration data of viability, weight, feed conversion, and age at slaughter.

In addition, all *r*HVT-NDV vaccinated groups generate higher values of financial income at slaughter age.

DISCUSSION AND CONCLUSIONS

Recently published research results (1,3) have demonstrated that the *r*HVT-NDV vaccine is quite able to induce some rapid and significant protection against a strong ND challenge in field and laboratory vaccinated commercial broilers.

The present results strongly indicate that such *r*HVT-NDV vaccine is also highly beneficial in regions free of velogenic ND where preventive vaccination with conventional live ND vaccines is practiced. Such beneficial effect most probably occurs for two main reasons: a) elimination of the normal post vaccination inflammatory reaction that occurs after the use conventional live ND vaccines and therefore eliminating the metabolic cost associated with it; and, b) elimination and/or prevention of "rolling" post ND vaccination reactions in the field, which not only are naturally growth detrimental by themselves but also many times, interact with reactions from the other respiratory vaccines (IBV and/or aMPV) causing more severe secondary bacterial infections and mortality in four to seven wk broilers. These two main reasons can be confirmed by the significantly lower histopathological lesion scores (Table 1) and higher productivity and economical results generated by the *r*HVT-NDV vaccinated broilers (Table 2).

In conclusion, the *r*HVT-NDV Newcastle Disease vaccine tested is effective and quite safe for vaccination of broilers in modern poultry production systems at both velogenic ND free and endemic countries.

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Table 1. Histopathology lesion scores at 14, 21, and 28 days of age from tracheas of broilers vaccinated with either an rHVT-NDV vaccine or live conventional vaccines agains ND.

	rHVT-NDV	Conventional live vaccine	rHVT-NDV	Conventional live vaccine	rHVT-NDV	Conventional live vaccine
	14 Days		21 Days		28 Days	
Company 1	0.38[a]	0.43[a]	0.53[a]	0.60[b]	0.69[a]	0.79[b]
Company 2	0.45[a]	0.52[b]	0.66[a]	0.86[b]	0.69[a]	0.99[b]
Company 3	0.20[a]	0.33[b]	0.35[a]	0.45[b]	0.56[a]	0.60[a]

Table 2. Productivity parameters and economical results from broilers vaccinated with either an *r*HVT-NDV vaccine or live conventional vaccines against ND.

Groups	Productivity and Economic Parameters				
	Slaughter Weight (g)	Mortality (%)	Feed Conversion (g/g)	Productivity Index	Extra income (US$) *
	Company 1 (mixed sex; slaughter age = 53.3 days)				
*r*HVT-NDV	3010[a]	5.0[a]	2.16[a]	255[a]	85.2
Phy.LMV.42 + La Sota	3050[a]	6.0[a]	2.26[b]	237[b]	
	Company 2 (mixed sex; slaughter age = 50.5 days)				
*r*HVT-NDV	3100[a]	6.3[a]	2.02[a]	285[a]	49.9
Phy.LMV.42	3100[a]	7.8[a]	2.05[a]	276[a]	
	Company 3 (females; slaughter age = 36.7 days)				
*r*HVT-NDV	1830[a]	3.5[a]	1.80[a]	272[a]	32
C2	1840[a]	3.9[a]	1.83[a]	264[a]	
	Company 3 (males; slaughter age = 50.7 days)				
*r*HVT-NDV	3190[a]	6.4[a]	2.03[a]	294[a]	16.8
C2	3180[a]	7.2[a]	2.04[a]	286[b]	

***** extra income per each group of 1000 broilers from the rHVT-NDV vaccinated group when compared with the income generated by the control group (conventional live vaccines).

EPIDEMIOLOGIC STUDY OF INFECTIOUS LARYNGOTRACHEITIS IN CENTRAL CALIFORNIA: 2000-2012

S. Carnaccini[A], M. Pitesky[B], R. P. Chin[A], G. Senties[C], B. Charlton[C], P. Woolcock[D], and H. L. Shivaprasad[A]

California Animal Health and Food Safety Laboratory System – Tulare[A], Turlock[C], and Davis[D] Branches, University of California, Davis
[B]California Department of Food and Agriculture, Animal Health Branch, 2800 Gateway Oaks, Sacramento, CA 95833

SUMMARY

Infectious laryngotracheitis (ILT) is a highly contagious viral respiratory disease of primarily chickens caused by Gallid Herpesvirus I. Chickens of all ages are susceptible and the disease is characterized by lacrimation, respiratory signs, and increased morbidity and mortality. Lesions of ILT include conjunctivitis, rhinitis/sinusitis, laryngitis and tracheitis, and occasionally bronchitis and airsacculitis associated with characteristic syncytia and intranuclear inclusion bodies. In October of 2005 an outbreak of ILT not distinguishable from the CEO-like vaccine strains began involving numerous mostly broiler flocks located in the Central Valley of California. Vaccinations with fowl pox LT-vaccine and CEO vaccines reduced the clinical signs of ILT but did not stop the outbreaks. Therefore, a coordinated industry effort was adopted in early 2008 by two companies to eradicate ILT, which proved to be highly successful. These efforts included extensive biosecurity audit, extended downtime between flocks placed in the vicinity to each other, high level of cleaning and disinfecting procedures, and vaccination with HVT-LT recombinant vaccines. The aim of the present study is to analyze the incidence of ILT, yearly and geographic distribution, age at which ILT was diagnosed, statewide surveillance efforts, and the scope of ILT cases by a spatial, temporal and spatial-temporal evaluation of the distribution of ILT in the Central Valley of California between the years, 2000-2012.

Between January 2000 and September 2012, 422 submissions were made to the California Animal Health and Food Safety laboratory System in Fresno, Tulare, and Turlock Branches to be tested for infectious laryngotracheitis. Of the 422 accessions, 213 were confirmed positive based on gross and microscopic lesions, fluorescent antibody test, PCR-RFLP, immunohistochemistry, serology, virus isolation, and sequencing. Although layer, breeder, and back yard

chicken flocks were included in this study, 70% of the flocks affected were broiler chickens. The age of the broiler chickens ranged between 18 to 49 d, with an average age of 35 d. Most of the cases diagnosed with ILT, about 76%, were in the years 2006 and 2007. From 2008 to 2012 ILT has appeared sporadically in the poultry flocks of Central Valley of California. At the onset of the initial outbreaks in 2005 most of these flocks had not been vaccinated for ILT but raised in the vicinity of layer flocks which were vaccinated with CEO-like vaccine.

Of the 422 accessions, only 302 provided address information of which 151 were diagnosed as positive for ILT and 151 negative for ILT. Geocoding of addresses and mapping was conducted using ArcGIS® v10. The 302 accessions represented 83 different locations. GIS mapping coupled with spatial, temporal, and spatial-temporal cluster analysis identified significant high rate spatial, temporal, and spatial-temporal clustering, meaning clusters where more ILT was observed than statistically expected. For example, Southern Stanislaus Co., Central and Southern Merced Counties and Southern Fresno Co. had areas with high rate spatial-temporal clusters. Interestingly, these were areas with dense broiler production reflecting the potential for vaccine based strains to spread to naïve broilers. On the contrary, low rate spatial-temporal cluster in the Southern Fresno Co. area shown to be between 2006 and 2010 probably due to the very low rate of ILT between 2008 and 2010. Temporal-Spatial clustering is the most consistent with vaccine induced CEO-like ILT. Of the 151 total positives, high-rate temporal spatial clustering accounted for 54 of the positive cases or 36% of the positives.

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EMERGENCE OF VARIANT INFECTIOUS BRONCHITIS VIRUS STRAIN 4/91 IN ONTARIO

E. Martin, M. Brash, J. Coventry, S. Hoyland, and D. Ojkic

Animal Health Laboratory, University of Guelph, Guelph, Ontario , Canada

Between 2002 and 2011, outbreaks of IBV in Ontario have been sporadic with both respiratory and nephropathogenic strains being detected in the field. A relatively small number of field IBVs were previously genotyped, and based on spike (S1) gene sequencing, Ontario viruses were highly related to variant IBVs circulating in the US. Since early 2012 an increased number of IBV-associated diseases in chicken flocks of multiple commodities have been reported. Clinical signs ranged from reduced weight gain and feed efficiency, to respiratory disease (gasping, coughing, sneezing, tracheal rales, nasal discharge) and depression. Histopathological findings included tracheitis, airsacculitis, pneumonia and urate nephrosis. Based on S1 gene sequencing, viruses from three outbreaks showed the highest percentage of identity to a variant IBV previously described in California. However, seven viruses appeared to be highly related to IBV strain 4/91 (793/b) that has not previously isolated in North America, but in Europe, Asia, and South America.

(The full-length article will be published.)

ISOLATION AND CHARACTERIZATION OF REOVIRUS FIELD ISOLATES FROM CLINICAL CASES OF VIRAL ARTHRITIS

H. Sellers, E. Linnemann, V. Gauthiersloan, and T. Collett

Poultry Diagnostic and Research Center, Department of Population Health,
College of Veterinary Medicine, University of Georgia
953 College Station Road, Athens, GA 30602 USA

ABSTRACT

From December 2011 until present, a significant increase in tendon submissions from clinical cases of tenosynovitis/lameness in broilers, ranging in age from 2.5-6 wk, were submitted to the Poultry Diagnostic and Research Center. In a majority of the cases, reoviruses were isolated from the tendons and characterized by RT-PCR of the sigma C followed by sequence analysis (genotyping). Genotypic analysis of the reovirus field isolates revealed two novel genotypes to date that were unrelated to current vaccine strains S1133, 2408 and 1733 (<50% amino acid identity). In addition, the closest match to reoviruses in the public and PDRC databases was approximately 80%, indicating that the field isolates belonged to a variant group of reoviruses unrelated to previously characterized isolates. Cross neutralizations were performed with the field isolates and S1133 and 2408 antiserum to determine serological relatedness. Little to no cross neutralization was observed providing initial evidence that S1133 and 2408 vaccines would not provide protective immunity against the variant field isolates.

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

MACROPHAGE FUNCTION CORRELATES WITH GENETIC RESISTANCE TO RESPIRATORY VIRAL DISEASE

Y. Drechsler[A] , E. Collisson[A], G. Banat[A] , C. Keeler[B], S. Tkalcic[A], W.E. Briles[C], and M. Dawes[A]

[A]College of Veterinary Medicine, Western University of Health Science, Pomona, CA
[B]Department of Animal and Food Sciences, University of Delaware, Newark, DE
[C] Northern Illinois University, deKalb, IL

SUMMARY

Breeding birds with resistant traits is ideal in controlling disease. Influenza (AI) and infectious bronchitis (IB) viruses are among the most contagious and difficult pathogens to control. Resistance to IBV-induced clinical illness and induction of AIV specific antibody has been correlated with genetically defined B haplotypes. The homozygous B2, B8 and B5 chicks were shown to have greater resistance while the B12 and B19 homozygous chickens were more susceptible to IBV associated illness. Since differences in resistance were early after infection, functions of macrophages, critical to both innate and acquired immunity, were compared among B haplotype-defined lines. Chicken peripheral blood macrophages were stimulated with poly I:C or IFNγ. As determined by NO production, macrophages from more resistant chicks were significantly more responsive to either stimulant. Macrophage mRNA expressions confirmed enhanced activity of immune pathways from more resistant birds. More vigorous innate immune responses may ultimately lead to enhance adaptive immunity.

INTRODUCTION

Infectious bronchitis virus (IBV) is a non-zoonotic, highly contagious respiratory virus of birds. IBV not only replicates in the trachea and lungs, but also the kidneys and gut. We have shown that birds with the homozygous B2, B5 and B8 haplotypes are more resistant to IBV while those with the homozygous B12 and B19 haplotypes following infection presented with more severe clinical illness (1). Interestingly, B2/B12 birds, heterozygous for the B2 and B12, overall appeared to be at least as resistant, if not more resistant than B2/B2 birds, suggesting a dominance of the resistant B2 phenotype. It has been reported that the severity and duration of illness

following Ark IBV strain challenge was not significantly different in IBV-vaccinated B2/B15 and B2/B21 birds (6). Although the initial respiratory signs of the B8/B8 birds were more severe early post-infection with IBV similar to that observed for the B19 and B12 homozygous chicks, the B8 homozygous birds displayed faster resolution of clinical illness after IBV infection than even the more resistant B2/B2, B5/B5 and B2/B12 birds.

Our classifications for resistance and susceptibility based on clinical illness also correlated with sequencing and descriptions of genetic clades of the chicken MHC B region; B2, B5 and B8 representing one gene cluster and 12 and B19 another (3). Furthermore, the sequences in the chicken B complex encompassing 14 gene loci from BG1 to BF2 have been shown to be identical at nine loci between the B12 and B19 homozygous haplotypes and at seven loci between the B5/B5 and B8/B8 (4); however, sequences of the B2/B2 haplotype were not found to be relatively close to the B5 and B8 or B12 and B19. Overall, the striking differences in disease resistance following IBV infection may be, at least in part, a direct consequence of differences in MHC Class I genes. Infection of congenic lines of birds with a highly pathogenic strain of AI suggested that resistance was more dependent on genes outside of the B locus. However, the strain of AIV used was lethal even in many of the more resistant birds (5). Thus, the pathogenesis is very different from an IBV strain that causes respiratory illness with low mortality.

The more clinically resistant B2/B2 and B8/B8 birds cleared IBV faster from all tissues examined (trachea, lungs and kidneys) compared with clearance from tissues of the B19/B19 birds. Tissues of the more susceptible birds may provide a more favorable environment for IBV infection. The greater susceptibility to clinical illness observed in B19/B19 birds could be a consequence of a more permissive environment for viral replication, or due to differences in the animal responses to infection, or a combination of both. Since variations in pathogenesis of IBV infection in different chicken haplotypes were observed early, it was speculated that innate immunity may be involved. As critical components of innate immunity, the current studies compared the activity of macrophages from B19/B19 and B2/B2 birds following differentiation, and following stimulation with poly I:C and the IFNγ response.

RESULTS

Response of macrophages to IFNγ and Poly I:C stimulation differed. Adherent cell concentrations were adjusted so that equivalent concentrations were cultured for macrophages derived from either B2 or B19 birds. The B2/B2 macrophages consistently differentiated faster than macrophages from B19/B19 birds.

Responses to poly I:C, which simulates an infection with an RNA virus, such as IBV, and responses to IFNγ, an interphase cytokine between innate and adaptive immunity, were compared for macrophages from either source. The measure of responsiveness was quantification of NO released from the exposed macrophages. The responses of B2 homozygous macrophages was consistently greater to both poly I:C and IFNγ than the responses of B19 homozygous macrophages. While the stimulation from both increased with time, background responses were also greater by 72 h of exposure to the stimulants. Therefore, further studies examined the expression of RNA at 48 h of stimulation.

RNA was collected from macrophages of B2 and B19 haplotypes after adherence and after three d of differentiation, as well as varying times after stimulation of macrophages. Samples were lysed in wells and processed with the RNeasy kit from Qiagen. RNA was sent on dry ice to Dr. Calvin Keeler at the University of Delaware for generation of libraries and sequencing. RNA quality was determined on a BioAnalyzer before library construction was allowed to proceed. Libraries were constructed for massively parallel cDNA sequencing (RNA-seq) using the Ovation RNA-Seq System and Encore NGS Library System (NuGen Technologies). Using uniquely tagged amplification primers (NuGen Technologies) libraries were multiplexed. High throughput sequencing was performed with an Illumina HiSeq 2000 and the results analyzed using a suite of analysis tools, including DAVID, Hierarchical clustering and KEGG Pathway Analysis.

RNA sequencing results. Differential gene expression was observed following IFNγ stimulated macrophages from B2 and B19 birds. Quantification of RNA expression indicated earlier and more robust stimulation of several genes and pathways in the B2 derived macrophages; for example, TLR and the apoptosis pathways and related genes in B2 macrophages at 1, 2, and 4 h post-stimulation. Several TLRs were differentially activated with more robust responses in the B2 haplotype. In addition, more B2 genes were upregulated in several pathways at all three times examined than in B19 stimulated macrophages. We are currently further analyzing the differential gene expression within the TLR pathway, which is evolutionarily conserved and a decisive determinant of the innate immune and inflammatory responses (8).

DISCUSSION

IFNγ is a potent activator of macrophages and higher production has been associated with disease resistance. These findings indicate that chickens with an enhanced IFNγ production are more resistant to certain infections. IFNγ enhances macrophage activation, expression of MHC and nitric oxide release, which aides in killing of pathogens and also increases activity of cytotoxic T cells and secretion of Th1 cytokines (7).

TLRs, critical for cells of innate immunity such as macrophages, are important pattern recognition receptors for pathogens (2). TLR binding by a pathogen leads to transcriptional activation of the NF B pathway, interferon-regulatory factors, and a large array of pro-inflammatory cytokines. The classic function of macrophage priming is to potentiate early innate immune responses, enhancing the host defense. This enhancing of the immune response has been implicated in antiviral and antibacterial responses (9) and IFNγ dependent priming of TLRs has been demonstrated to enhance TLR expression. Our results show that macrophages from B2 and B19 chickens were stimulated differently by IFNγ. B2 macrophages may be more efficiently primed and therefore provide a more effective innate immune response, accounting for their improved disease resistance to IBV.

Due to the emergence of drug resistant, as well as more virulent pathogens, genetic resistance to disease poses an advantage over the use of antimicrobials. Even though parameters of immune competence of the host are known, such as antibody production, phagocytic activity, nitric oxide release or cytokine production, the genetic and molecular basis for disease resistance is still not entirely understood. Future studies should identify genes that are directly responsible and thus can be selected for disease resistance.

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FIELD EXPERIENCES WITH AN ARGENTINE VARIANT INFECTIOUS BRONCHITIS VIRUS AS A LIVE VACCINE

M. Delamer

C.C 15 – Tortuguitas - Argentina
Email: mateodelamer@arnet.com.ar

SUMMARY

For more than 15 years, a multiage commercial eggs layers complex of about one million birds suffered the permanent circulation of an untypyfied infectious bronchitis virus. Different programs with Mass + Conn + inactivated commercial or autogenous vaccines failed in preventing egg drops, loss of inner and shell quality at or near the peak of production. Finally, as a last resort it was decided to expose or "vaccinate" new pullets with a mild field strain recently isolated. Different flocks of pullets from 12 wk of age to others with 50% per day egg production were "vaccinated" by spray or drinking water. None of

the previous production problems typical of IB infection appeared in more than nine years with several millions of birds immunized. We concluded empirically that a variant strain that wasn´t totally controlled by standard vaccines was causing the field problems. This was recently proved by molecular techniques. (A. Rimondi *et al*.) More recently, an acute and severe sanitary problem appeared in a multiple age broiler complex which usually had between 4-5% total mortality that jumped to 15- 25%. A variant IB virus was isolated. The universal experience dictates that the only solution in these cases is total depopulation of the farm and strong cleaning and disinfection of the house and equipment. Surprisingly, a prevention program with application of a very attenuated Mass vaccine at day old by spray at the hatchery, plus the Argentine variant strain via drinking water at two wk, totally controlled the field problems that usually appeared at 35 d of age and the mortalities returned to 4-5 % without any disinfection nor depopulation practiced. Our experiments and results may show that knowing exactly the nature of the field bronchitis virus circulating in a particular area, region, or industrial avian complex, it is possible to solve the problems by priming the chickens at day old with a very mild Mass vaccine plus the additive effect of a specific tailor made attenuated variant strain vaccine.

AVIAN NEPHRITIS IN COMMERCIAL BROILER CHICKENS

S. Ul-Haq and S. J. Ritchie

Canadian Poultry Consultants
30325 Canary Court, Abbotsford, British Columbia, Canada V4X 2N4

SUMMARY

Commercial broiler chickens from multiple flocks were submitted for necropsy to Canadian Poultry Consultants with a complaint of higher than normal mortality, ranging from one to four percent by the third wk of age. Gross post-mortem examination revealed proventriculitis, severe hemorrhagic nephritis, and cream colored toneless intestinal tracts. Swollen pale kidneys with distended tubules and visceral gout were also seen in birds less than seven d. Overall, flocks less than 18 d were most significantly affected. Avian nephritis virus (ANV) was identified by polymerase chain reaction (PCR) in multiple tissues, either with or without the presence of a significant number of bacteria. Although the diagnostic investigation suggested the possible involvement of ANV, further work is required in order to be conclusive.

INTRODUCTION

Avian nephritis is a viral problem of young chicks. The causative agent, ANV, primarily targets kidneys, potentially resulting in significant mortality. As isolation and identification of ANV is challenging, there have been few field reports of the disease associated with ANV infection (3). This investigation was initiated following the observation of a high frequency of excessive mortalities associated with post mortem lesions consistent with avian nephritis in commercial broiler chicken flocks.

The main objectives of this study were:

- To evaluate for the presence of ANV in the affected commercial broiler flocks
- To assess the age distribution of ANV in the affected birds
- To determine the presence of ANV in different tissues of typically affected birds
- To assess the local geographical distribution of ANV

MATERIALS AND METHODS

Commercial broiler chickens submitted to Canadian Poultry Consultants for necropsy with a complaint of higher than normal mortality were the cases considered for inclusion into this investigation. The case definition was a flock in which the significant gross lesions were hemorrhagic nephritis, cream colored toneless intestines, and proventriculitis. A complete post-mortem examination was done and gross post-mortem lesions were described. Fresh proventriculus, liver, heart, lungs, kidneys, intestines (duodenum, jejunum and ileum), and bursa were retrieved and sent to the Animal Health Centre, BC Ministry of Agriculture laboratory for further diagnostic workup.

RESULTS AND DISCUSSION

Avian nephritis virus was found in two to 30 d old commercial broiler chickens. In some cases producers of the affected flocks observed chicks huddling, loose droppings, and a slight dip in weight

gain from four to eight d. Typically, the first wk mortality was under or around one percent. Beginning in the second wk, however, a sudden increase in mortality was seen, which peaked by 13-15 d and eventually declined by 18 d without any intervention. Overall, the mortality ranged from one to four percent by the third wk of age.

Necropsy of the affected birds revealed proventriculitis, severe hemorrhagic nephritis, and a cream colored toneless intestinal tract. Swollen pale kidneys with distended tubules and visceral gout were also seen in birds less than seven d. The ANV was detected by PCR in multiple tissues from the affected birds. The presence of significant bacteria in these tissues was not consistent. ANV was found to be widespread in Fraser Valley commercial broiler chicken flocks, with no specific geographical distribution.

In previous studies it has been observed that one day old chicks are the most susceptible to ANV, but chickens of all ages may be infected. Transmission readily occurs by direct or indirect contact (2). Egg transmission has been suggested on the basis of field observations (1).

Although detailed diagnostic investigation suggests the possible involvement of ANV in the cases described here, further investigation is required in order to draw firm conclusions.

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CURRENT AND FUTURE TRENDS IN VETERINARY DIAGNOSTICS

J. Pedersen

U. S. Department of Agriculture, Animal and Plant Health Inspection Service, Veterinary Services, National Veterinary Services Laboratories, Ames, IA, USA

SUMMARY

Nucleic-acid, or genomic-based diagnostic procedures such as PCR, are increasingly becoming the standard for the detection and surveillance of poultry diseases. Molecular tests can detect non-culturable organisms, provide rapid results for fastidious slow-growing organisms, and are sensitive enough for the detection of organisms present in low numbers. PCR is often more sensitive and specific than conventional tests, economical and versatile when combined with sequencing or restriction fragment length polymorphism. Genomic sequencing is an essential component of diagnostics as well as the research needed for the development of new and better diagnostic tests. Sanger based sequencing has been part of veterinary diagnostics for several decades, but next-generation sequencing (NGS) technology has opened up a new chapter in the book. More sequence data is generated in a fraction of the time, for less cost and without the requirement of amplicon specific primer PCR (9). PCR and nucleic-acid tests have found a strong and permanent place in diagnostics, however standard or conventional methods are still necessary and will remain part of the total diagnostic picture.

On the human health side, point of care tests is a strong trend in diagnostics. For animal health these tests are referred to as pen-side, and include the group of tests that require little or no laboratory infrastructure. Rapid screening tools provide needed information to field investigators. Flu DETECT® (Synbiotics, Kansas City, MO) and VetScan® (Abaxis, Union City, CA) are pen-side tests for the detection of type A influenza. The tests are highly specific but are less sensitive than either virus isolation or real-time PCR (rPCR). A new rapid fully integrated and automated platform for the detection of nucleic acid is Cepheid's GeneExpert (2, 8). The molecular analyzer isolates, purifies, and concentrates nucleic acid from multiple sample types which is then followed by DNA amplification and detection performed in a fully

integrated and automated manner. The user simply loads the cartridge and the system does the rest.

Probe-based or real-time PCR (rPCR) is a primary test in most diagnostic labs. Two common methods for detection of products in rPCR are non-specific fluorescent dyes (SYBR® Green) and sequence-specific DNA probes labeled with a fluorescent reporter (1, 9). SYBR Green chemistry is more economical than Taqman and can be used as a screening test, but is synonymous with conventional PCR gel electrophoresis detecting non-specific amplification when not combined with melt curve analysis. Probe based PCR is not only rapid, but provides target-specific analysis resulting in increased specificity and sensitivity. Taqman and molecular beacon probes are the most commonly used probe-based chemistries (9). rPCR is conducted in thermal cyclers with fluorescence detection modules which report the amount of fluorescence at each cycle. Gel electrophoresis is no longer needed, reducing the amount of hands-on time, reducing the possibility of laboratory contamination, and providing non-subjective sequence specific result interpretation. Up to five different targets can be detected by some multiple channel real-time instruments, enabling multiplexing. High specificity and the ability to multiplex are advantages of Taqman probes while the disadvantages are the initial cost of the probe and assay design which may not be trivial. Molecular beacon and minor-groove binder (MGB) probes are more recent modifications of rPCR. Molecular beacons are highly-specific. If the target sequence does not match the beacon sequence exactly, hybridization and fluorescence will not occur; an excellent tool for allelic discrimination experiments. MGB probes have lower background signal resulting in better precision and the MGB moiety stabilizes probe hybridization raising Tm and reducing probe length requirement (1, 12). PCR has supplemented or replaced cross virus neutralization (VN) assays for strain differentiation of infectious bursal disease virus and replaced the hemagglutination-inhibition and VN for genotyping the spike gene of infectious bronchitis virus (5, 10, 11). RT-PCR has replaced lengthy multiple passage VI processes for avian metapneumovirus, a virus that has proven difficult to culture.

With the advent of capillary electrophoresis based Sanger sequencing, scientists are able to sequence an entire genome or the gene of interest. Sanger sequencing is used to determine the amino acid sequence of the fusion and hemagglutinin gene of Newcastle disease virus and avian influenza virus, respectively, for molecular pathotyping. Nucleotide sequencing technology is widely adopted in laboratories around the world and is listed as an acceptable test for many diseases by the World Organization for Animal Health (OIE). Sanger sequencing technology is hampered by inherent limitations including cost and speed (6, 7). To overcome these barriers, an entirely new technology was developed – Next-Generation Sequencing (NGS), a fundamentally different approach to sequencing. The newer technologies constitute various strategies that rely on a combination of template preparation, sequencing and imaging, and genome alignment and assembly methods. On the research side, a major advance offered by NGS is the ability to produce an enormous volume of data cheaply. On the diagnostic side, a distinct advantage is the capability to sequence an organism for which there is insufficient knowledge to perform primer-based PCR (6).

DNA microarray is a collection of microscopic DNA spots synthesized directly onto to a solid surface in an orderly arrangement, or "array." Microarray is routinely used in research to measure gene expression and to genotype regions of a genome. A panviral microarray has been developed for the identification of taxonomically conserved viral protein microdomains (4). A similar concept and design (ViroChip) was used to characterize the severe acute respiratory syndrome (SARS) coronavirus (4). The panviral microarray was used to detect Reston ebolavirus (REBOV) in domestic swine in the Philippines experiencing severe outbreaks of porcine reproductive and respiratory disease syndrome. An unidentified virus was isolated and subjected to microarray analysis which produced positive signals for the REBOV L gene. As demonstrated by the identification of the SARS and REBOV, DNA microarray is a diagnostic tool for emerging diseases.

Historically the indirect enzyme-linked immunosorbent assay (ELISA) has been the main screening method for serosurveillance due to sensitivity and ease of automation. Charles River Services (Wilmington, MA) has developed a Multiplexed Fluorometric ImmunoAssay (MFIA) for serosurveillance (3). Antigen is covalently linked to suspended polystyrene microspheres. Analysis is conducted with an xMAP 96-well-microplate suspension microarray fluorescence reader (Luminex®, Billerica, MA). Microspheres come in multiple distinct color sets, allowing for multiplex reactions. Beads pass one at a time through the detector where the bead's color set which corresponds to a particular analyte is read (3). MFIA is not currently being used for standard diagnostic testing, but current diagnostic trends favor multiplexing procedures that reduce hands-on time and use automated readers.

Currently the trend in diagnostic surveillance testing favors procedures that are compatible with multiplexing, allowing for the detection of multiple diseases with one test run, providing rapid single day test results with automated instrumentation.

Multiplexing allows the laboratory to use one enzyme chemistry, extraction kit and instrument for all tests in a disease group. While multiplexing does help reduce cost and provide an overall diagnostic picture, it is important to veterinary diagnostics that in-house multiplex assays be fully evaluated for sensitivity and specificity, more is not always better.

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MODIFIED TECHNIQUE FOR HATCHER AND ENVIRONMENTAL FLUFF TESTING

C. Warren, S. Weber, and R. Crespo

Avian Health and Food Safety Laboratory-Washington Animal Disease Diagnostic Laboratory, Washington State University, 2607 West Pioneer, Puyallup, WA 98371

SUMMARY

Hatchery hygiene is recognized as an important factor in healthy poultry production. Hatcheries typically test for total bacterial loads, fungal counts (specifically *Aspergillus fumigatus*), and presence of *Salmonella* spp. Testing methods include open plate environmental testing, specific surface swabbing, and microbiological examination of hatchery fluff. Hatcher fluff was one of the first sample choices introduced and it is still used today (3, 4). Chick fluff is tested after each hatch to monitor effective cleaning practices so that actions can be taken accordingly to prevent the spread of bacteria and fungus.

Standard fluff testing procedure requires mixing 0.5 g of fluff sample into 25 mL of sterile water. Then 0.1 mL (1:50) is pour plated to Sabouraud Dextrose Agar (SDA). The mixture is further diluted to 1:5000 for total bacteria counts in Plate Count Agar (PCA) and Violet Red Bile Agar. This method is time consuming and cumbersome. To facilitate faster turnaround times and reduce costs, the Avian Health and Food Safety Laboratory have validated a modified protocol for microbiological analysis of fluff. This protocol combines techniques from tried hatcher testing protocols and quicker high through put methods commonly used in the food industry.

Volume vs. weight. Because submissions are collected by the client, the sample integrity varies greatly. Eggshells and larger feathers are normally included in the submitted samples. These non-fluff items change the weight of the sample dramatically, and consequently the amount of actual fluff tested may vary and skew test results. To combat this issue, the

AHFSL uses a tablespoon (5), which is approximately 0.5 g of pure fluff. Volume allows for quicker, and more selective sampling.

Filter vs. standard bags. When the sterile water is added to the fluff, the sample becomes a thick slurry. This makes testing difficult and chunks of fluff may be introduced in various quantities to the cultures. To avoid this problem, whirl-pak filter bags (Nasco, WI) are now used. These filter bags allow the fluff to be washed by the sterile water and remain sequestered when aliquots are removed during testing.

Petrifilms vs standard agar plates. The use of pour plates (plate count agar) requires the technician to devote a block of time to making and using them without interruption. To free up space in incubators and technician time, the food safety industry regularly uses Petrifilms (3M, MN) to perform bacterial and fungal counts (1, 2). These plates have been designed to replace pour plates and spread plates. These Aerobic, Yeast/Mold, and Coliform Petrifilms are also easier to read, with more uniform colony shapes and indicator dyes for quick colony identification.

By using this modified protocol, it is possible to provide more effective and efficient results to clients while reducing the turnaround time or the need for expensive automation.

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USE OF NEXT GENERATION SEQUENCING PLATFORM TO DO FULL GENOME SEQUENCING OF PATHOGENIC BACTERIA

F. C Leung

School of Biological Sciences
The University of Hong Kong

SUMMARY

The next generation sequencing platform technology has transformed the human and bacterial genome sequencing. Such platform technology has shortened the human genome sequencing from years down to less than one year, and bacterial genome sequencing from years down to months, maybe weeks. So far such platform technology is available only to large research group and genome research and core service centers. The introduction of the 454Jr and MiSeq has again changed the paradigm where these table top units can be placed in single Principle Investigation research laboratory.

Full and complete (no gaps) bacteria genomes are necessary to study the evolution of the bacteria. We have taken on the challenge of developing a working protocol using these new types of table top personal sequencers, 454Jr and MiSeq where we do full and complete bacterial genome sequencing in less than one month. I will report our learning experiences using this new platform technology in doing full bacterial genome sequencing.

(The full-length manuscript will be submitted to *Veterinary Microbiology* for consideration for publication.)

HIGHLY PATHOGENIC AVIAN INFLUENZA H7N3 OUTBREAK IN MEXICO

A. Heneidi

Director of Epidemiology and Risk Analysis
SENASICA-SAGARPA. Mexico

BACKGROUND

In May 1994 the first official identification in Mexico of the low pathogenic avian influenza virus (LPAIV) was made. This led to investigation of this disease in the national poultry production. As a result, the serologic presence or virus isolation of subtype H5N2 was detected in almost half of the national territory. Therefore, a national program for its control and eradication was established.

Currently, this program keeps activities on epidemiological surveillance in the poultry industry, backyard self-consumption poultry, fighting birds, as well as in slaughterhouses by official serological and virological sampling. From June 2011, the regulation established for the avian influenza virus (AIV) of mandatory notification (H5 and H7) or notifiable AIV (NAIV) was reinforced and improved in some other aspects with diagnostic techniques and the epidemiological surveillance activities, harmonizing them with the international guidelines of the World Organization for Animal Health (OIE).

Based on this, certification of compartments free from NAIV was started at national level, which included, since its very beginning, a large proportion of poultry farms located in the State of Jalisco (which were later affected by the highly pathogenic avian influenza subtype H7N3), determining that in these farms subjected to official serological and virus sampling, with samples being processed in official laboratories, between April and May 2012, there was no evidence of the circulation of any NAIV.

However, in June 18, 2012, authorities from Secretariat of Agriculture, Livestock Production, Rural Development, Fishery and Food (*Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación* or *SAGARPA*, by its name and initials in Spanish) received a telephone call with a notification from Tepatitlan, State of Jalisco that reported high mortality in layer birds in the zone.

Between June 19th and 20th, three diagnostic laboratories authorized for the diagnosis of avian influenza by the National Service of Agro Alimentary Health, Safety and Quality (*Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria* or *SENASICA*) notified and submit to the National System on Epidemiological Surveillance (*Sistema Nacional de Vigilancia Epidemiológica*) eight hemagglutination isolates negative to Newcastle disease virus and that did not correspond to the AIV subtype H5.

On June 19th, official sampling was started on farms reported with an increase of mortality and suggestive clinical evidence of avian influenza (AI), as the first step in the preparation of the diagnosis of the situation. At this phase of the investigation, it was considered the possibility of vaccine application according to the extension and magnitude that the sanitary problem may present.

Between June 20th and 24th, the *SENASICA* official laboratories confirmed the presence of a highly pathogenic avian influenza virus (HPAIV) Type A, subtype H7N3, an exotic virus for the national poultry production. On June 22nd, reports to the OIE were issued and between 22nd and 24th of the same month, the zoosanitary information was published on the *SENASICA* web page.

On June 25th, based on the carried out official sampling, an internal quarantine was established in the Municipalities of Tepatitlan and Acatic, both in the State of Jalisco, where the three index cases were identified. Quarantine was implemented according to what wasestablished in Title IV, Articles 54, 56, 63, and 64 of the Federal Law on Animal Health. Simultaneously, not being biological products available in the international market, a program for the production of a national vaccine against AIV subtype H7N3 was started by *SAGARPA* using a LPAIV subtype H7N3 isolated from wild ducks in 2006, with the support of the National Manufacturer of Veterinary Biologicals (*Productora Nacional de Biológicos Veterinarios* or *PRONABIVE*). Three other private laboratories participated in the manufacturing of the biological product, resulting in the releasing of the first vaccine batch of 10 million doses on July 26th. The vaccine was tested for safety and efficacy (potency) in México by the National Center for Animal Health (*Centro Nacional de Salud Animal* or *CENASA*) and later by the USDA.

From June 25th to August 20th, the epidemiological sampling performed by *SENASICA* in different poultry production units under risk, allowed to identify the presence of the HPAIV H7N3 in 44 commercial farms, with a susceptible population of

11.4 million commercial layers located in eight municipalities in the State of Jalisco.

Between June 27th and July 5th, eight mobile federal inspection posts were installed for the control of the movement of birds, their products and byproducts within the quarantine region, which were in charge of the *SENASICA* official personnel, supported by the Federal Police and later by the National Defense Secretariat (*Secretaría de la Defensa Nacional* or *SEDENA*), who stayed in duty until the closure of the operative.

On July 2nd, it was published on the Official Gazette of the Federation (*Diario Oficial de la Federación* or *DOF*) the agreement by which the National Mechanism for Emergency in Animal Health (*Dispositivo Nacional de Emergencia de Salud Animal*) was activated under the terms of Article 78 of the Federal Law on Animal Health; and on July 8th, the epidemiological surveillance areas were established containing focal, perifocal and buffer areas, currently including approximately 20,000 km^2.

On August 20th, and as part of the first vaccination phase, 81.7 million doses were delivered for their application in 338 poultry Production Units. On a second vaccination phase, around 58.2 million doses were delivered, and by the end of October, as part of a third vaccination stage, 21.7 million doses were delivered, completing a total of 161.6 million doses against AI subtype H7N3, which allowed the control and eradication of the HPAIV H7N3 in the region of Los Altos, in the State of Jalisco.

IMPORTANCE OF POULTRY PRODUCTION IN MEXICO

Poultry production in Mexico represents an important business in the national livestock production, with a participation of 63.45% (including egg, poultry meat and turkey meat). From this, egg production outstands with 29.52% of the national livestock production for 2011, reaching 2,538,137 tons produced, as shown in Table 1.

The importance of the poultry sector in Mexico, especially in the table egg production, lies on its high level of production, which scores it in the fifth place worldwide and Mexico as the first consumer of table eggs.

The State of Jalisco is the most important state producing table eggs in Mexico, providing 55% of the domestic production, as shown on Table 2. This is the reason for the huge impact in sanitary, economic, commercial, social, and politic aspects resulted from this outbreak of HPAIV subtype H7N3 in the region called Los Altos in the State of Jalisco.

In conformity with the inventories of the Mexican Poultry Producers Association (*Unión de Avicultores de México*), the population exposed and under risk of this outbreak was more than 80 M layers and around 17 million broilers at the cycle.

POSSIBLE ORIGIN OF THE OUTBREAK

It has been identified that within the last years, outbreaks of NAIV (H7N3, H7N2, H5N2, and H5N1) occurred in the USA, Canada, Europe, and Asia, and have been caused by direct or indirect contact between domestic/commercial birds with infected wild birds.

During the epidemiological surveillance activities in migratory waterfowls performed in Mexico in 2006, a LPAIV subtype H7N3 was isolated from a migratory wild duck in the center of the country. Between 2007 and 2008, activities in the north of the country were carried out, identifying by partial sequencing the presence of hemagglutinins H5, H6 and H9, while the amino acid sequencing indicate that such viruses corresponded to LPAI viruses.

It is important to emphasize that the natural flow of migratory waterfowls infected with AIV subtype H7N3 occur in the entire American continent, mainly along the Pacific coast, according to what has been published by different authors. During the Jalisco outbreak, between August and October 2012, official epidemiological sampling and additional specific sampling were carried out and the HPAIV H7N3 was isolated from endemic wild non-aquatic birds.

As above mentioned, since 1994 an epidemiological surveillance program has been established in the country for NAIV, and from June 2011 official epidemiological sampling and additional specific sampling in commercial farms have been carried out in order to give recognition to compartments free from NAIV including the farms in the State of Jalisco, and specifically in the region of Los Altos, where the outbreak took place. Analyses indicated that in this affected region no presence of NAIV was identified until April-May 2012 (according to what indicated in the official re-sampling). It is important to indicate that in many of the compartments previously recognized as free from any NAIV, a HPAIV H7N3 was detected between June and August 2012.

It is possible that a LPAIV H7N3 has been circulating in migratory waterfowls and then transmitted to endemic non-aquatic fowls that finally may have entered in contact with commercial birds; or that the virus may have been transmitted directly or indirectly to commercial birds and simultaneously to endemic non-aquatic wild birds. The official epidemiological investigation is still in process.

MAIN EPIDEMIOLOGICAL INDICATORS OF THE OUTBREAK

The preliminary epidemiological analysis identifies the main epidemiological indicators in the directly affected population that correspond to a general morbidity rate of 25.20%, as well as a mortality rate of 10.33%, with a mortality rate of 40.98%, having affected commercial layer and heavy breeder birds.

From the 44 commercial poultry farms in the State of Jalisco where the *SENASICA* personnel isolated the HPAIV H7N3, 54.5% of isolations occurred in the municipality of Tepatitlan de Morelos, 15.9% in Acatic, 13.6% in Teocaltiche, and 16% in the rest of the region.

It is important to mention that during the HPAIV H7N3 outbreak that occurred in the region of Los Altos in the State of Jalisco, only commercial layers and heavy breeder birds were affected. From the total identified foci, 86.4% of virus isolates occurred in commercial layers and 13.6% in heavy breeders.

The HPAIV H7N3, in addition to being isolated from commercial layers and breeders, was isolated from endemic wild birds at the beginning of August from a Great-tailed Grackle (*Quiscalus quiscula* (Icteridae)), or *Zanate Común* by its name in Spanish; and by the end of September from a Barn Swallow (*Hirundo rustica* (Hirundinidae)), or *Golondrina Común* by its name in Spanish. Both isolations occurred in the municipality of Tepatitlan de Morelos. The general rate in affected wild birds was 91.7% morbidity, 75% mortality, and 81.8% mortality rate.

EPIDEMIOLOGICAL SURVEILLANCE AND OUTBREAK CLOSURE

On November 16th, 2012, the official veterinary services from *SENASICA* declared the HPAIV subtype H7N3 outbreak as concluded in the State of Jalisco, Mexico. After an 88 d period of the last occurance of the last virus isolation in the commercial poultry production; this is equivalent to 4.2 incubation periods in conformity with what established by the OIE. By December 28th, 2012, a 133 d period had passed since the last virus isolation, equivalent to 6.33 incubation periods.

Since the beginning of the outbreak until its official closure on November 16th, 2012, during the epidemiological surveillance activities, 42 municipalities in the State of Jalisco have been sampled, with a total of 712 poultry production units from which 513 corresponded to commercial farms and 199 were backyard self-consumption producing properties, including fighting birds.

The personnel who participated in the HPAIV H7N3 control and eradication operative was formed by 87 technicians from which, 64 were *SENASICA* official veterinarians, seven were temporarily hired, and 16 were from the Jalisco Committee for the Livestock Production Promotion (*Comité de Fomento y Protección Pecuaria de Jalisco*).

National epidemiological surveillance. So far, until December 28, 2012, including the State of Jalisco, a total of 2,263 production units has been sampled in the country, with an inventory of around 287.3 million birds. Sampling was done by obtaining of 199,158 serologic and virus samples, with no detection of the presence of the HPAIV H7N3 out from the eight affected municipalities in the State of Jalisco.

Calculation of economic loss. According to the information provided by the Mexico National Poultry Producers Association, the HPAIV H7N3 outbreak has left damages to the poultry industry of at least 8,617 M pesos, equivalent to nearly 663 million US dollars.

The main affects suffered by the poultry producers were the mortality and the slaughtering of the layers, importantly in the municipalities of Tepatitlan and San Juan de los Lagos, both in the State of Jalisco. This situation resulted in a reduction in the egg production, a reduction in the income, and a loss of employment. The bird loss is estimated at 22.4 million birds (includes mortality, the depopulation of infected flocks and of healthy flocks as a preventive measure) and the loss of 7,688 jobs, along with the resulting lack of supply and increased prices and imports.

CONCLUSIONS

The HPAIV subtype H7N3 outbreak, which occurred in Mexico at the beginning of June 2012, only affected the northeastern zone of the western State of Jalisco, and was controlled and eradicated by the *SENASICA* Mexican veterinary services within an approximate period of five months, beginning from the official notification date (June-November 2012).

The epidemiological surveillance activities already carried out in the country, allowed in 2006 the isolation of a LPAIV H7N3 from wild ducks in a swamp in the State of Mexico in the central part of the country, identifying the circulation of this subtype of AIV in migratory waterfowl. This isolate was later used for the manufacturing of the Mexican killed vaccine that favored the eradication of the pathogenic virus.

The HPAIV H7N3 was only isolated from commercial layer birds, breeders, and from endemic wild birds.

SENASICA implemented new diagnosis techniques within a term of seven to ten d, which allowed to count with HI test and PCR specific for the subtype H7N3, allowing the identification and

prevention of the spreading and establishment of the virus in other poultry production zones in the country.

The official control of movement of birds, poultry products, and byproducts implemented in the zone under quarantine by *SENASICA* with the support of the army and the federal police, restricted the movement of poultry merchandises with high risk of contamination and likely dissemination of the pathogenic virus.

The potential, the infrastructure, and the experience of the Mexican official technicians, allowed the production of a killed vaccine against the AIV H7N3 within a period of approximately 30 d, and the application of more than 161 milliondoses in about 90 d with vaccination squads formed by the poultry producers, favoring the control and preventing the spreading of the virus to susceptible flocks.

The creation of a vaccine bank (at least 50 million doses) and a bank of working seed was carried out for any future contingency. Vaccination will be suspended and an emergency vaccination program will be in place, in case it is needed.

The HPAIV H7N3 outbreak which occurred in the State of Jalisco, Mexico, had no zoonotic implication in other domestic animals or in public health, similar to the HPAIV H5N2 outbreak occurring in Mexico in 1994 and 1995. There was also no implication with the current LPAIV H5N2, which has affected some commercial and backyard farms in Mexico. Transmission to other domestic animal species, such as swine, was not detected; nor was it detected in human beings.

Derived from the worldwide experience in the outbreak control and eradication, it is necessary to reinforce the biosecurity and Good Livestock Production Practices in poultry production units, as well as keeping and counting with more epidemiological surveillance activities in poultry farms, backyard produced birds, migratory and endemic birds.

A major use of molecular epidemiology in the epidemiological research and surveillance activities is needed that would allow evaluating risk factors and identifying the presence of endemic virus of NAIV in migratory waterfowl, and in consequence, to be able to prevent or estimate possible genetic modifications that may favor the mutation of the AIV to HP as well as the identification of the determinant and underlying factors for the risk of exposition of commercial and domestic birds.

The mechanisms aimed to alert poultry producers shall be kept and reinforced in order to have a prompt notification on suspected avian diseases, as well implementing a specific biosecurity contingency plan, if required.

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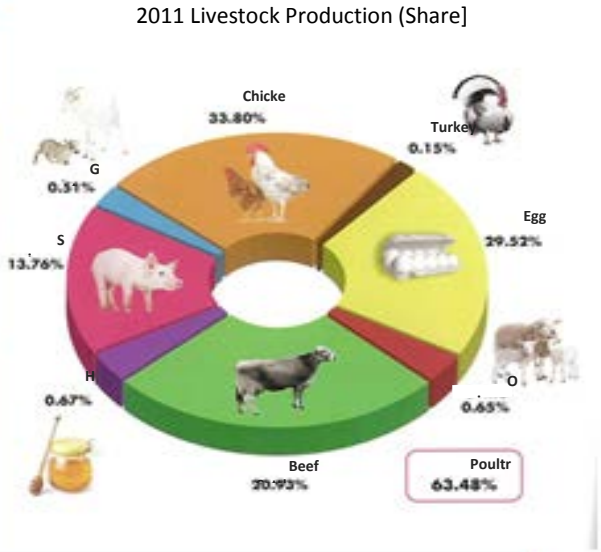
Table 1. SHARE OF POULTRY PRODUCTION IN 2011 LIVESTOCK PRODUCTION

Type of Product	Production Volume (ton)	Million Pesos	Share (%)
Egg	2'538,137	35,450	29.52
Chicken	2'906,214	58,386	33.80
Turkey	13,103	694	0.15
Total	5'457,454	94,530	63.48

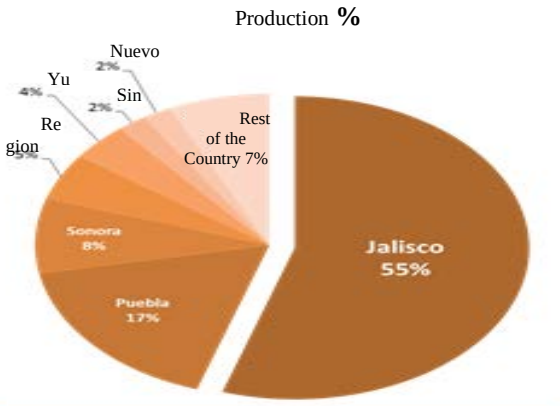


Volume of National Livestock Production
8'598,478 tons

Source: Compendio de indicadores económicos del sector avícola 2012, Unión Nacional de Avicultores.

Table 2. Main States Producers of Egg

State	Production %
Jalisco	55
Puebla	17
Sonora	8
Region Lagunera	5
Yucatan	4
Sinaloa	2
Nuevo Leon	2
Rest of the	7



Mexico is in 5th place in egg production world wide

EFFICACY OF INACTIVATED AVIAN INFLUENZA H7 VACCINES AGAINST MEXICAN H7N3 HIGHLY PATHOGENIC AVIAN INFLUENZA VIRUS CHALLENGE

D. Kapczynski[A], M. Pantin-Jackwood[A], Y. Ricardez[B], S. Guzman[B], E. Spackman[A], D. Suarez[A], and D. Swayne[A]

[A] Exotic & Emerging Avian Viral Diseases, Southeast Poultry Research Laboratory, USDA-ARS, Athens, Georgia 30605

[2]Exotic Diseases of Animals (CPA), SENASICA, Mexico

A recent outbreak of highly pathogenic avian influenza (HPAI) H7N3 was reported in Jalisco, Mexico, beginning in June of 2012. To date, more than 11 million birds have died or been slaughtered in an effort to stop the spread of disease. In response to the outbreak, vaccine efficacy trials were recently performed to determine if U.S. and Mexican-origin inactivated H7 vaccine would protect birds from clinical disease and shedding of virus. In the first set of experiments, four phylogenetically-related U.S. low pathogenic avian influenza (LPAI) isolates, either H7N2 or H7N3, were formulated into inactivated emulsion vaccines and injected into seven wk old SPF birds. These isolates contained between 92-97 amino acid similarity to the hemagglutinin gene of the challenge virus (A/chicken/Jalisco/CPA1/2012 H7N3). The APHIS-approved H7 vaccine antigens were included into this experiment for testing. Birds were challenged at ten wk of age with 106 EID50 per bird delivered via intranasal route. Results demonstrate that three of the four H7 vaccine isolates tested provided 100 % protection, whereas the fourth isolate provided 90 % protection. In the second experiment, a Mexican-lineage LPAI H7N3 isolate from wild birds, with 98 % sequence similarity to the HPAI virus, was formulated into an inactivated vaccine and applied to two wk old birds. Birds were challenge as previously described and demonstrated 100 % protection from challenge. All vaccines tested reduced shedding of virus compared to sham vaccinated birds. Taken together, these results indicate that both U.S. and Mexican vaccine isolates can provide protection to poultry against this recent HPAI H7N3 virus.

(This work has been submitted for publication.)

IMMUNOSUPPRESSIVE EFFECTS OF "VARIANT" INFECTIOUS BURSAL DISEASE VIRUS IN BROILERS IN CANADA

S. Kurukulasuriya[A], D. Ojkic[B], T. Zachar[A], S. Popowich[B], S. Tikoo[C], and S. Gomis[A]

[A]Department of Veterinary Pathology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Saskatchewan, Canada
[B]Animal Health Laboratory, Laboratory Services Division, University of Guelph, Canada
[C]Vaccinology and Immunotherapeutic program, School of Public Health, University of Saskatchewan, Saskatoon, SK. Canada S7N 5E3

ABSTRACT

Infectious bursal disease virus (IBDV) is a highly immunosuppressive disease of young chickens which causes significant economic losses to the poultry industry. Protection against IBDV in broilers is achieved by hyperimmunization of their parents. Recent studies have demonstrated that the majority of IBDV strains circulated in Canada are variant strains of IBDV and are capable of immunosuppression in broilers. The objective of this study was to investigate the immunosuppressive effects of variant IBDV strains isolated in Canada in commercial broilers and SPF birds. Groups of SPF and commercial broilers were orally administered with five (*i.e*. NC171 "like", Del-E "like" , 05SA8, Prezetto-BR or 586 "like") variant strains of IBDV at one d or three d post-hatch. The percentage of bursal weight to body weight (BW: BW), bursal histopathology, and antibody titers were taken at d 19 and 35 post-hatch. Groups of SPF and commercial broilers that were not infected with IBDV had a percentage of BW: BW of 0.58± 0.16 and 0.19 ± 0.04 respectively. In contrast, SPF and commercial birds infected with IBDV had a percentage of BW: BW of 0.12±0.02 and 0.09 ± 0.08 respectively. Severe bursal atrophy was noted on microscopic examination of bursae in both SPF and commercial broilers at 35 d post-hatch. The average antibody titer against IBDV was over 8,000 in SPF birds at 35 d post-hatch and similarly, the average antibody titer against IBDV was over 4,000 in commercial broilers at 35 d post-hatch.

This study demonstrates that variant strains of IBDV circulated in Canada were capable of crossing the maternal antibody barrier and causing severe bursal atrophy in commercial broilers. In order to demonstrate immunosuppression of broilers following infection with variant IBDV, broilers were infected with a virulent strain of *E. coli*. Variant IBDVs increased the mortality of broilers at a significant level following *E. coli* infection.

INTRODUCTION

Infectious bursal disease virus (IBDV) belongs to the family Birnaviridae, an acute and highly contagious disease of two to six wk old chickens (1,2). The most severe consequence of IBDV infection is the functional loss of the bursa of Fabricius (BF). Subtypes of IBDV emerged that could not be controlled by immunization with vaccines prepared from classic IBDV strains, and were called variants (4). These IBDVs escape the immunity elicited by classic vaccines (5-6). Variant strains do not cause obvious clinical disease, but induce severe immunosuppression. The immunosuppression resulting from an IBDV infection is the underlying cause of many cases of respiratory and enteric disease in chickens and vaccination failures. Vaccination with attenuated virus is widely used worldwide to control IBD in broilers (7). One of the major problems with attenuated IBD vaccines is their sensitivity to maternally derived antibodies which are always present at the time of vaccination. One way to approach this issue is the use of less attenuated "intermediate" vaccines, but these vaccines can themselves cause a degree of vaccine-induced lesions in the BF (8). Because the IBD virus is resistant to many disinfectants and environmental factors, once a poultry house becomes contaminated, the disease tends to recur in subsequent flocks. Control of IBD has been complicated by the recognition of variant strains of the IBD virus in broiler barns in many provinces of Canada (9). The objective of this study was to identify virulence of variant IBDV strains in Canada.

MATERIALS AND METHODS

IBDV variant strains (NC171 "like", Del-E "like", 05SA8, Prezetto-BR or 586 "like) were isolated from bursa of Fabricius of commercial broiler chickens in Saskatchewan, Canada. Identity of variant IBD strains was conducted by sequencing of VP2 region of IBDV strains at the Animal Health Laboratory, University of Guelph. Following identification, bursal homogenates were prepared and individual strains were

propagated in SPF birds. Briefly, six-day- old SPF birds were orally inoculated with ($1x10^3$-$1x10^4$ $TCID_{50}$ per bird) variant IBDVs and bursae were harvested at 19 d post-hatch. Following harvesting of bursae from SPF birds sequencing was conducted to confirm the genotype of IBDV. These variant IBDV stocks were used in all animal experiments conducted in commercial broiler chickens. The percentage of bursal weight to body weight (BW: BW), bursal histopathology and antibody titers were taken at 19 and 35 d post-hatch.

In order to study immunosuppressive affects of variant IBDVs, groups of broilers (n=20) were infected with individual variant IBDV strains at one or six d post-hatch. A virulent isolate of *E. coli* was intramuscularly administered at 20 d post-hatch. Mortality, clinical signs, and bacterial isolations from air sacs were conducted for seven d following *E. coli* challenge.

RESULTS AND DISCUSSION

Groups of SPF and commercial broilers that were not infected with IBDV had a percentage of BW: BW of 0.58± 0.16 and 0.19 ± 0.04 respectively. In contrast, SPF and commercial birds infected with IBDV had a percentage of BW: BW of 0.12±0.02 and 0.09 ± 0.08 respectively. Severe bursal atrophy was noted on microscopic examination of bursae in both SPF and commercial broilers at 35 d post-hatch. The average antibody titer against IBDV was over 8,000 in SPF birds at 35 d post-hatch and similarly, the average antibody titer against IBDV was over 4,000 in commercial broilers at 35 d post-hatch. Birds infected with individual variant IBDV strains prior to *E. coli* challenge had significantly higher mortality. This study demonstrates that variant strains of IBDV circulated in Canada were capable of crossing the maternal antibody barrier and causing severe bursal atrophy in commercial broilers.

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MONITORING *IN OVO* SINGLE BROILER VACCINATION AGAINST IBD WITH AN IMMUNE-COMPLEX IBD VACCINE IN SPAIN FROM 2009 TO 2012

J.L. Balaguer[A], R. Sales[A], C. Garcia[B], A. L. Tudón[B], P. Catalá-Gregori[B], F. Lozano[C], and M. Paniago[C]

[A]CEVA Animal Health, Barcelona, Spain
[B]Centro de Calidad Avícola y Alimentación Animal de la Comunidad Valenciana CECAV, Castellón, Spain
[C]CEVA Animal Health, Libourne, France

ABSTRACT

Auditing proper vaccine preparation and vaccination technique at the hatchery is critical to obtain consistent and uniform immunization of broiler flocks, particularly when using a single *in ovo* vaccination against infectious bursal disease (IBD). The objective of this study was to monitor the result of an *in ovo* single broiler vaccination against IBD using an immune-complex IBD vaccine. A total of 20,610 serum samples were collected from over 1,400 broiler flocks raised in the Cataluña region of Spain during a four year period (2009 to 2012). Approximately 10 to 15 serum samples randomly taken from broiler flocks between 26 to 69 d of age were analyzed serologically using a commercially available indirect ELISA. Additionally, bursa samples were also randomly collected in 2012 for RT-PCR analysis to detect if the

vaccine virus strain (Winterfield 2512) was present. The serological monitoring showed raising antibody titers as broilers aged; ranging most of them between 6000 and 8000 by 50 d of age and a reduction in the coefficient of variation down to 21% at 47 d of age in the year 2012. The RT-PCR results showed 100% presence of the vaccine virus strain in the bursa tissue of the vaccinated flocks examined.

INTRODUCTION

Proper vaccine handling at the hatchery and its efficient administration to each day-old chick, or embryonated chicken egg, is critical to obtain consistent and uniform flock immunization, especially when using a single vaccine dose to protect against a specific disease during the life of the bird. This is the case for the protection against infectious bursal disease (IBD) by administering via *in ovo*, a single dose of an immune-complex IBD vaccine, to 18 d old chicken embryos. Evaluation of the serological response to this vaccine administration during the broiler grow out period in conjunction with the detection of the vaccine virus strain in the bursal tissue will determine the efficiency and uniformity of the vaccination process at the hatchery.

OBJECTIVE

The objective of this study was to monitor, during 2009 to 2012, the serological response of commercial broilers raised in the Cataluna region of Spain, to the immune-complex IBD vaccine administered at the hatchery via *in ovo* using a specific commercially available IBDV ELISA kit in conjuction with bursal sampling for the vaccine IBDV strain (Winterfield 2512) detection in the bursa by RTT-PCR and sequencing.

MATERIALS & METHODS

Broiler flocks. Over 1,400 broiler flocks raised in the Cataluña region of Spain within 2009 -2012 period, which received a single *in ovo* vaccination against IBD using an immune-complex IBD vaccine (Cevac TRANSMUNE®). They were monitored at different ages (from 26 to 69 d of age) for their serological response.

Parameters measured. A total of 20,610 serum samples (approximately 10 to 15 blood samples per flock) collected from the selected broiler flocks were serologically evaluated by using a commercially available IBD ELISA kit (Idexx). Most of the blood samples were taken at the processing plant, but other sampling was conducted during the grow out period. Additionally, random sampling of bursas were analyzed by RT-PCR for the detection and sequencing of the infectious bursal disease virus present in the bursal tissue.

RESULTS

Serology. The IBD ELISA results obtained from the serum samples collected during the four year period showed a raising trend as the broilers grow reaching in the majority of flocks an ELISA geometric mean (GM) titer ranging between 6,000 and 8,000 (Fig. 1 and 3). The coefficient of variation (CV) decreased over the years showing the lowest percent value down to 21% at 47 d of age in the year 2012 (Fig. 2).

RT-PCR. Results of bursa tissues submitted for RT-PCR analysis and sequencing showed 100% detection of the IBDV vaccine strain (Winterfield 2512) in the immune-complex vaccinated flocks (Table 1).

DISCUSSION

Monitoring proper vaccine handling at the hatchery and its efficient administration to each day-old chick or embryonated chicken egg is critical to obtain consistent and uniform flock immunization, especially when using a single vaccine dose to protect against a specific disease during the life of the bird. This field serological monitoring helped to evaluate hatchery efficiency in the administration of a single *in ovo* vaccination using an immune-complex IBD vaccine and confirmed the quality of the vaccine administration.

The virus strain in the immune-complex IBD vaccine used in this study reaches the bursa at variable ages depending on the level of maternally-derived antibodies present at hatch. This individual broiler variation in vaccine "take" is reflected in the serological GM titers and percent of CV; it stabilizes at approximately 38 to 40 d of age.

The considerable number of serum samples evaluated with the commercially available ELISA test, most of them at slaughter time, helped to outline the GM titer range at different broiler ages. Additionally, the reduction in the coefficient of variation confirmed the consistency and improvement of the proper hatchery vaccination. Finally, the detection of the IBDV vaccine strain (Winterfield 2512) in 100% of the bursa tissues submitted for RT-PCR analysis validate the quality of the hatchery vaccination technique over the years.

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Table 1. Broiler flocks from different farms from a poultry company which received a single *in ovo* vaccination with an immune-complex IBD vaccine evaluated by PCR and serology in 2012.

		PCR		Serology	
Flock	Age	Result	Sequence	GMT	%Cv
1	41	Positive	W2512	6798	17
2	34	Positive	W2512	5768	9
3	33	Positive	W2512	3162	59
4	30	Positive	W2512	ND	ND
5	33	Positive	W2512	ND	ND
6	33	Positive	W2512	ND	ND
7	35	Positive	W2512	5176	41
8	40	Positive	W2512	6953	29
Average	34.87	100%	100%	5571	31

Fig. 1. ELISA GM Titers in Vaccinated Broilers with an Immune-Complex IBD Vaccine (2009 - 2012]

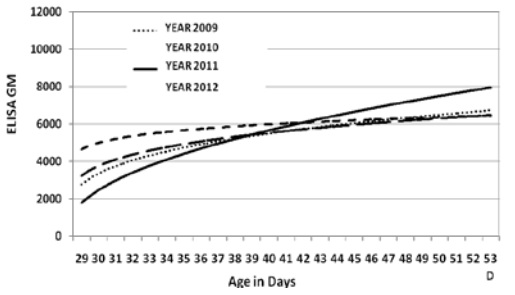
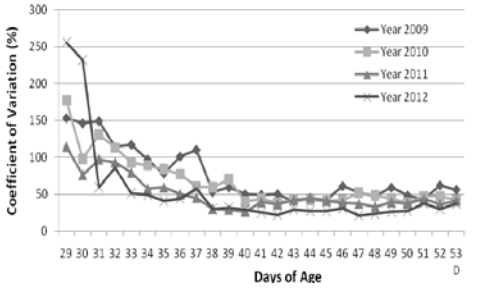
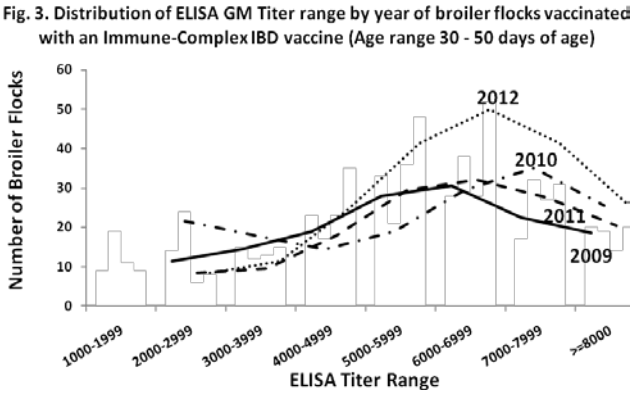


Fig. 2. Serological Coefficient of Variation in Vaccinated Broilers with an Immune - Complex IBD Vaccine (2009 - 2012)





SEROLOGICAL DETECTION OF *MYCOPLASMA SYNOVIAE* IN CHALLENGED AND CONTACT EXPOSED CHICKENS USING A NEW ELISA

G. F. Slacum[A], B. van Leerdam[B], and B. van Dam[B]

[A]BioChek USA Corp, 3 Southgate Road, Scarborough, ME 04074
[B]BioChek BV, Burg. Bracklaan 57, 2811 BP Reeuwijk, The Netherlands

SUMMARY

Early detection of new *Mycoplasma synoviae* (MS) infections through monitoring programs is essential in controlling and eradicating MS infections. Historically, monitoring programs for MS have been based on the use of rapid plate agglutination (RPA), hemagglutination inhibition (HI), and enzyme-linked immunosorbent assays (ELISAs). However, opinions vary regarding the sensitivity of the RPA and ELISA. Previous studies in which the ELISA showed lower sensitivity were conducted with commercially available MS ELISAs produced using whole cell antigen. Recently, a new MS ELISA based on a recombinant antigen with excellent specificity (>98%) has been approved for use in the U.S.

In this study, the sensitivity of the recently approved MS ELISA was compared to MS RPA for the detection of MS from seven through 21 d post-challenge (dpc). The sensitivity of the ELISA (BioChek) and RPA was tested using different lots of each assay on the same samples derived from broiler breeders challenged at four wk of age with MS strains WVU 1853 and K5664 and from comingled broiler breeders.

Both the Mg/Ms ELISA and RPA detected positive birds in the WVU 1853 challenged group starting seven dpc. However, the ELISA detected a greater number of positive birds than the RPA. Positive birds in the K5664 group were detected in a small percentage of birds starting at seven dpc by ELISA and nine dpc by RPA. In the birds exposed to strains WVU 1853 and K5664 by contact, the ELISA detected positive birds starting at seven dpc, while the RPA detected positive birds starting at 14 dpc.

ACKNOWLEDGEMENTS

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EVALUATION OF MINIMUM INHIBITORY CONCENTRATION (MIC) OF STRAINS OF *MYCOPLASMA GALLISEPTICUM* (MG) ISOLATED FROM UNITED STATES POULTRY INDUSTRY

R. Gilbert[A], N. Ferguson-Noel[B], and S. Catania[C]

[A] Elanco Animal Health, 2500 Innovation Way, Indianapolis, IN 46140
[B] Poultry Diagnostic & Research Center, 953 College Station Rd., Athens, GA, 30602-4875
[C] Istituto Zooprofilattico Sperimentale delle Venezie, Viale dell'Università 10, 35020 Legnaro (PD), Italy

INTRODUCTION

The minimum inhibitory concentration (MIC) is the lowest concentration of an antibiotic that will inhibit the visible growth of a microorganism in *in vitro*. The MIC can be interpreted to determine which antimicrobial agent might be effective. Therefore, MIC values can be a tool for the veterinarian to make an appropriate selection of antimicrobial agent to use in a disease treatment. A lower MIC for a given antibiotic can be an indication of a better therapeutic choice than if the isolate has a high MIC.

Mycoplasma gallisepticum (MG) is an important poultry pathogen that is associated with chronic respiratory disease and severe loses in production in both layer and meat type birds. When flocks become infected, antibiotics are often used to control the disease and minimize the shed from hen to progeny. The purpose of this study was to evaluate the antibiotic susceptibility of MG strains isolated recently from the various sectors of the US poultry industry, in order to advise poultry veterinarians to make appropriate antimicrobial therapy decisions.

MATERIALS AND METHODS

The ten MG strains were isolated and characterized at the Poultry Diagnostic & Research Center in Athens, Georgia. These isolates were selected from cases submitted within the past five years. Strain selection was based on the region of the US and the type of bird. Although none of the flocks were vaccinated, three of the samples (2, 4, and 8) were classified as 'vaccine like" based on their genetic characteristics. Table 1 gives the history of each MG strain selected.

Once selected, the strains were re-isolated to confirm viability and lyophilization was used to preserve the isolates during shipping. For re-isolation, the samples were inoculated in Frey's modified broth and agar and incubated at 37 °C. Mycoplasma isolates were identified using direct immunofluorescence.(1) The lyophilization was conducted using one mL of actively growing cultures, that were pre-frozen at 70 °C and lyophilized using a Labconco® Freeze Dry System (FreeZone 4.5) according to the manufacturers recommendations.

Once the strains arrived at the lab (Mycoplasma Unit, Istituto Zooprofilattico Sperimentale delle Venezie in Italy), propagation of strains and MIC tests were performed using the Mycoplasma Experience® Media without inhibitors. The antimicrobial susceptibility testing method is an internal procedure of Mycoplasma Unit of IZS Venezie (PDP DIA 103-Measurement of minimum inhibitory concentration in mycoplasmas of veterinary interest). This internal procedure is based on the guidelines of Hannan (2) and international guidelines for human mycoplasmas (3). The antimicrobial agents are commercially available in microtitre plates (Sensititre®). Plates were incubated at 37 ± 1 °C. Each strain was tested in duplicate and the MG reference strain (ATCC 15302) was included in the study. Each plate contained a positive and negative control as well. Antibiotics and their relative concentrations are shown in Table 2. MIC plate readings were done 24 to 48 h after inoculation, when the positive control well showed a typical acidification and color change, revealing the mycoplasma growth. MIC breakpoints used in this study are reported in Table 3 (4) (5). It is noted that the breakpoints used are specific to the studies and methods reported by the authors. Since there are no internationally recognized mycoplasma interpretive criteria available from organizations such as the Clinical and Laboratory Standards Institute, the data interpretations should be considered as advisory for veterinarians.

RESULTS AND DISCUSSION

In all strains tested, the positive control well showed obvious growth after 24 to 48 h of incubation; whereas no growth was observed in the negative control well. According to the procedure, the plates were read when the positive control well showed growth, indicated by a color change. For the antibiotics tested, the MIC for the ATCC quality control strain

was similar to that observed in prior studies in the laboratory and thus the test was considered valid. The MIC values are the lowest concentrations (µg/mL) where no mycoplasma growth occurred, MIC results are available in Table 4. Calculations of the MIC_{50} (the MIC able to inhibit growth in 50% of the strains tested) and MIC_{90} (the MIC able to inhibit growth in 90% of the strains tested) are reported in Table 5.

The tested MG strains had the lowest MIC results for tylosin and erythromycin. Based on Table 3, these results were below the stated breakpoint for each antimicrobial agent, suggesting an appropriate treatment choice. The MIC results for oxytetracycline ranged from 1µg/mL to 8µg/mL. Based on the breakpoint for Oxytetracycline, this antimicrobial agent would also be a viable treatment option. The MIC results for Lincomycin suggested that only Sample 3 would be considered resistant.

SUMMARY

MIC testing gives the unique opportunity to evaluate whether phenotypic antibiotic resistance may have developed. This information, along with the veterinarian's field experience, is essential for judicious use of antimicrobials and a successful therapeutic outcome for treated animals.

This study focused on the antimicrobial susceptibility of MG field strains from the US Poultry Industry. The data from this study supports the continued use of tylosin, erythromycin, oxytetracycline and lincomycin as treatment options for MG. Since other countries may use antimicrobials differently or more frequently, contributing to a difference in resistance patterns, this study should be repeated using MG strains that are local to that area. This type of study may provide a greater understanding of what antimicrobial would be the best treatment of choice in each country.

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Table 1. MG sample identification.

Sample ID#	Year	Region	Bird type	Clinical signs	Vaccine like[A]
1	2010	AR	Broiler Breeder	Not available	No
2	2010	GA	Broiler	Not available	TS-11-like
3	2010	IN	Turkey	Yes	No
4	2010	GA	Broiler	Not available	TS-11-like
5	2009	PA	Layer breeder	No	No
6	2009	WV	Layer	No	No
7	2009	GA	Turkey	Yes	No
8	2009	PA	Turkey	Not available	F-strain-like
9	2009	GA	Broiler	Yes	Not available
10	2009	GA	Broiler	Yes	Not available

[A]As determined by targeted sequencing of mgc2 and IGSR (6,7)

Table 2. Antimicrobial agents tested at various concentrations (µg/mL).

	Tylosin	Lincomycin	Erythromycin	Oxytetracycline
A	0.12	0.12	0.12	0.12
B	0.25	0.25	0.25	0.25
C	0.5	0.5	0.5	0.5
D	1	1	1	1
F	2	2	2	2
G	8	8	8	8
H	32	32	32	32

Table 3. Antimicrobial agent breakpoints used in this study.

Antimicrobial Agent	**Breakpoint (µg/mL)**		
	Sensitive	Intermediate	Resistant
Tylosin (Hannan et al., 1997)	≤1	≤ 2	≥ 4
Lincomycin (Kempf et al., 1989)	≤ 2-8	≤ 8	> 8
Erythromycin (Kempf et al., 1989)	≤1-4	≤4	> 4
Oxytetracycline (Hannan et al., 1997)	≤ 4	8	≥ 16

Table 4. MICs (µg/mL) of 10 field *Mycoplasma gallisepticum* strains**.**

Sample ID	Tylosin	Lincomycin	Erythromycin	Oxytetracycline
1	<0.12	8	<0.12	8
2	<0.12	2	<0.12	8
3	<0.12	32	<0.12	8
4	<0.12	2	<0.12	2
5	<0.12	8	<0.12	8
6	<0.12	8	<0.12	8
7	<0.12	2	<0.12	2
8	<0.12	8	<0.12	2
9	<0.12	2	<0.12	1
10	<0.12	8	<0.12	2

Table 5. MIC_{50} and MIC_{90} (µg/mL).

Antimicrobial Agent	MIC 50	MIC 90
Tylosin	<0.12	<0.12
Lincomycin	8	8
Erythromycin	<0.12	<0.12
Oxytetracycline	2	8

DETECTION OF MG AND MS DNA IN SAMPLES SUBMITTED TO THE GEORGIA POULTRY LAB IN 2012 USING IDEXX REAL TIME PCR REAGENTS

K. Roza, W. Witbeck, P. Lopez, J. Ferrell, and P. Tyrrell

IDEXX, One Idexx Drive, Westbrook, ME, 04092

SUMMARY

Detection of DNA from pathogenic avian mycoplasmas can be challenging due to the sophisticated techniques required to achieve accurate results. Inhibition of the reaction as a result of sample contamination can occur at any step throughout the process. Most real time PCR (qPCR) assays fail to identify if inhibitors have affected the reaction and positive results may be errantly classified as negative. The Georgia Poultry Lab uses commercially available IDEXX real time PCR reagents to detect Mg and Ms DNA. These reagent sets contain an internal control, meant to detect the presence of inhibitors in the

reactions. In this paper, results generated from September to December of 2012 are reviewed. The use of this qPCR format involving hybridization probes and an internal control in each individual well is evaluated and discussed.

INTRODUCTION

The common species of mycoplasmas which are pathogenic to domestic chickens are *M. gallisepticum* (Mg) and *M. synoviae* (Ms). In many areas of the country these are reportable conditions which require governmental intervention and hold severe consequences for flocks found positive for these diseases. Acceptable methods of detection for these pathogens are outlined by the National Poultry Improvement Plan Auxiliary Provisions (NPIP).

Detection of Ms and Mg has historically been via serology (hemagglutination inhibition [HI]), serum plate agglutination (SPA) or enzyme-linked immunosorbent assay (ELISA)). Positive serologic results are often followed via detection of the organism with culture. Real time PCR is emerging as a highly accurate technique for confirmation of the presence of Ms and Mg DNA and is gaining in popularity. In September 2012, the NPIP technical committee approved the use of the IDEXX Real-time PCR reagent sets for Mg and Ms under section 147.52 of the NPIP guidelines (1).

Testing results generated by a commercial diagnostic laboratory between September and December of 2012 will be discussed here. This lab used the IDEXX reagents for their Mg and Ms testing and followed strict Standard Operating Procedures to provide the most accurate and efficient detection methods possible. The DNA samples used in their testing were processed via a boiling procedure and run in pools of five initial samples per reaction.

The results of the PCR were interpreted using two data points, the crossing point and melting temperature (Tm). The simultaneous generation of two data points for each reaction is made possible by the use of hybridization probes in these reagents. This allows for qualification and differentiation of the DNA segments of interest. The DNA sequences used to select the probes and primers were based on amplicon sequences reported by S. Kleven and Z. Raviv (2). The addition of melting curve detection provides increased diagnostic sensitivity and confidence in test results.

In each reaction an internal control was included, adhering to recommendations by the OIE for PCR use for detection of mycoplasmas (3) . This control is included in the IDEXX reagent sets. This unique template control is used to assure that no reaction inhibition occurs and that conditions were adequate to allow for DNA amplification. This provides a much higher level of confidence in negative results. Also, a negative template, negative control, and a weak positive control were used with each run to assure fidelity of the results and provide a baseline for comparison of sample values.

Positive results were determined based on the demonstration of a crossing point prior to 40 cycles of amplification, and a melting temperature within 2˚C of the target temperature, which was established for each assay by IDEXX and is published on the Certificate of Analysis. The internal control of each negative reaction was evaluated to assure that amplification had not been inhibited, which would cause a false negative result. If the internal control did not show adequate amplification, as determined by the Roche LightCycler 480 software, an alternative extraction protocol was performed and the samples were re-tested with internal control again present.

MATERIALS AND METHODS

Test samples. The samples tested were submitted to the lab from various commercial poultry flocks, often as part of the NPIP program. All samples were assigned an accession number and logged prior to testing. The samples were pooled into sets of five, and a boiling method was used to extract the DNA material from the cells. Five hundred µL of each pool was aliquoted to be used for this testing. The remaining volume was frozen for storage in case further testing was needed. The aliquots were processed using a protocol of micro-centrifugation for two min at 13,000 RPMs. The supernatant was poured off and 400 µL of nuclease free water was added to each tube. The samples were then heated to 100-110˚C for ten min, and centrifuged at 13 RPM for two, followed by rapid cooling for five min. Any sample not run immediately was stored frozen at -18℃ to -22℃ until use. Samples which contained visible debris or blood contamination were rejected from testing based on a high likelihood of reaction inhibition (4). In any reaction in which a retest was necessary, a commercially available DNA extraction kit was used to extract a new sample from the stored pool.

Assay conditions. All samples were run according to reagent set instructions, using IDEXX MG Detection Reagents, and MS Detection Reagents. Each reaction consisted of 4 µL of the applicable IDEXX master mix, 6 µL nuclease-free PCR grade water, 2 µL of detection reagent and 5 µL DNA sample. One µL of internal control positive reagent and 2 µL of internal control detection mix were also added to each well. Plates were prepared under a biosafety hood using sterile technique to avoid well to well contamination and samples and positive control were

added to the plate in a secondary location. Cycling conditions were as follows:

Activation: 95°C, 10 min, one cycle.

Amplification (45 cycles): 95°C, 20 sec; 60°C, 20 sec, single acquisitions; 73°C, 15 sec.

Melting Curve: 95°C, one min; 45°C, one min; 80°C, 0.14°C/sec ramp rate, continuous acquisitions.

All testing discussed here was performed on a Roche LightCycler 480 Real-time PCR instrument with a 96-well block, using the protocol recommended by IDEXX. The same cycling program is used for both the MG and MS assays with these reagents, allowing for simultaneous testing of both assays within a single run.

RESULTS

For this presentation, we reviewed a total of 158 pooled reactions, generated between September and December of 2012. This correlates to 790 individual samples (see Table 1). Each of the samples was tested for both Ms and Mg according to lab protocol. Of these samples, seven Mg samples and 12 Ms samples generated positive results.

The data in Tables 2 and 3 represent samples which the internal control failed to amplify. Reactions shown in bold indicate positive samples which would have been missed without the use of the internal control. Each of these was retested after a commercial extraction kit was used on the stored remainder of the initial pool. Upon retesting, the internal control successfully amplified for all reactions, indicating that an inhibiting agent was initially present in these cases.

DISCUSSION AND CONCLUSIONS

In a previous presentation at this meeting, we showed that the IDEXX real time PCR reagent sets for MS and MG demonstrated excellent sensitivity and specificity on internal validations. In this work we presented the results of samples tested in a commercial diagnostic lab that specializes in poultry testing and how the use of the internal control provided by IDEXX increased the sensitivity & confidence level of their testing.

Using the IDEXX real time PCR reagent sets, the Georgia Poultry Lab was able to detect the presence of mycoplasma DNA in samples submitted to their lab. Special attention was paid to the samples in which the internal control did not amplify in an adequate manner on the initial run. This failure to amplify by the internal control suggests that the reaction contained inhibiting agents which would interfere with the PRC ability to detect and amplify the target DNA strain if present. This provided a clear indication for re-testing. The lab chose to use an alternate extraction method for the samples which were retested.

Our results indicate that in the cases where inhibitors were present, IDEXX's PCR reagent set allowed for identification and correction of this problem. As seen in the provided data, in some cases, negative samples which were retested due to the lack of internal control amplification, became positive upon retesting. This indicates a sample which would have been incorrectly identified had an internal control not been used. For this reason we recommend the use of an internal control in each PCR reaction to increase confidence and sensitivity.

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Table 1. Total number of samples evaluated.

	Ms	Mg
Total # Reactions	158	158
Positive	12	7
Negative	146	151

Table 2. MG data collected over a four month period.

Sample	Result	IC Results	Retest Results	Retest IC
1	Neg	Neg	Neg	Pos
2	Uncertain	Neg	Neg	Pos
3	Neg	Neg	Neg	Pos
4	Neg	Neg	Neg	Pos
5	Neg	Neg	Neg	Pos
6	Neg	Neg	Neg	Pos
7	Neg	Neg	Neg	Pos
8	Neg	Neg	Neg	Pos
9	Neg	Neg	Neg	Pos
10	Neg	Neg	Neg	Pos
11	Neg	Neg	Neg	Pos
12	Neg	Neg	Neg	Pos
13	Neg	Neg	Neg	Pos
14	Neg	Neg	Neg	Pos
15	Neg	Neg	Neg	Pos
16	Neg	Neg	Neg	Pos
17	Neg	Neg	Neg	Pos
18	Neg	Neg	Neg	Pos
19	Neg	Neg	Pos	Pos
20	Neg	Neg	Neg	Pos
21	Pos	Neg	Pos	Pos
22	Pos	Neg	Pos	Pos
23	Neg	Neg	Neg	Pos

Table 3. MS data collected over a four month period.

Sample	Result	IC Results	Restest Results	Restest IC
1	Neg	Neg	Neg	Pos
2	Neg	Neg	Neg	Pos
3	Neg	Neg	Neg	Pos
4	Neg	Neg	Neg	Pos
5	Neg	Neg	Neg	Pos
6	Neg	Neg	Pos	Pos
7	Pos	Neg	Pos	Pos
8	Neg	Neg	Neg	Pos
9	Neg	Neg	Neg	Pos
10	Neg	Uncertain	Neg	Pos
11	Neg	Neg	Neg	Pos
12	Neg	Neg	Neg	Pos
13	Neg	Neg	Neg	Pos
14	Neg	Uncertain	Neg	Pos
15	Neg	Neg	Neg	Pos
16	Neg	Neg	Neg	Pos
17	Neg	Neg	Neg	Pos
18	Neg	Neg	Pos	Pos
19	Neg	Neg	Neg	Pos
20	Neg	Neg	Neg	Pos
21	Pos	Neg	Pos	Pos

EFFECT OF EGGSHELL APEX ABNORMALITIES ON STORAGE OF EGGS FROM CHICKENS NATURALLY INFECTED WITH *MYCOPLASMA SYNOVIAE* AND INFECTIOUS BRONCHITIS VIRUS

F. Santos, M. Brandão, N. Soares, C. da Silva, L. Machado, D. Abreu, V. Pereira, and E. Nascimento

Departamento de Saúde Coletiva Veterinária e Saúde Pública, Faculdade de Veterinária, Universidade Federal Fluminense, RJ 24230-340, Brazil. Email: elmiro@vm.uff.br

SUMMARY

In a free-range chicken farm, positive to infectious bronchitis by ELISA and *Mycoplasma synoviae* (MS) by ELISA and PCR, there were analyzed 58 normal and 79 eggs with eggshell apex abnormalities (EAA). The effect of EAA on egg storage, at 23-25^{o}C and humidity of 55-70%, for normal and EAA eggs was evaluated by comparing means for eggshell strength (4.24Kgf; 3.41Kgf), eggshell percentage (10.20%; 9.97%), shell weight (6.60g; 6.53g), egg apex thickness (0.4004mm; 0.3854mm), side (0.4074mm; 0.4039mm) and bottom (0.3745mm; 0.3699mm), egg weight loss (1.86g; 2.35g), and Haugh units (HU) on the fifth storage week (45.54;38.29). All egg data was obtained in a Digital Egg Tester 6000 (Nabel, São Paulo, Brazil). Eggshell thickness from EAA eggs was affected only in the apex, not in the middle and bottom of the egg. EAA eggs, from *M. synoviae* and infectious bronchitis infected layer hens, significantly affected eggshell strength, egg weight loss, egg apex thickness, and HU ($P<0,05$) by Student´s t-test.

INTRODUCTION

Deleterious effects of infectious bronchitis virus (IBV) are well known, causing decrease in external and internal egg quality, as well as drop in egg production (2); however, a new pathology in the eggshell was described in 2008 affecting flocks of hens in the Netherlands and was called eggshell apex abnormalities (EAA) (4). Eggs with EAA have rough and thin shell, increased translucence, which increases cracking and breaking. The abnormalities are at the top of the egg (pointed end), up to approximately two cm from the apex, and almost always have a well-defined region. The production of EAA eggs is associated with MS infection in the oviduct of chicken layers. This disease was first described in white hens kept in cages, but then was described in brown laying hens in cages and in other production systems (5, 7). The proportion of egg affected in a flock may be from 0.1% to 25%. (1, 5). Recently, the occurrence of EAA was reported in other countries such as Italy, Germany, and Great Britain (1, 6, 7).

The main cause of economic losses due to the EAA is due to the weakness of the shell and the consequent increase in cracks and breaks. The egg loss generates labor costs in separating eggs with abnormalities and machine cleaning due to broken eggs. Affected flocks can lose two to three eggs per hen and decrease in larger size eggs. It is estimated that in a flock with five percent of eggs with abnormalities, there is a loss in gain of three percent in a productive period of 30 to75 wk (4).

The main factors affecting egg quality during storage are temperature and relative humidity, which influence the loss of carbon dioxide and water vapor by the egg. The exchange of these gases is also related to quality of the eggshell. When the shell is thin or has defects, this exchange is facilitated (3).

The objective of this work was to assess the effect of egg storage at controlled environment on EAA eggs compared to apparently normal eggs, both egg groups obtained from the same flock.

MATERIALS AND METHODS

The eggs used in the study were from a 3,000 laying hen free-range farm with chickens ranging from 24 to 65 wk of age. The producer's initial complaint was a strong drop in egg production. Samples were collected and it was confirmed the infection by MS and negativity to *Mycoplasma gallisepticum* (MG) by ELISA, isolation, and PCR. By ELISA, seroconversion to MS and IBV was observed after three wk from the first sera collection (IDEXX Laboratories, Westbrook, ME, USA).

In order to perform the egg storage test, 58 eggs regarded as normal and 79 regarded as EAA were collected. Eggs were stored for four wk in a controlled environment at a temperature of 23 to25^{o}C and relative humidity of 55 to 70%. Eggs were separated into five groups of similar weight and analyzed at d 2, 7 14, 21, and 28 of lay. Quality parameters evaluated were eggshell strength, shell weight, egg apex thickness (measured at three points; apex – pointed end, side, and

bottom), egg weight loss, and Haugh units (HU). Averages were compared by Student´s t-test at confidence interval level of 95%. All egg data were obtained in a Digital Egg Tester 6000 (Nabel, Kyoto, Japan).

RESULTS

The parameter means for normal and EAA eggs, respectively obtained, were: eggshell strength (4.24Kgf; 3.41Kgf), eggshell percentage (10.20%; 9.97%), shell weight (6.60g; 6.53g), egg apex thickness (0.4004mm; 0.3854mm), side (0.4074mm; 0.4039mm), bottom (0.3745mm; 0.3699mm), egg weight loss (1.86g; 2.35g), and Haugh units (HU). The average results of HU are represented in the Figure 1. It was found that significant differences between EAA and normal eggs in the parameters of eggshell strength, eggshell thickness at the apex, weight loss, and HU on the fifth storage wk.

DISCUSSION

The average eggshell strength of EAA eggs was 20% lower than normal eggs. This difference was lower than that previously described (4) of EAA and normal eggs from chickens challenged for MS (32%) and not challenged (44%). Co-infection with IBV may have affected all eggs, diminishing the perception of the isolated effect of EAA.

Eggshell apex thickness averages was the only catagory with significant differences. While no differences were found for side and bottom eggshell thickness, eggshell percentage, and shell weight, it could be concluded that this was responsible for the great loss in the eggshell strength of these eggs.

There is no description in prior literature on the influence of EAA in the quality of eggs during storage. The higher weight loss by EAA eggs probably may have occurred due to the smaller apex eggshell thickness, hence increasing the rate of water vapor passage, facilitating the water loss by the egg.

Regarding HU averages, in the first period of analysis no differences were found between values from EAA and normal eggs. A previous study also did not find differences between HU results on a field study (5). However, it was noted a trend of greater reduction in the quality of EAA eggs during the period of analysis, with a significant difference observed at d 28. For the same reasons of the higher weight loss of EAA eggs, it may have occurred with CO_2 loss, rising pH of albumen faster than normal eggs, therefore, decreasing HU values.

Egg producers must be alert to the occurrence of new abnormalities in eggs that causes losses not only during processing due to an increase of cracks and breaks, but also during transport to the consumer and by losses in product quality during its shelf life.

CONCLUSIONS

The presence of EAA in table eggs, of free-range layers positive to MS and IBV, can cause a greater decrease in quality during storage than that which occurs in eggs without abnormalities.

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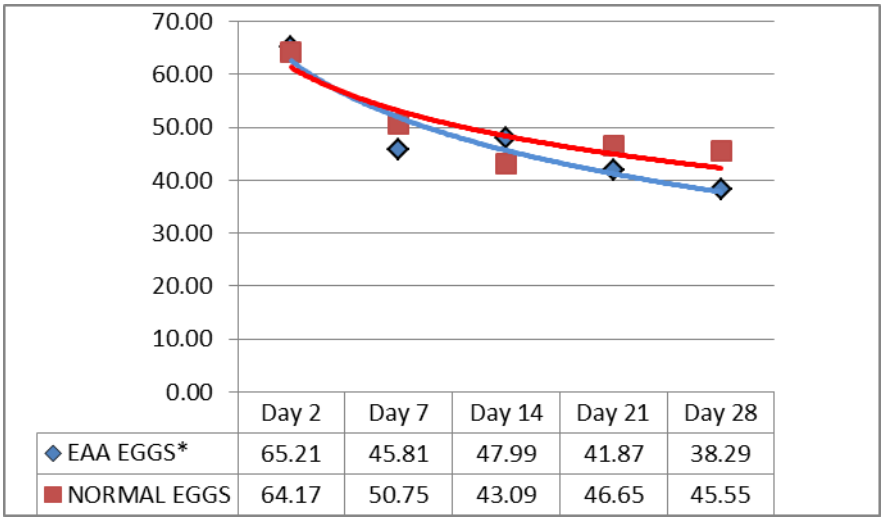
Figure 1. Average of Haugh units obtained by analysis of EAA (eggshell apex abnormalities) and normal eggs in different days of storage after laying.



A METABOLIC DRIFT LIVE ATTENUATED *SALMONELLA* GALLINARUM VACCINE AGAINST FOWL TYPHOID

A.A. Shehata[A,B], H.A. Sultan[B], H.M. Hafez[C] , and M. Krüger[A]

[A]Institute of Bacteriology and Mycology, Faculty of Veterinary Medicine, Leipzig University, An den Tierkliniken 29, D-04103 Leipzig, Germany
[B]Poultry and Rabbit Diseases Department, Faculty of Veterinary Medicine, Minoufiya University, Egypt
[C]Institute of Poultry Disease, Berlin University, Berlin, Germany

SUMMARY

The present study relates to the development of a live fowl typhoid (FT) vaccine candidate against *Salmonella* Gallinarum (SG), having two independent attenuating markers based on metabolic drift (MD), spontaneous chromosomal mutations. Such vaccine candidate fulfills the security demand of the World Health Organization (WHO). The vaccine proved to be genetically stable, safe, and effective in experimental studies**.** This attenuated vaccine strain is designed sufficiently invasive enough to induce immunity in chickens, and to protect against experimental SG challenge. The vaccine can be administered orally which is an easy and cost effective alternative to injection. Also, MD mutants could be differentiated from wild strains using culture media containing antibiotics involved in the genetic changes. The full-length article was published in Avian Diseases Journal (5).

INTRODUCTION

Fowl typhoid (FT) is a systemic disease, caused by *Salmonella enterica serovar* Gallinarum biovar Gallinarum in domestic poultry, which results in septicemia. Infection in chickens occurs at all ages and is characterized by severe hepatomegaly and splenomegaly accompanied by liver with bronzing aspect, anemia, and septicemia. Mortality and morbidity rates due to FT may reach up to 80%**.** Attenuated live *Salmonella* vaccine candidates have received considerable attention as they confer solid immunity, and it can produce systemic and mucosal immunity in the gut when administered orally because it directly stimulates the gut-associated lymphoid tissue (GLAT) and enhances production of IgA. Metabolic drift (MD) mutations induced clone-specific gradually reduced colony sizes, which are inversely correlated with the degree of the reduction in colony size. Live vaccines for *Salmonella* Typhymurium (Vac T) and *Salmonella* Enteritidis (Vac E) were previously

developed by Linde and coworkers based on the MD mutation method (3). These mutants have different attenuating markers which increase stability (incidence of back mutation). Accordingly, in the present study, a live attenuated MD vaccine against SG was developed and the protective efficacy against wild type challenge was evaluated in commercial layer chickens.

MATERIALS AND METHODS

The streptomycin (Sm) or rifampicin (Rif) metabolic drift mutants of an SG wild strain (Z34/11) were generated as previously described method (3). Briefly, single antibiotic-resistant mutants were generated by spatulating approximately 10^{10} cfu of fresh SG on Caso agar supplemented with 500 µg Sm or 300 µg Rif per mL. Plates were incubated aerobically at 37 °C for 48 h. Small resistant colonies were passaged once on antibiotic supplemented Caso agar, then on antibiotic-free Caso agar. Stable diminished colony sizes after at least 50 passages on Caso agar served as a criterion of stability and considered single marker MD mutants. The Sm and Rif attenuating marker clones were designated as SG-Sm and SG-Rif, respectively. For isolation of double marker mutants, approximately 10^{10} cfu fresh cultures of single mutants SG-Sm or SG-Rif were used for isolation of second MD markers. The SG-Sm and SG-Rif were spatulated on Caso agar containing either 300 µg Rif or 500 µg Sm per mL, respectively. The same procedures were carried out for resistant colonies to obtain stable mutants containing two MD attenuating markers as mentioned above. The resulting MD mutants containing containing two attenuating markers were designated SG-Sm-Rif or SG-Rif-Sm. To test the safety and protective efficacy, 140 one d old commercial layer chicks (Hyline) were assigned to seven groups of 20 chickens. Group treatment is shown in Table 1. Safety was assessed based on clinical signs, mortality rate, bacterial re-isolation, and gross lesion scores. At seven and 14 dpv, five chickens from each group were humanely euthanized and examined for presence of necrotic foci of the livers and spleens: the lesion scores were carried out according to Matsuda and co-workers (4). For bacterial recovery from internal organs, liver and spleen (one g of each) were collected from euthanized birds at seven and 14 dpv, and the viable bacterial cells were then determined quantitatively on Caso-agar. A sample was assumed as negative when it still was negative after enrichment in Selenite broth (Roth, Germany) for 16 h. The SG re-isolation was confirmed by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF). To distinguish the mutants from the wild type strain, specimens collected from vaccinated birds were cultured on Caso-agar supplemented with antibiotic which involved in the genetic changes. Two wk post-vaccination, ten birds from groups No. 2-7 were challenged orally with 2×10^6 cfu of SG wild strain (homologous strain) and kept under observation for 15 days. Five chickens per group were humanely euthanized, necropsied weekly for bacterial recovery and post mortem examination. Protection was evaluated based on clinical signs, mortality, Hp score and bacterial recovery from internal organs.

RESULTS AND DISCUSSION

Five MD mutant strains could be isolated and designated; SG-Rif1, SG-Sm6, SG-Rif1-Sm4, SG-Sm6-Rif10, and SG-Rif1-Sm10. The colony sizes in relation to wild type strain were reduced to 50%, 40%, 30%, 30% and 20%, respectively. The probability of a back mutation can almost be excluded as the reduced colony sizes were stable after at least 50 passages on culture media. The SG mutant strains behaved as attenuated strains in comparison with wild type strain in commercial layer chickens (Table 1). Chickens that received SG-Rif1 or SG-Sm6 (one marker vaccine) showed residual gross lesions in the liver and spleen but significantly lower ($P<0.001$) than positive control chickens (infected with *SG* wild type strain). The degree of attenuation depends on the number of attenuating marker (3). However, vaccinated groups with double marker vaccine demonstrated no significant differences in the mean gross lesion values compared to the negative control group, which resulted in zero (Table 1). To assess systemic infection, the recovery of SG from internal organs as liver and spleen was tested. Wild type strain could be isolated from the liver and spleen at 7 and 14 dpi. The recovery of SG mutant strains in these organs was significantly lower than the wild type strain ($p< 0.0001$). Following challenge with 10^6 cfu wild type homologous strain, all developed mutants except Rif1-Sm10 showed highly significant protection against homologous challenge. All non-vaccinated challenged chickens demonstrated severe depression and showed high mortality rates (6/10) (60%). The incidence of back mutation per one attenuating marker may reach 10-9, in relation to the observed frequency of back mutations of spontaneously attenuated *Shigella*-tested in volunteers, which was about 10^{-9} (1). Two markers SG mutants assure safety and the probability of a back mutation can almost be excluded (The incidence of back mutation is 10^{-18}).

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Figure 1. Bacterial recovery from liver and spleen. Specimens collected from euthanized birds and dead birds if found. Viable bacterial determined quantitatively on Caso-agar and expressed in $\log_{10}$ cfu ± SD /g.

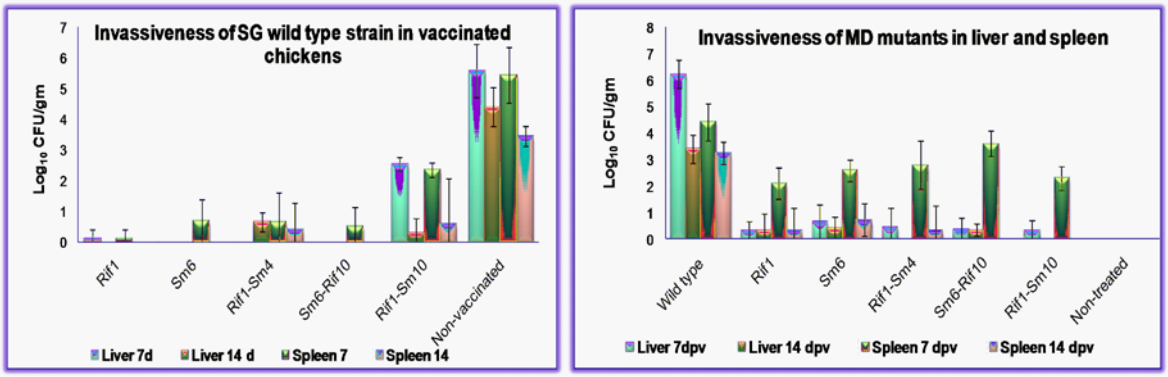

Table 1. Safety and gross lesion scores of different MD mutants compared with *SG* wild type strain.

Group No.	Strain/ MD –mutant[1]	Relative colony size[2]	Mortality for 15 dpv (n=20)	Gross lesions scores (n= 5)[3] (mean ± SD)			
				Liver		Spleen	
				Enlargement	Necrotic foci	Enlargement	Necrotic foci
1	Wild type	100%	8	3±0.7	2±0.1	2.4±0.6	2.5±0.4
2	Rif1	50%	0	0.3±0.3*	0.4±0.2*	0.5±0.3*	0
3	Sm6	40%	0	0.5±0.7*	0.5±0.5*	0.5±0.3*	0
4	Rif1-Sm4	30%	0	0	0	0	0
5	Sm6-Rif10	30%	0	0	0	0	0
6	Rif1-Sm10	20%	0	0	0	0	0
7	Non-vaccinated	-	0	0	0	0	0

[1]Chickens in group 1 inoculated orally with $2X10^6$ cfu wild type strain (positive control). Chickens in groups 2-6 vaccinated orally with $2 X 10^8$ cfu of different MD mutants. Group No.7 non-vaccinated negative control.
[2]The relative size of MD mutants in relation to wild type strain.
[3]Gross lesions score calculated as described by Matsuda and coworkers **(4).**
Values were expressed as means ± SD.
*Asterisks denote significant decrease ($p< 0.001$).

A RETROSPECTIVE STUDY OF CAUSES OF MORTALITY IN BACKYARD CHICKENS IN NORTHERN CALIFORNIA

A. Mete, F. Giannitti, B. Barr, L. Woods, and M. Anderson

California Animal Health and Food Safety Laboratory System (CAHFS), Davis, CA, 95617-1770

A five-year retrospective study was conducted to characterize the spectrum of diseases causing mortality in 1301 backyard chickens submitted to the CAHFS laboratory in Davis, California. Infectious diseases were diagnosed in the majority (60.4%). Viral diseases comprised 50% of the infectious entities followed by bacterial diseases with an incidence of 39%. Marek's disease in the viral group and *Escherichia coli* in the bacterial group were the most commonly diagnosed infectious diseases. Zoonotic agents including *Aspergillus* spp., *Salmonella* spp., *Listeria* spp., *Mycobacterium* spp., *Candida* spp. and *Baylisascaris* spp. were detected in 46 (3.5%) birds. Among non-infectious conditions, fatty liver hemorrhagic syndrome and reproductive tract adenocarcinoma were the leading causes of mortality. This analysis provides an overview of backyard chicken diseases for practitioners and avian pathologists working with backyard poultry as well as serving as a surveillance report for commercial poultry houses in the vicinity.

(The full-length paper is pending publication in *Avian Diseases*.)

SAFETY AND EFFICACY OF A COMBINED AVIAN ENCEPHALOMYELITIS – FOWL POX – PIGEON POX LIVE VIRUS VACCINE AGAINST AE AND FOWL POX VIRUS CHALLENGE IN LAYER TYPE CHICKENS

G. Sarma, B. A. Kersting, S. Jamison, and G. Spina

Hygieia Biological Laboratories
P.O. Box 8300, Woodland, CA95776
Email: gsarmax@aol.com

ABSTRACT

Avian encephalomyelitis (AE) virus infection in laying and breeding flocks causes a marked drop in egg production, decrease in egg hatchability, and high mortality in young infected chicks. Fowl pox (FP) virus causes cutaneous lesions (dry pox) or diptheritic lesions (wet pox) in infected chickens. Dry pox produces scabs and lesions around the comb, wattle, ear lobes, and eyes. Wet pox lesions are mostly seen in the oral cavity and the upper respiratory tract, especially the larynx and trachea. Wet pox causes higher mortality in infected flocks resulting in significant economic loss. Pigeon pox (PP) vaccine appears to provide better cross protection against some wet pox field strains. Therefore, in problem areas, chickens are often vaccinated with a mixture of FP and PP vaccines to control wet pox induced mortality. Although there have been reports of increased efficacy associated with various field mixtures of fowl pox and pigeon pox vaccines, the process of vaccination is labor intensive and time consuming since pigeon pox vaccine has to be separately reconstituted and then mixed with fowl pox vaccine prior to vaccination. In the present study, we evaluated a combination AE + FP + PP vaccine for its safety and efficacy in chickens aged eight wk or older. A total of 124 chickens were vaccinated with AE + FP + PP vaccine via wing web and challenged with virulent strains of avian encephalomyelitis and fowl pox viruses. Protections against fowl pox and avian encephalomyelitis virus challenge were 100% and 97% respectively. The laboratory studies indicated the vaccine to be safe for use in chickens aged eight wk or older.

INTRODUCTION

Vaccination of poultry with either FP or PP vaccines appears to generate cross-protective immune response against the other (1, 3, 4). However, the immunity induced by either vaccine alone may not be sufficient enough to protect against variant strains.

Outbreaks of FP have emerged in some previously vaccinated flocks leading to virulent field strains (variant strains) that were not controlled by use of commercial FP vaccine alone (2). The virulent strains often cause increased mortality due to respiratory obstruction (wet pox or diphtheritic form of the disease). Vaccinating chickens with both FP and PP vaccines appears to provide better protection against some hot field strains of the virus. Therefore, poultry producers in pox endemic areas routinely vaccinate their flocks with both FP and PP vaccines so as to obtain a greater spectrum of protection against the disease. For vaccinating chickens, poultry producers mix various FP containing products with PP vaccine and administer the mixed product via wing web route. However, mixing of separate products has some disadvantages including preparation error, longer time requirement for vaccine rehydration and mixing, and eventual loss of virus titer, inconsistency in vaccine potency and product contamination during preparation and processing. In the present study, we evaluated a combination vaccine containing AE+ FP + PP in a single preparation for its safety and efficacy in layer type chickens aged eight wk or older.

MATERIALS AND METHODS

A total of 124 chickens aged eight wk or older were vaccinated with the AE+FP+PP vaccine via wing web using a double needle wing web stab. The freeze dried vaccine was rehydrated with sterile diluent following the directions for use circular accompanying the product. The experiment consisted of two trials. In each trial, a group of chickens originating from the same source and flock was kept as unvaccinated challenged controls. At four to six d post vaccination, all vaccinated and control chickens were checked for vaccine induced "take" at the site of vaccination. All vaccinated chickens were observed daily for 21 d post vaccination (DPV) for development of any vaccine associated adverse reactions, mortality, and appearance of pox lesions on the comb, wattle, eyelids, and other non-feathered areas of the body. Chickens were also observed for development of clinical signs of avian encephalomyelitis including ataxia, circling, depression, paralysis, sudden death, tremors, or torticollis.

Vaccinated and positive control chickens were challenged against AE and FP at 21 DPV. The AE challenge was done intracerebrally and fowl pox challenge virus was administered through wing web route using a double needle stab. The AE challenged chickens were observed for 21 DPC for AE clinical signs such as ataxia, circling, depression, paralysis, sudden death, tremors, or torticollis. Any chicken showing clinical signs of AE and/or mortality was considered positive or unprotected. The FP challenged chickens were observed for ten DPC for clinical signs of FP such as appearance of nodular lesions at the site of challenge virus inoculation, as well as lesions or scabs at the non-inoculated, non-feathered areas of the body including the comb, wattles, eyelids and oral mucosa. Any chicken showing lesion at the challenge virus inoculation site or elsewhere was considered positive or unprotected.

RESULTS AND DISCUSSION

The vaccine was found to be safe for wing web administration in chickens aged eight wk or older. This was evidenced by the absence of any vaccine associated adverse reactions, absence of secondary pox lesions at the non-inoculated sites, and absence of AE clinical signs or mortality during the three wk post vaccination observation period (Table 1). A further evaluation of the safety of the vaccine under field conditions is in progress.

When examined at four to six DPV, 100% of the vaccinated chickens revealed good vaccine "take" at the site of vaccination. The percentages of protection against AE and FP challenge are shown in Table 1. The vaccine induced 100% protection against FP challenge in the vaccinated chickens. Protection in the unvaccinated challenged controls was 0%. The average percentage protection against AEV challenge was 97% in the vaccinated chickens and 0% in the unvaccinated challenged controls.

CONCLUSIONS

The AE – FP – PP vaccine, when tested in chickens aged eight wk or older, was found to be safe and efficacious against both FP and AE virus challenge.

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Table 1. Efficacy of AE+FP+PP vaccine against challenge with AE and fowl pox viruses.

Treatment groups	No. of birds vaccinated	No. of birds challenged		% Protection (average)		Post vaccination "Take" at the site of vaccination (%)
		AE	FP	AE	FP	
Vaccinated with AE+FP+PP vaccine	124	60	62	96.7	100	100
Unvaccinated challenged controls	0	61	62	0	0	0

The numbers in the table represent data from two trials.

USE OF A NOVEL WATER ADDITIVE TO EXTEND FLOCK PRODUCTION PARAMETERS

N. Reimers

SUMMARY

Improving shell quality, hen day production, hen housed production, and hen mortality is a continual goal in production medicine. Flocks with high production, good shell quality, and low mortality are well suited for non-molting programs. Farmers regularly test novel products and new management practices to achieve this desired aim. Water is an ideal route for product distribution as it allows for rapid addition of products through a route that is under the direct control of the farmer.

Nine flocks located on three premises representing four cohorts were included in this study. Data was collected over a 37 m period. Control flocks were provided with ad-lib city or well water. Treated flocks were provided with ad-lib city or well water and the additive injected by a proportioner on a continuous basis. No treatment was administered at the pullet ranch. Flocks were enrolled at 19 wk of age.

Farms A, C and D represent two premises and each cohort contained two flocks of caged white hens fed a conventional ration – one treated and one control on each farm. Farm A and C were Hyline W-36s. Farm D was Lohman LSLs. These farms are hand gathered and the flocks were molted at different times.

Farm B represents a single premises and a single cohort containing three flocks of cage-free, ISA Browns fed a conventional ration – two treated flocks and one control. Farm B is belt gather and hand packed. Flocks at Farm B were not molted.

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

Table 1. Results of water treatments.

Farm	Age at Molt	Age at Depopulation	Extra Eggs/Hen Housed for Treated Flocks (Age)
A	Control – 71 weeks Treated – 81 weeks	Still in lay at 96 weeks when data collection concluded for this presentation	-4.11 (96 weeks) *Treated Group in molt 3.82 (80 weeks) 5.05 (70 weeks)
B	N/A	81 weeks	27.10 (81 weeks)
C	Control - 1st 68 weeks, 2nd 109 weeks Treated – one molt 102 weeks	136 weeks	43.91 (136 weeks)
D	Control – 69 weeks Treated – no molt	99 weeks	5.62 (99 weeks)

FIELD CASES AND EXPERIMENTAL REPRODUCTION OF NECROTIC HEPATITIS AND SPLENOMEGALY IN BROWN LAYER CHICKENS ASSOCIATED WITH *COXIELLA*-LIKE ORGANISMS

C. Gabriel Sentíes-Cué[A], B. Charlton[A], L. Woods[B, C], and B. Murphy[C]

California Animal Health & Food Safety Laboratory System (CAHFS), Turlock Branch[A], Davis Branch[B]
University of California, Davis, 1550 N. Soderquist Road, Turlock, CA, 95381
[C]Department of Pathology, Microbiology, and Immunology, School of Veterinary Medicine, University of California, One Shields Avenue, Davis, CA, 95616

SUMMARY

In 2010 and 2011, multiple submissions from a brown layer operation were received at the Turlock Branch of the California Animal Health and Food Safety Laboratory System. The principal concerns in the clinical history included increased mortality and a drop in egg production. At necropsy, the most significant findings were multifocal necrotic hepatitis and splenomegaly. Aerobic and anaerobic bacteriology, special stains, and viral isolation attempts on hepatic tissue and direct electron microscopy examination of hepatic sections and bile were unsuccessful in identifying an etiology. Transmission electron microscopy and molecular analysis of hepatic sections suggest that a *Coxiella*-like organism was associated with the hepatic lesions.

In order to reproduce the disease, experimental inoculations of 26-wk-old, brown layer chickens by intraperitoneal, intravenous, and oral (by gavage) routes (IPH, IVH, and OH groups, respectively) with hepatic tissue homogenate generated from the initially affected birds were performed . For each of the groups inoculated with hepatic tissue homogenate, control chickens were inoculated with sterile saline solution in parallel. All the birds were observed daily for any clinical signs during 15 d before euthanasia, necropsy examination, and sample testing. Mild lethargy starting on the third day post inoculation was seen in two birds of the IPH group. PCR results for *Coxiella*-like infection, gross lesions, bacteriology, and chicken infectious anemia virus (CAV) serology by the ELISA tests of the hepatic homogenate inoculated groups are presented in Table 1. No virus was isolated from liver tissue pools of the IPH, IVH, and OH groups. The PCR results for *Coxiella*-like infection performed on the same type of samples taken from the control groups were all negative. No significant gross lesions were found in any of the birds of the control groups. All the birds of the control groups were serologically positive for CAV by the ELISA test, except one bird of the orally inoculated group.

Positive PCR results for *Coxiella*-like infection on spleen, liver, blood, and cloaca samples from birds inoculated with liver tissue homogenate of sick birds indicates that a *Coxiella*-like infection was able to establish a systemic infection in 26-wk-old brown layers and shed in the feces regardless of the inoculation route used in this experiment. Although no specific pathogen was detected in the liver exhibiting multifocal necrotizing hepatitis, splenomegaly seen in some birds of the IPH and IVH groups suggests that *Coxiella*-like infections may be able to cause significant pathological changes. Since *Pasteurella multocida* was isolated from the ovary and *Escherichia coli* was isolated from the oviduct of liver-homogenate inoculated birds, it could be speculated that *Coxiella*-like infection may be a contributing factor for the pathological manifestation of preexisting subclinical infections in 26-wk-old brown layers. No correlation was found between positive CAV serology and *Coxiella*-like PCR results. *Coxiella*-like infections in birds have been reported previously in a toucan and psittacines (1,2), but to the author´s knowledge this is the first report of a naturally occurring *Coxiella*-like infection in commercial chickens and its experimental reproduction.

(The full article will be submitted to *Avian Diseases*.)

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Table 1. PCR results, gross and microscopic lesions, and CAV serology in the hepatic homogenate inoculated groups.

		IPH grp.	IVH grp.	OH grp.
Positive PCR Results	Cloacal swab	1/5	1/5	1/5
	Liver	1/5	2/5	2/5
	Blood		1/5	1/5
	Spleen	5/5	5/5	5/5
Gross and Microscopic Lesions	Multifocal necrotizing hepatitis	1/5		
	Splenomegaly	1/5 moderate, 1/5 mild	2/5 moderate	
	Lymphocytic necrosis in splenic white pulp	3/5 moderate		1/5 moderate, mild 2/5, very mild 1/5
	Lymphocytic infiltration in splenic capsule	1/5 mild		
	Splenic congestion		2/5	
	Oophoritis	1/5*	1/5*	
	Yolk peritonitis	1/5		
	Involuted ovarian follicles		1/5	
	Salpingitis			1/5**
Positive CAV Serology (ELISA)		5/5	5/5	5/5

* *Pasteurella multocida* isolated.
** *Escherichia coli* isolated.

DIAGNOSIS OF CHLAMYDIA IN COMMERCIAL TURKEYS IS LIKE OPENING A CAN OF WORMS

H. L. Shivaprasad[A], S. Carnaccini[A], M. Bland[B], M. Lee-Dutra[C], R. Aaziz[D], R. Moeller[A], P. Blanchard[A], and K. Laroucau[D]

[A]California Animal Health and Food Safety Laboratory System - Tulare Branch, University of California, Davis, Tulare, CA 93274
[B]Cutler Veterinary Associates International, P. O. Box 1042, Moorpark, CA, 93020
[C]California Department of Food and Agriculture, Animal Health Branch, Tulare, CA 93274
[D]University Paris Anses, Animal Health Laboratory, Bacterial Zoonoses Unit, French Food Safety Agency (AFSSA), 94706, Maisons-Alfort, France

SUMMARY

Chlamydiosis is a naturally occurring contagious systemic and zoonotic disease of various species of birds including turkeys, caused by *Chlamydophila psittaci*. Several outbreaks of chlamydiosis in turkeys, as well as in employees of the turkey processing plants have been described in the United States. Chlamydiosis

in turkeys is characterized by respiratory, nasal and ocular signs, and increased morbidity and mortality. The lesions of chlamydiosis in turkeys include conjunctivitis, airsacculitis, pericarditis, perihepatitis and pneumonia. Chlamydiosis can be diagnosed by fluorescent antibody (FA) test, isolation in chicken embryos, cell culture, complement fixation test, immunohistochemistry, and PCR.

An unusual outbreak of chlamydiosis occurred in 15,000 13 wk old turkeys organically grown in an open range housing system. The disease was characterized by unilateral or bilateral swelling above the eye due to inflammation of the nasal glands in 3 to 5 % of the birds. Except for a slight drop in feed and water consumption, the birds did not exhibit any respiratory signs. An increased mortality in the flock was not detected. Chlamydiosis was confirmed by FA and immunohistochemistry of the nasal glands in the early stages of the disease. Other samples, such as conjunctiva, lungs, air sacs, heart, liver/spleen, and feces were negative for chlamydia by FA in 23 birds submitted for necropsy over several wk. Chlamydiosis, further confirmed by PCR, was isolated in chicken egg embryos and typed as *Chlamydophila psittaci*; it was genotyped as genotype B by multilocus sequence variable number of tandem repeats (VNTR) analysis (MLVA), multilocus sequence typing (MLST) analysis, and *omp*A gene sequencing. The isolate was found to be similar to the reference CP3 *C. psittaci* strain isolated from a pigeon.

In order to maintain the "organic" status of the flock, the owner did not want to treat the turkeys with antibiotics. Food Safety Inspection Services (FSIS) of USDA refused to process the turkeys based upon historical information. The flock was quarantined by the California Department of Agriculture (CDFA).

This was an unusual outbreak of chlamydiosis with low risk of transmission. Based on the laboratory tests performed, the disease was not systemic and was confined only to the nasal glands. Approximately 3000 turkeys with clinical signs of swelling above the eye were culled. The remaining turkeys were processed by FSIS without any incidence.

With possible significant economic losses, the owner faced a serious dilemma as to whether to treat or not to treat for chlamydia. In addtion to treatment options, handling and disposal of infected birds and the basis of refusal by the FSIS to process the turkeys will be discussed. Also, the prophylactic measures taken by those exposed to chlamydia, the roles of the laboratory, the university, the county, and the state public health officials and OSHA personnel, CDFA and the logistical challenges of necropsying birds in the laboratory will be presented. Importantly, the significance of this unique chlamydia affecting the nasal glands in the turkeys in the context of testing, zoonotic potential, processing of turkeys, source and transmission, and outcome of the disease will be presented.

SUMMARY OF DISEASES DIAGNOSED IN BROILER CHICKENS SUBMITTED TO THE CALIFORNIA ANIMAL HEALTH AND FOOD SAFTEY LABORATORY SYSTEM, 2010-2012

S. Carnaccini[A], R. Chin[A], G. Senties[B], B. Charlton[B], D. Mills[B], P. Woolcock[C], and H. L. Shivaprasad[A]

California Animal Health and Food Safety Laboratory System, Tulare[A], Turlock[B], and Davis[C] Branches

SUMMARY

Approximately 290 to 300 million broiler chickens are grown per year in California. The majority of these chickens are White Plymouth Rock and White Cornish breeds and they are processed commonly around 42 d of age. A small percentage of these chickens are also being raised as antibiotic free and organic chickens, but their numbers are increasing. California also raises about four to five million Brown/Red and Silkie chickens and they are being sold in the live market when they are between eight and 20 wk of age; their numbers are also increasing.

Four diagnostic laboratories of the California Animal Health and Food Safety Laboratory System (CAHFS) provide diagnostic services to the poultry and livestock industries of California. Most of the broiler industry is located in the central valley of California and laboratories located in Tulare and Turlock provides diagnostic services to them. Data on the number accessions (submissions) and pathologic conditions observed in broiler chickens submitted between January 2010 and December 2012 to the Tulare and Turlock branches of CAHFS were analyzed. The vast majority of the chickens examined were White Cornish and White Plymouth Rock breed

chickens but the data on Brown/Red and Silkie chickens were also included. Diseases and conditions were diagnosed based on gross and microscopic lesions, serology (for AI, NDV, IBV, IBDV, MG, and MS and occasionally for reovirus, CIAV, ILT, and AE.), bacterial/mycological and virus isolations, parasitology, biotechnology (RT-PCR for AI, IBV, IBDV, NDV, etc. and sequencing for IBV), negative stain electron microscopy for enteric viruses on the intestine, and toxicology including nutritional analysis on the feed.

The number of accessions reviewed for the period 2010 and 2012 were 832 necropsy cases and 658 non-necropsy (mostly for serology, virology, bacteriology, etc.) cases. The summary provided here is for necropsy cases only. The total numbers of broiler chickens examined for this period were 9800. The age of the chickens examined ranged from one day old to over 16 wk of age. The most common reasons for submissions of chickens to the laboratories for disease diagnoses were increased mortality, respiratory signs, unevenness in the flock, loss of weight/runting stunting syndrome, and digestive problems. Other signs such as ill thrift, anorexia, depression, locomotor problems, increased condemnations at the processing plant, neurological signs, leg problems, sudden death, flock monitoring etc. were reported less frequently.

The most frequent diseases or conditions diagnosed during this period were bursal disorders, with IBDV as one of the probable causes; coccidiosis, most of the time in association with necrotic enteritis, *Clostridium perfringens*; colibacillosis, infectious bronchitis, in particular Cal 99 serotype was frequently isolated; proventriculitis; enteritis of unknown cause, probably due to a viral infection; omphalitis (yolk sac and umbilical infections), most commonly diagnosed in baby chicks; gangrenous dermatitis and cellulitis, associated with *E. coli* or *C. perfringens* and occasionally *Staphylococcus aureus*; bordetellosis (*Bordetella avium* and occasionally *B. hinzii*); toxicosis, most of the time due to ionophores such as monensin, salinomycin and nicarbazin, and sodium; miscellaneous diseases included tibial dyschondroplasia, femoral head necrosis , tracheal and bursal cryptosporidiosis, dehydration, renal urate deposit (gout), starve out, trauma, cannibalism, eye abnormalities, hepatic disorders, hernia, etc.

EVIDENCE OF SEROTYPE CROSS PROTECTION AGAINST FOWL CHOLERA BY VACCINATION WITH SIDEROPHORE RECEPTORS AND PORINS®

C. Carver, D. Emery, D. Straub, and B. Wileman

Epitopix, LLC. 1801 Biotech Ave NE, Willmar, MN 56288
*Corresponding author: Charlie.Carver@Epitopix.com

SUMMARY

Fowl cholera is a highly contagious, lethal disease affecting both wild and domestic fowl. *Pasteurella multocida* is the etiologic agent responsible for disease, and conventional fowl cholera vaccines are widely used to protect commercial chicken and turkey flocks. For typical commercial bacterins to be effective, whole cells of three or four common serotypes are included. These vaccine formulations are serotype-specific, thus only protecting against the serotypes included in the vaccine. Many poultry systems have decided to experiment with autogenous whole-cell bacterins that contain serotypes not represented in the available commercial vaccine formulations to protect against these other serotypes.

Epitopix has developed a fowl cholera vaccine using the company's Siderophore Receptor and Porin (SRP®) technology that in this study offered protection against a heterologous challenge. The vaccine was produced using a *Pasteurella multocida* SRP bacterial extract from a serotype 3x4 strain in a water-in-oil adjuvant. Vaccinate and control chickens were challenged IM with serotype 1 *Pasteurella multocida*, strain X-73, and observed for mortality for 14 d. Results of 100% protection in vaccinated and 100% mortality in unvaccinated controls are evidence of protection across serotypes.

BACKGROUND

A necessary characteristic of any bacterial pathogen is the ability to survive and proliferate successfully within its host. This proves difficult due to the hostile environment the bacterium encounters during host invasion, which includes conditions such as limited oxygen availability, osmotic and pH stress, the presence of antimicrobial enzymes, and nutrient

restriction. Many of these obstacles are overcome by the protective role of the major pore-forming porin proteins located in the outer membrane of gram negative bacteria that allow solute diffusion or mediate active transport. There are four major groups of porins identified: the general porins; the monomeric porins; the specific porins; and the TonB-dependent, gated porins (iron-siderophore receptor proteins). The different types of porins allow the pathogen to survive in many different environments. For example, iron is one of the primary nutrients in limited supply during microbial invasion of a host species because the iron is complexed with high affinity, host iron-binding proteins such as transferrin in blood, lactoferrin in secretory fluids, ovotransferrin in albumin, and ferritin within cells. Since nearly all species of bacteria require iron for electron transport and as co-factors for essential metabolic enzymes, the low availability of iron within a host is an initial barrier to infection that microorganisms must overcome in order to proliferate. Bacterial pathogens have developed strategies for obtaining iron from their hosts. A common method bacteria employ to acquire iron is the secretion of small iron-chelating molecules, siderophores, which bind iron with high affinity and "steal" iron directly from the host binding proteins. The outer membrane porins that serve as iron-siderophore receptor proteins bind the iron-bound siderophores and interact with other membrane-associated proteins to internalize the iron. In addition, the general porins also serve a major role during infection; these proteins regulate the pathogen's internal solute concentration in the changing osmotic environment in host tissues. The siderophore receptor and other porins, collectively referred to as the SRPs, are surface exposed, highly conserved and expressed in high copy number on the outer membrane. These traits make SRPs good candidate antigens for vaccine development.

MATERIALS AND METHODS

General protocol. The vaccination/challenge protocol was adapted from 9CFR 113.117 potency testing for Pasteurella Multocida Bacterin, Avian Isolate, Type 1(1)).

Chickens. Specific Pathogen Free leghorn chickens were obtained from Charles River Laboratories, (North Franklin, CT) and grown to 22 wk of age. The chickens were divided into three groups of ten with equal numbers of males and females in each group. Groups were identified by color-coded leg bands. All chickens were housed together and provided antibiotic-free feed and water ad libitum.

Vaccination. Chickens were vaccinated IM in the breast muscle at 22 wk of age and again 21 d later. Group 1 chickens were vaccinated with SRP vaccine prepared from a 3x4 serotype *Pasteurella multocida* isolated from a turkey on a commercial farm in Minnesota. Group 2 test subjects were vaccinated with a homologous whole-cell bacterin as a positive control. Group 3 birds served as unvaccinated negative controls.

Challenge. The challenge organism was obtained from USDA-APHIS Veterinary Services, Center for Veterinary Biologics and designated serotype 1 challenge strain X-73. The challenge strain was passed twice in healthy chickens to increase virulence prior to challenge. The resulting culture was identified to species via Oxiferm II tubes (Becton Dickenson, Heidelberg, Germany) and frozen in trypticase soy broth with 10% glycerol. The frozen seed was grown overnight, passed into fresh broth, grown for approximately three h, and diluted in tryptose broth to a target 5000cfu/mL final concentration.

All birds were challenged in the breast muscle with a target 2500 cfu (in 0.5 mL) freshly grown and diluted culture 14 d following the second vaccination. Challenge cultures were kept on ice throughout the challenge. Immediately following challenge, an unused vial of challenge material was transported back to the lab and plated onto TSA II 5% sheep blood agar to confirm inoculum level.

Observations. Chickens were observed for mortality daily by the research farm specialist. Any moribund birds were euthanized per the Center for Veterinary Biologics Testing Protocol SAM 607 (2).

RESULTS

Mortality in the unvaccinated control group was 100% (10/10). No mortality was observed in the heterologous SRP vaccinated group or the homologous whole-cell vaccinated group. The challenge preparation level was confirmed to be 2400cfu/dose by plate count.

DISCUSSION

Fowl cholera is present worldwide and can be transmitted by common birds and animals. Infection of flocks can lead to high mortality in general, and negative productivity in layers and breeders specifically. While treatment and removal of infected birds is required for control, strict biosecurity and vaccination programs are central to the prevention of large outbreaks of disease in commercial flocks.

Commercial vaccines for fowl cholera generally come in two types: killed bacterins and live attenuated vaccines. Killed bacterins are serotype specific; success of vaccination depends largely on whether the vaccine contains the right components for the serotypes involved in the outbreaks. Attenuated live vaccines

have short lived immunity so the need for multiple revaccinations is common. Attenuated live vaccines may also cause illness in some birds post-vaccination. Better protection has been offered by autogenous killed vaccines using serotypes specific to the farm site but they do not protect against serotypes that are not present in the vaccine.

The SRP technology has been used in several commercial poultry operations for over a decade in autogenous form to successfully control fowl cholera, and is currently used in the conditionally licensed bovine *Salmonella* Newport and *Escherichia coli* Bacterial Extract vaccines produced by Epitopix. The *E. coli* vaccine was the first vaccine in the U.S. to demonstrate a reduction in *E. coli* O157 shedding and prevalence (3). *Salmonella* Newport Bacterial Extract vaccine effectively helps control infection and fecal shedding of *Salmonella* Newport, resulting in reduced disease incidence and improved herd performance (4).

The present study provides evidence that *Pasteurella multocida* SRP protects across a heterologous serotype of virulent *Pasteurella multocida*. This technology may be effectively applied in USDA licensed or autogenous vaccine formulations and shows promise to protect against multiple serotypes of *Pasteurella multocida* in commercial poultry operations.

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TRADITIONAL CAGES FOR LAYERS *VS.* ENRICHED CAGES OR SLAT FLOOR

D. Gallazzi[A], V. Cesari[B], P. Guarneri[A], G. Grilli[A], and I. Toschi[B]

[A]Dipartimento di Scienze Veterinarie e Sanità Pubblica
Università degli Studi di Milano. Via Celoria 10 – 20133 Milano, Italy
[B]Dipartimento di Scienze Agrarie e Ambientali - Produzione, Territorio, Agroenergia
Università degli Studi di Milano. Via Celoria 2 – 20133 Milano, Italy

ABSTRACT

In 15 layers farms, ten with traditional cages (690737 hens), four with enriched cages (688929 hens), and one with slat floor (14498 hens), egg production, number and weight of eggs, FCR and mortality were registered during a whole production cycle (from 18 to 56 wk of age). Hybrid line, origin of pullets, environmental and hygienic conditions, light programs, and diets were the same in all farms.

On the average, the comparison between traditional and enriched cages shows that there were not significant differences on productive performance with number and weight of eggs, feed intake, and feed conversion ratio. The mortality was significantly lower in hens housed in enriched cages in comparison to those reared in traditional cages, showing a higher level of welfare in animals housed in enriched cages.

Preliminary results registered on laying hens housed on slat floor seem to reduce productive performance, even if the lower number of defective eggs could be a positive aspect of this housing system.

INTRODUCTION

The use of conventional cages was prohibited by European Union on January 1, 2012 for their negative effects on the welfare of hens (2).

With the ban of traditional battery cages, characterized by at least 550 cm^2 per hen of cage area, some research was performed to study the effects of different housing systems on productive performance of laying hens. Enriched (or modified) cages, with at

least 750 cm^2 of cage area per hen (600 cm^2 of which shall be usable), are the most commonly used cages on commercial egg production farms, even if scientific data regarding its effect on egg quality and productive performance of laying hens are few. Recently, alternative systems, such as slat floor, are becoming more prevelant on commercial layer farms, but information concerning its influence on egg production is not yet available.

The aim of this preliminary trial was to compare productive performance of laying hens reared on traditional cages, enriched cages, and slat floor.

MATERIALS AND METHODS

The study was performed on 15 layer farms located in Northern Italy: Ten layer farms using traditional cages, four commercial facilities using enriched cages, and one layer farm using slat floor system.

In the farms using traditional and enriched cages, a multiple-tier systems was used (from four to ten floors), while in the alternative housing system, hens were housed in slatted floor with at least one third of the ground surface occupied by litter (wood shavings).

The same strain of laying hens was used and laying hens were moved to layer houses at the same age (16 wk) in all housing systems. Commercial diets with similar formulation and chemical composition were given by the same supplier; water and food were available *ad libitum.* Environmental conditions and light program were similar among the different layer facilities. Productive data about feed consumption and then number and weight of eggs were registered during the whole production cycle. Egg production was calculated as kg of egg produced per hen and feed conversion ratio (FCR) was calculated as kg of feed consumed per kg of egg produced. The collected eggs were classified as "normal"or "defective eggs."

Moreover, mortality was daily registered. Data recorded were analyzed using Student's t-test.

RESULTS AND DISCUSSION

The mean values of variables according to the different cage systems are reported in the Table 1, while preliminary data recorded on slat floor system are commented in the text.

In the present study, the comparison between traditional and enriched cages shows that there are no significant differences in productive performance. Number of eggs and average egg weight, in fact, were similar between the two different housing systems (310 *vs*. 324 and 62.5g *vs*. 61.5 g, respectively for traditional and enriched cages). Feed intake, comparing traditional and enriched systems, did not show significant differences; FCR results were the same in both housing systems (2.04 kg feed/kg eggs). These values seem to suggest that the use of enriched cages did not worsen productive performance of laying hens (4).

Preliminary results registered on laying hens housed on slat floor, instead, seem to suggest a lower egg production (18.8 kg) and egg weight (60.2 g) and a higher FCR (2.12 kg feed/kg eggs). This data is in accordance with some studies (1, 5) that reported egg production of laying hens was higher in conventional cages than those housed in alternative systems such as aviaries, floor pen, or free range. In our preliminary study , the number of defective eggs was lower in slat floor system, even if other productive cycles are necessary to confirm this result. The mortality was lower (P=0.064) in hens housed in furnished cages in comparison to animals reared in conventional cages (4.97 *vs* 8.25 %). This result seems to confirm a greater welfare of animals housed in this housing system (3).

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Table 1. Comparison between different housing systems on productive performance of hens during the production cycle (means ± SD).

		Traditional	Enriched	Student's T
Number of eggs	number per hen	310±12.3	324±14.3	0.106
Average egg weight	g	62.5±1.92	61.5±1.95	0.393
Egg production	kg/hen	19.5±0.81	19.9±0.92	0.431
Feed intake	g/d	110±3.09	108±1.25	0.393
FCR	kg feed/kg eggs	2.04±0.08	2.04±0.07	0.909
Mortality	%	8.25±3.14	4.97±0.20	0.064

EFFECT OF HEAP SIZE, SHAPE, COVERING, AND TURNING ON LITTER PASTEURIZATION DURING IN-SHED PARTIAL COMPOSTING

AFM F. Islam*, M. Mahmoud, S.K. Burgess, K.G. Renz, and S.W. Walkden-Brown

School of Environmental and Rural Science, University of New England, Armidale NSW 2351
*Corresponding author. Email: fislam2@une.edu.au

SUMMARY

Limited availability and high cost of chicken bedding materials are driving some broiler producers to reuse litter for multiple chicken batches in Australia. Pasteurization of litter between batches by heaping or windrowing for up to ten d can greatly assist in reducing pathogen carryover to the next batch of chickens. We have previously reported litter temperatures of 50–70 °C inside broiler litter heaps between three and ten d after heaping, depending on depth within the heap (1). The surface of the heaps remained relatively cool (20–40 °C) and turning at d three significantly increased temperatures after turning. Larger heaps are generally hotter than long windrows (2), which are easier to make. There are reports in the USA that both increased moisture level (3) and covering (4) of litter can elevate heap temperatures significantly. It has been shown recently that using a tarpaulin to cover the heap can elevate heap temperatures between 5 and 10°C, particularly closer to the surface (5). Pasteurization of litter has been shown to eliminate, or greatly reduce, the infective load of viral pathogens and coccidia in the litter (3). However, current industry demand is for short batch turnaround times of six d or less with a strong preference to avoid turning litter during pasteurisation if possible. Thus there is demand for quick and uniform pasteurisation of litter without turning.

The objective of the current study was to investigate the effects of covering, heap size and shape (heap vs windrow) on intra-heap temperatures with a view to reducing the time to maximum temperature, increasing the maximum temperature achieved, slowing the decline in temperature after the peak, and achieving greater uniformity of temperatures in the heap particularly near the surface.

The experiment utilized a 4 (size/shape) × 2 (cover) × 2 (turn) factorial design with two replicates of each treatment combination. Three heap sizes and a windrow were prepared. Polypropylene tarpaulin covers were or were not applied to litter heaps/windrows. Half of the covered and half of the uncovered heaps/windrows were turned at d three using a bobcat front-end loader. The experiment was conducted on three identical tunnel ventilated sheds on a NSW farm in late spring having a temperature range of 15-37°C. The litter material was pine shavings and had previously had one batch of chickens reared on it. The average initial moisture content was 18±0.74%. Twenty four litter heaps and eight windrows were prepared in total. Heap volumes were: large (~27m^3), medium (~9m^3), and small (~3m^3). The size of the windrows were ~3m^3, using an equivalent volume as

the small heaps. Windrows were, about 7.8 m long, 2.27 m wide at the base and 0.84 m high.

Once the heaps were formed, four to eight iButton temperature data loggers (Maxim Integrated, San Jose, CA, USA) were inserted in each heap at four depths from the surface (5, 25, 50 and 100 cm). In the case of the small heaps and windrows, the deepest iButton depth was approximately 80cm. Time of insertion and the location of each iButton were recorded. All iButtons were set to record temperature hourly. Twelve heaps, three from each size and four windrows, were covered with large tarpaulins covering the entire heap/windrow to ground level. At d three, 12 heaps and four windrows (half of them were covered) were turned and re-covered the covered heaps/windrows. At d seven of the experiment, all iButtons were collected and temperature data retrieved for analysis.

Temperature data were analysed by ANOVA to test the effects of heap size/shape, cover, turning and depth with their interactions on the maximum temperature achieved or temperature achieved at 24 h intervals. Significance of differences between levels within an effect was tested using student's T test. Least squares means ± standard errors of means or simple temperature profiles without analysis are presented.

There were significant effects of heap size/shape ($P < 0.009$), depth ($P < 0.0001$) and turning ($P = 0.006$), but not covering ($P = 0.09$) on the maximum temperature achieved with a significant interaction between the effects of heap size and turning ($P < 0.001$). There was no difference in maximum overall temperature in large (60.4±0.53ºC), medium (60.5±0.53ºC) and small (60.6±0.54ºC) heaps, however, windrows (57.9±0.64ºC) had lower overall temperatures than heaps (Figure 1, left). Higher maximum temperatures were recorded at depths of 25cm (62.8±0.56ºC) and 50cm (62.9±0.57ºC) than 5cm (57.3±0.57ºC) and 100cm (56.6±0.58ºC). Turning increased maximum temperature in windrows and medium heaps but not in large and small heaps (Figure 1, right).

Further exploration of the data revealed that temperatures within the heap or windrow increased most rapidly near the surface, between 5cm and 50cm. At a depth of 100cm, the temperature increased slowly and was still increasing at d seven in the large and medium heaps (Figure 2). Turning the heaps/windrows at d three greatly reduced the temperatures inside the litter mass; however, by d seven, the temperature was higher at all depths in the turned heaps/windrows (Figure 3) than in unturned ones (Figure 2).

This study demonstrated that at depths of 5-50cm, the temperature inside the heap rose quickly, rising above 56^{o}C within 72 h and could reach above 60^{o}C within seven d. On the other hand, temperatures deep inside the heap (eg. 100cm from the surface) increased much more slowly, taking up to seven d to reach 56^{o}C and still increasing. The overall rate of temperature increase in the smaller heaps was more rapid than medium and large heaps. Therefore, heap size should be optimized according to available time of pasteurization. If turnaround times are short and rapid pasteurization is required, smaller heaps will be better and will provide higher temperatures overall than windrows.

The more rapid increase in temperatures observed at shallower depths was probably due to availability of oxygen enabling a rapid aerobic thermophilic process quickly. However, high temperatures were not sustained for as long at 5cm depth, presumably due to drying out of the litter material, and/or heat loss due to reduced insulation. Deep inside the heap moisture availability was less likely to become limiting, but low oxygen availability probably created anaerobic or semi-anaerobic conditions where the decomposition process was partially anaerobic.

Unlike previous reports (5), the use of tarpaulin covers did not influence heap temperatures. This may be due to the low initial litter moisture content in the current experiment which was lower than that in the earlier reports (5).

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Figure 1. Effect of heap size/shape on the maximum temperature achieved at various depths (left) and the effect turning on heaps of various sizes/shapes. *Columns not sharing the same letter are significantly different within the same heap size/shape.

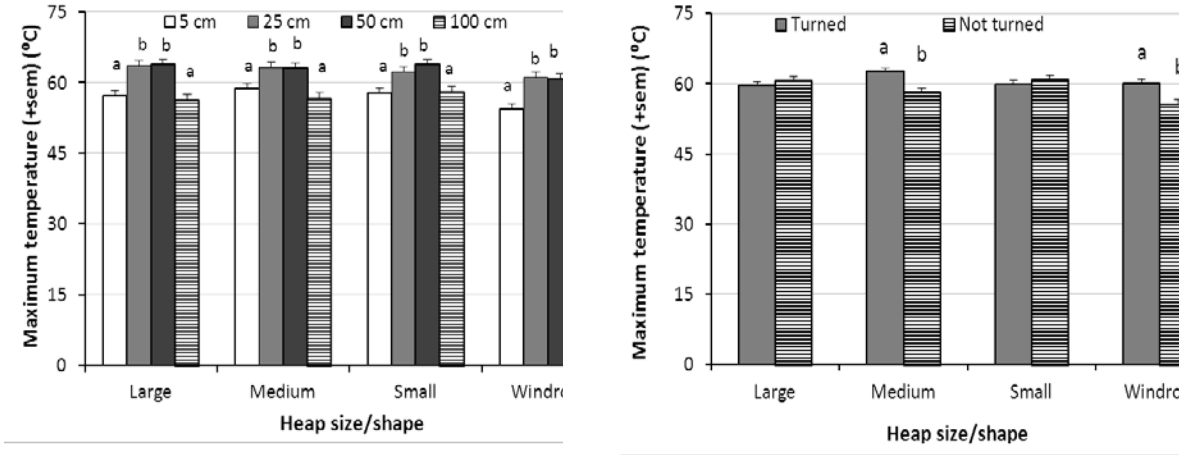


Figure 2. Temperature profiles over time at various depths in large (top left), medium (top right) and small heaps (bottom left), and windrows (bottom right) without turning.

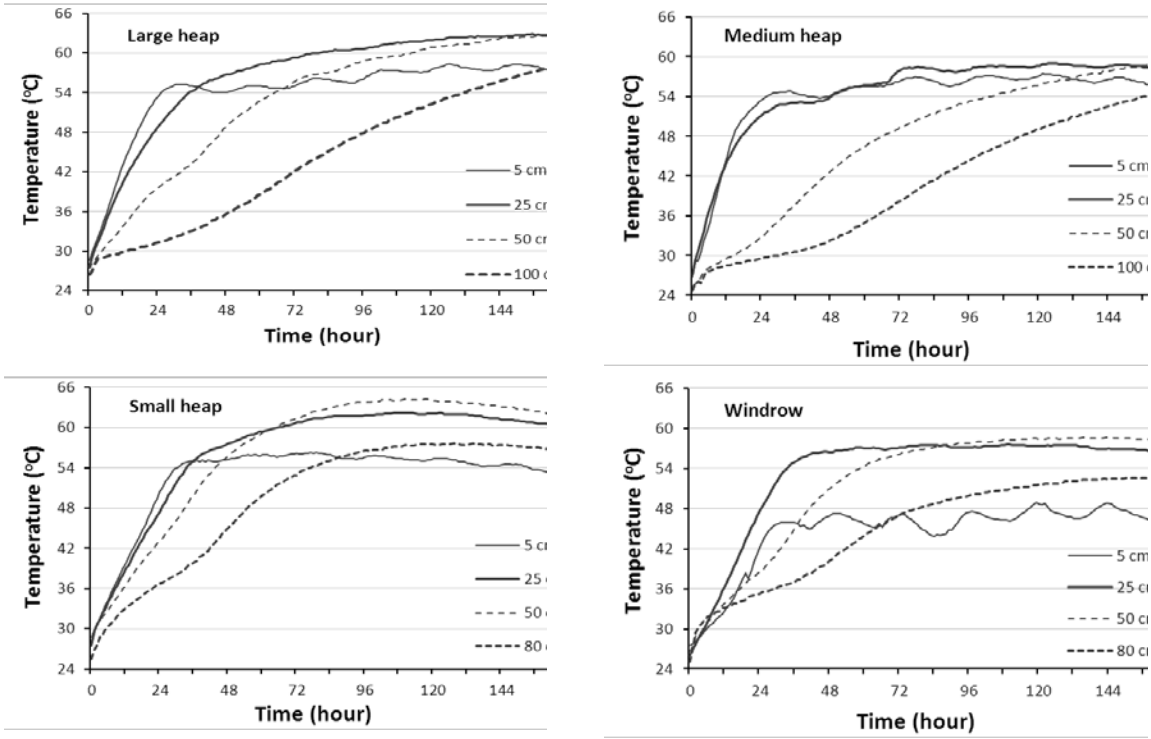
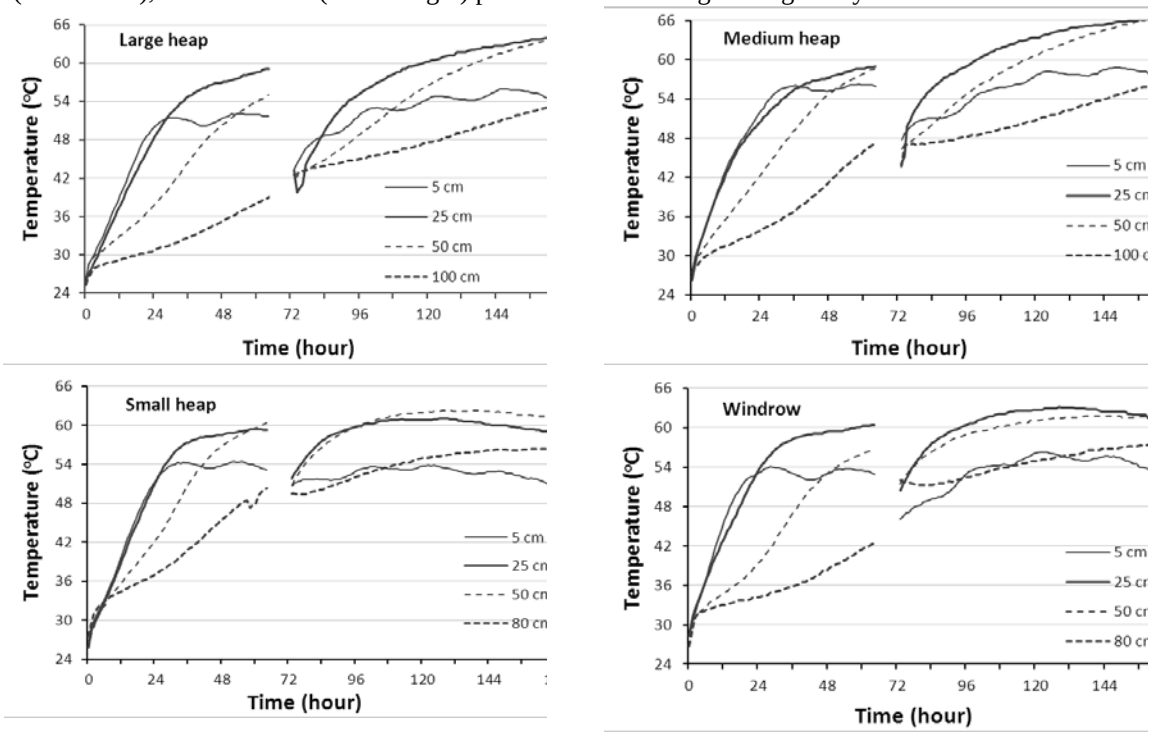


Figure 3. Temperature profiles over time at various depths in large (top left), medium (top right) and small heaps (bottom left), and windrows (bottom right) prior to and following turning at day 3.



PREVALENCE OF *SALMONELLA* ENTERITIDIS AND *SALMONELLA* TYPHIMURIUM IN BROILER CHICKENS IN ALBERTA

C Mainali[A]*, M. McFall[A], S. Otto[A], R. Cassis[A], G. Gensler[A], R. King[A], D. Peters[A], J.A. St. Amand[B], and C.B. Christianson[B]

[A] O. S. Longman Building, 6909-116 Street Edmonton, Alberta, Canada T6H 4P2
[B] 97 East Lake Ramp N.E, Airdrie, Alberta, Canada T4A 0C3

* Author for correspondence: Chunu Mainali, Department of Agriculture and Rural Development
Tel: (780) 415-9624; Fax: (780) 427-1437
E-mail:chunu.mainali@gov.ab.ca

ABSTRACT

A baseline study was conducted from October 2007 to March 2008 to estimate the prevalence of *Salmonella* spp., *S.* Enteritidis (SE), and *S.* Typhimurium (ST) at chick placement and at slaughter of broiler chickens in Alberta, Canada. Fifteen chick pads at placement were sampled from each of 459 flocks (n=6885). From these, 22%, 10% and 3% were positive for *Salmonella* spp., SE and ST respectively. Additionally, five pooled cecal and five pooled neck skin samples from each of 502 flocks (n=2510 each) were collected at the processing plants. Ceca had a prevalence of 35%, 5% and 4%; whereas neck skin had a prevalence of 47%, 3% and 16% for *Salmonella* spp., SE and ST respectively. Prevalence for *Salmonella* spp. at the flock level was 53% for chick pads, 56% for cecal samples, and 69% for neck skins. This study provides baseline prevalence information for *Salmonella* spp., SE, and ST in broiler chickens at the beginning and at the end of the production cycle in

Alberta, which serves as a bench mark for the future research.

INTRODUCTION

Salmonella is an important pathogen in both humans and animals. In humans, salmonellosis is considered one of the most frequent bacterial foodborne diseases, posing a major public health concern in industrialized countries (1). Poultry and poultry products are considered a major source of *Salmonella*-related foodborne disease in humans (2, 3). Each year approximately 6000 – 12,000 cases of salmonellosis are reported in Canada (4). The *Salmonella* spp. most often reported as the cause of human illness in Canada are ST and SE (5). In Alberta, poultry infection with SE, ST, or *S.* Heidelberg is provincially reportable under the Animal Health Act and Reportable and Notifiable Diseases regulation (6).

Alberta Agriculture and Rural Development, together with Alberta Chicken Producers, is seeking and planning to implement a prevention and control program focused on SE and ST. Baseline prevalence information is essential to set targets and to develop control policies and programs. The purpose of this study was to estimate the prevalence of *Salmonella* spp., SE, and ST at different stages of broiler chicken production in Alberta.

MATERIALS AND METHODS

Selection of study population. To estimate the prevalence of *Salmonella* spp. with an allowable error of 3.5% and 95% confidence, samples from 414 broiler flocks were needed. A flock is defined as group of birds of the same age raised by the same producer that were placed or processed on the same day.

Sample collection. Fifteen chick pads were randomly collected from each of 459 flocks from three different federally registered hatcheries before chicks were placed on farms. Each chick pad sample was individually folded and placed in a whirl-pak bag and was treated as one sample. Chick pads were placed in coolers and shipped to the Agri-Food Laboratories Branch in Edmonton for *Salmonella* testing.

Sixty paired ceca were collected from each of 502 flocks by using a systematic approach in the evisceration area at three different federally inspected plants. Content from paired ceca were pooled in groups of 12 following a strict protocol to avoid cross-contamination between samples, totaling five pooled samples from each flock. In addition, from the same flock, 60 independent neck skins were collected at the end of line before the birds entered the chiller. The neck skin samples were collected in whirl-pak bags, pooled in groups of 12 for a total of five pooled samples per flock from each of 502 flocks. All the samples were placed in coolers and shipped to the Agri-Food Laboratories Branch in Edmonton for *Salmonella* testing.

Sample analysis. Chick pad samples were processed by adding 225 mL of pre-enrichment, buffer peptone water (BPW) to each bag. All samples were mixed in a shaker for 30 min at a low speed. Cecal content samples were well mixed and 12 g were transferred into 108 mL of BPW.

The chick pad and pooled cecal content samples were then incubated at 35 ± 2° C for 20 to 24 h. Following incubation, 1mL of BPW was transferred to Tetrathionate (TT) selective enrichment broth and incubated at 35 ± 2° C for 20 to 24 h. The TT broth was vortexed and three 0.1 mL aliquots were transferred to three different sites, on a Modified Semisolid Rappaport-Vassiliadis plate (MSRV) (EMD Chemicals, Darmstadt, Germany). The plates were incubated at 42 ± 2° C for 48 h for detection of motile *Salmonella* species. DNA was extracted from the growth (halos) on MSRV plates using the Magnesil® KF Genomic System. Each extract was screened for *Salmonella* spp. and those testing positive were subsequently tested for SE and ST using real-time PCR assays.

Neck skin samples were processed by adding 225 mL of pre-enrichment, BPW to each sample bag and incubating at 35 ± 2° C for 24 h. After incubation, 0.1 mL of BPW was inoculated into Rappaport-Vassiliadis (RV) and TT broths and incubated at 42.5˚ C for 24 h. A sub-sample of 0.5 mL of each incubated selective enrichment broth was transferred to a test tube and mixed. A 5 µL aliquot from combined selective enrichment broths were screened for *Salmonella spp.* with BAX system® (DuPont™, Mississauga, ON). *Salmonella* positive samples were further analyzed for SE and ST by real-time PCR.

Statistical analysis. Data were compiled in Excel spreadsheets (Microsoft Corporation, Redmond, Washington, USA) and descriptive statistics including prevalence and 95% confidence intervals (CI) for each sample type were determined at sample and flock levels using SPSS 16 (SPSS, Inc., Chicago, IL). If any of the 15 chick pad and five cecal and neck skin samples per flock tested positive for *Salmonella* spp., then the whole flock was considered positive for *Salmonella* spp.

RESULTS

In total, 6885 chick pads from 459 flocks (15 chick pads from each flock) at placement and 2510 each of pooled ceca and neck skin samples (12 paired ceca or 12 neck skins in each pooled samples) from

502 flocks at slaughter were collected from October 2007 until March 2008.

Sample level prevalence. Out of 6885 chick pads, 1538 were positive for *Salmonella* spp. Similarly, out of 2510 pooled cecal or neck skin samples, 879 cecal and 1190 neck skin samples were positive for *Salmonella* spp. The percentage distributions of *Salmonella* spp., SE, and ST with 95% confidence interval for different sample types are shown in Table 1.

Flock level prevalence. Out of 459 flocks, 243 flocks had at least one *Salmonella* positive chick pad, giving a flock level prevalence of 52.9% (95% CI, 48.3 to 57.6). Similarly, out of 502 flocks tested at processing, 284 had at least one *Salmonella* positive cecal sample and 349 had at least one *Salmonella* positive neck skin samples giving a flock level prevalence of 56.5% (95% CI, 52.1 to 61.0) and 69.5% (95% CI, 65.3 to 73.5) respectively.

DISCUSSION

The sample level prevalence of *Salmonella* from cecal samples obtained in a previous study (7) and reported by the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) was lower than the prevalence reported in this study (8). However, this study indicated that the prevalence of SE in all sample types was lower in Alberta when compared with the Canadian national prevalence of 19% reported by the CIPARS (8).

Flock level prevalence of *Salmonella* spp. in this study ranged from 52.9% for chick pads to 69.5% for neck skin samples. The flock level prevalence of *Salmonella* spp. observed in this study is similar to those reported in other studies (7, 9) examining cecal samples at slaughter. However, this study reports a higher level of flock prevalence on the basis of skin samples (69.5%) compared to ceca (56%), which may be due to cross contamination during processing at slaughter.

This study provides baseline prevalence information for *Salmonella* spp., SE and ST in broiler chickens at the beginning and end of the production cycle in Alberta which serves as a bench mark for the future research.

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

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Table 1. Percentage distribution and 95% confidence interval of *Salmonella* spp., *S.* Enteritidis, and *S.* Typhimurium for different sample types of broiler chickens in Alberta, Canada.

Sample	***Salmonella* spp.** (No. of Positives) % (95% CI*)	***S.* Enteritidis** (No. of Positives) % (95% CI*)	***S.* Typhimurium** (No. of Positives) % (95% CI*)
Chick Pad n = 6885	(1538) 22.3 (21.4 - 23.3)	(718) 10.4 (9.7 - 11.2)	(234) 3.4 (2.9 - 3.8)
Cecal n = 2510	(879) 35.2 (33.2 - 36.9)	(120) 4.8 (3.9 - 5.7)	(102) 4.1 (3.3 - 4.9)
Neck Skin n = 2510	(1190) 47.4 (45.4 - 49.4)	(75) 2.9 (2.4 - 3.7)	(400) 15.9 (14.5 - 17.4)

*Confidence interval.

PCR MULTIPLEX FOR DIFFERENTIAL DIAGNOSIS OF *MYCOPLASMA GALLINARUM, M. GALLISEPTICUM, AND M. SYNOVIAE*

M. Soares, E. Nascimento, D. Abreu, C. Eiras, F. Santos, C. da Silva, R. Gouvêa, L. Machado, and V. Pereira

Departamento de Saúde Coletiva Veterinária e Saúde Pública/Faculdade de Veterinária, Universidade Federal Fluminense, Rua Vital Brazil Filho, 64, Niterói, RJ: 24230-340, Brazil.
Email: elmiro@vm.uff.br

SUMMARY

Mycoplasma gallinarum (MGa) is considered nonpathogenic to fowls, but it has been regarded pathogenic when associated with Newcastle and infectious bronchitis viruses. Respiratory diseases with these agents plus *M. gallisepticum* (MG) and *M. synoviae* (MS) are frequent. Specific primers for MG, MS, and MGa, all in 30 pmol concentration were attempted in Multiplex PCR (MPCR). After standardization with standard MGa, MG, and MS, the MPCR was applied in tracheal swab samples from chicken layers with respiratory disease, yielding positive amplicons for MGa (three samples), MS + MGa (seven samples), MG + MS (one sample), and MS (two samples). As to these mycoplasmas, MPCR was effective in differential diagnosis.

INTRODUCTION

The poultry industry is considered one of the most important economic sectors in agribusiness (4). However, intensification of production increases the risk of spread of infectious diseases, especially those affecting the respiratory system of birds. Avian mycoplasmosis is considered of great economic impact for losses attributable to infection by MG and MS (1). MGa has been considered nonpathogenic, but this species is frequently isolated from the respiratory tract of chickens at all ages and can aggravate respiratory problems when associated with vaccine viruses of Newcastle disease and infectious bronchitis (2). This study aimed to use the Multiplex-PCR for simultaneous detection and differentiation of concomitant infections by MG, MS, and MGa.

MATERIALS AND METHODS

A total of 13 samples of trachea were obtained with swabs from laying hens in two farms in Rio de Janeiro state. A standard MG strain (ATCC 19610), a vaccine strain MS (MS-BAC®, Fort Dodge Animal Health Ltda.), and a standard MGa strain (GM67StrainPG16) were used as a positive control. The DNA sample was extracted by the phenol-chloroform method (3). We used specific primers for MG (MG-f 5 ' CGT GGA TAT CTT TTC TAG CAG CTGC 3' and MG-r 5 'AGT TAT GTA GCA CAG TTC AAT GCA T 3' - 732 bp), for MS (MS-f 5 'GAG AAG CAA AAT AGT GAT ATC A 3' and MS-r 5 'CAG TCG AAG TCT GCC TTA ACA A 3' - 207 bp) and for MGa (MGa-f 5 'GCA GTT ATA TAT GGA

AAC AAC 3' and MGa-r 5 'AGT ATA GGG TTA CAA CCC CC 3' - 293 bp) in the same concentration of 30 pmol. The reactions, in a 50 μL container were 25.65 μL of PCR ultrapure water, 5 μL of 10X PCR buffer, 3 μL MgCl2 (50mM), 4 μL of dNTP mix (0.25 mM each), 1 μL of each primer in concentration described, 6 μL of DNA extracted and resuspended in TE buffer, and 0.35 U Taq Polymerase (2.5 U / μL). The Multiplex-PCR was performed under the following conditions: 94° C / 5 minutes followed by 40 cycles of 94° C / 1 minute, 55° C / 1 min and 72° C / 2 minutes, with a final extension of 72° C/10 minutes and 4° C for 30 seconds. After electrophoretic run the agarose gel was stained with 1.5% ethidium bromide and the results were visualized in UV light transilluminator.

RESULTS AND DISCUSSION

It was possible to visualize the amplicons produced in different sizes from each primer, identified as the species, being allowed to vary for each sample. The visualization of agarose gel allowed the diagnosis of only three positive samples for MGa, seven positive samples for MS and MGa, one positive for MG and MS, and two positive for only MS. This simultaneous diagnosis was obtained in eight diseased birds from chicken flocks with respiratory problem. From these results it is demonstrated that Multiplex PCR is a fast and efficient tool in the differential diagnosis of mixed infections by different species of mycoplasmas.

CONCLUSIONS

Multiplex PCR enabled simultaneous diagnosis of MGa and MS, and MS and MG, present in samples collected from live poultry, shortening the search time diagnosis and reducing costs.

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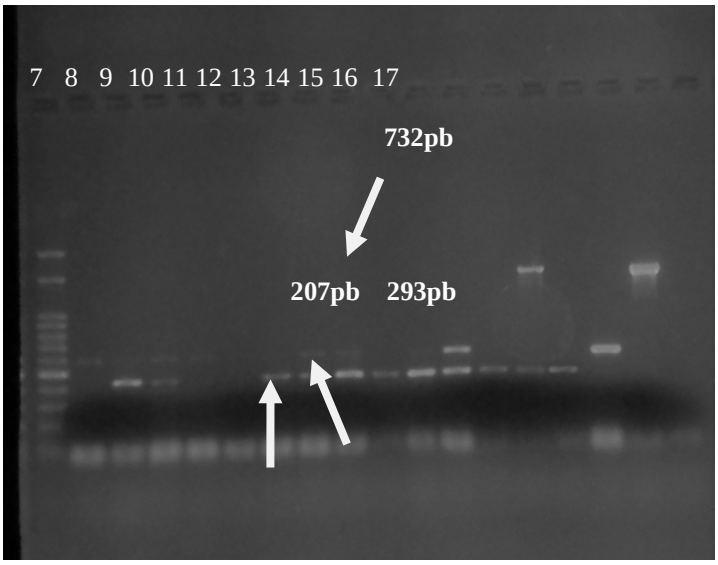
Figure 1. Multiplex PCR results in the diagnosis of *Mycoplasma synovie* (MS, 207pb), *Mycoplasma gallinarum* (MGa, 293pb) and *Mycoplasma gallisepticum* (MG, 732pb) in chickens. 1 - ladder, 50bp; samples 2, 5 and 6 - Positive only MGa; Samples 3, 4, 7, 8, 9, 11 and 12 - positive for MG and MS; samples 10 and 13 - positive only for MS; sample 14 - positive for MG and MS, 15 - positive control for MS; 16 - positive control for MGa; 17 - positive control for MG, and 18 - negative control.



MYCOPLASMA GALLISEPTICUM BY PCR IN GLUCOSE FERMENTING MYCOPLASMA ISOLATES FROM MAGELLANIC PENGUINS (*SPHENISCUS MAGELLANICUS*) IN BRAZIL

M. Barreto, J. Pires, M. Lemos, M. Silva, L. Ogino, J. Almeida, V. Pereira, and E. Nascimento

Departamento de Saúde Coletiva Veterinária e Saúde Pública/Faculdade de Veterinária, Universidade Federal Fluminense, Rua Vital Brazil Filho, 64, Niterói, RJ: 24230-340, Brazil
Email: elmiro@vm.uff.br

SUMMARY

Mycoplasmas have been reported in several species of birds, including *M. sphenisci* from penguins captured on the shore of Rio de Janeiro, Brazil,, as well as in other animals. Pathogenic mycoplamsas can cause respiratory, joint, and reproductive diseases besides predisposing to secondary infections and transmitting to domestic fowl. A total 17 Magellanic penguins (*Spheniscus magellanicus*) rescued on the shore of Rio de Janeiro Brazil, were placed in the Wildlife Rehabilitation Center of the Estácio de Sá University, Campus Vargem Pequena. On clinical examination, the penguins were dehydrated, weak, and some exhibited discrete dyspnea. The objective of this study was to describe the culture recovery of eight isolates of glucose fermenting mycoplasma from tracheal samples of these 17 penguins. In six of these isolates, the positivity to *Mycoplasma gallisepticum* was confirmed by PCR.

INTRODUCTION

Mycoplasmas have been reported in several species of birds, including domestic and wild ones (1, 3, 4, 11). They can cause respiratory, articular, and reproductive diseases besides creating a favorable environment for the development of secondary infection (5, 8, 9). In aquatic birds, there are reports of isolation of mycoplasmas in ducks (6), swans, geese, night heron, and penguins (4, 14). In penguins, there

are few reports on *Mycoplasma* spp. isolation; the first isolation and characterization of a new species, *Mycoplasma sphenisci*, being described by Frasca and colleagues (4). This new species was isolated from jackass penguins (*Spheniscus demersus*) that lived in captivity and showed signs of respiratory disease, characterized by a recurrent choanal discharge of a thick mucous with caseous aspect, besides chronic halitosis. Microorganisms genetically very similar to *M. sphenisci* (> 99%) and *Mycoplasma inners* (96.8%) were identified in fecal samples of Adela penguins (*Pygoscelis adeliae*) from the Ross Sea coast in Antarctica (1). In Brazil, the Magellanic penguin (*Spheniscus magellanicus)* is the most known type of penguin, found in several beaches of the Brazilian coast, mainly beaches of Rio de Janeiro state from July to October. These birds arrive on the Brazilian coast very weakened in most cases due to adverse factors during the exhausting trip to Brazil. The penguins referred here were rescued in Rio de Janeiro beaches and taken to the Wildlife Rehabilitation Center of the Estácio de Sá University, Campus Vargem Pequena, Rio de Janeiro, RJ, Brazil. Tracheal swabs were taken for *Mycoplasma* spp. diagnosis.

MATERIALS AND METHODS

Oropharyngeal swabs were collected from 17 penguins on September 2012. The obtained samples were transported to the laboratory in 2.0 mL of modified Frey's medium (8) with glycerol (1, 2) and stored at -20°C until use. In the laboratory of Nucleus for Diagnosis of Mycoplasmosis (NUDMC)/Nucleus for Animals of Laboratory (NAL), Federal Fluminense University, the samples were diluted up to 10^{-10}, seeded on Frey`s liquid and solid media, and incubated at 37°C. Growth was followed until changes from the red color given by the phenol red indicator were accomplished. The agar plates were incubated into a jar to attain anaerobic condition and observed under stereomicroscope (40x to 80x) for colony visualization daily for 21 d. Once color liquid medium was changed, aliquots were seeded on agar plates for growth confirmation. The colonies grown on plates were stained by the method of Dienes (2) and subject to sterol requirement by using a disk soaked with digitonin procedure (11). Eight isolates were sent to the Molecular Epidemiology Laboratory, Department of Collective Veterinary and Public Health, for polymerase chain reaction (PCR) to confirm the presence of *Mycoplasma* spp. with the primers MGSO-TGC ACC ATC TGT CAC TCT GTT AAC CTC and GPO3- GGG AGC AAA CAG GAT TAG ATA CCC T for 270 bp amplification and *Mycoplasma gallisepticum* with the following primers: MG-B1 (5'-CGT GGA TAT CTT TAG TTC CAG CTG C- 3') e MG-B2 (5'- GTA GCA AGT TAT AAT TTC CAG GCA T- 3') (9b); MG 13r (5'- GCT TCC TTG CGG TTA GCA AC- 3'); MG 14f (5'- GAG CTA ATC TGT AAA GTT GGT C- 3') (15). The methodology of DNA extraction and purification described by Sambrook and colleagues (13) and Zeugin and Hartley (16) was used.

RESULTS

Of the cultured samples, 47% (08/17) yielded colonies with typical "fried egg" shape after two to three d of incubation. These isolates fermented glucose, changing the color of the medium from red to yellow within 48 to 72 h. By Diene`s test, the isolates were grouped into class Mollicutes by presenting colonies with deep blue colored center, surrounded by light blue color. Growth was inhibited in the presence of digitonin, confirming their sterol dependence. The eight typical mycoplasma colonies producing isolates, when subjected to PCR, by generic proceedure yielded amplicons of the expected 270bp. For *M. gallisepticum* specific PCR, the amplicons produced were, respectively, 481bp (10) and 185bp (14) in size. The presence of microorganism of the genus *Mycoplasma* and species *gallisepticum* was diagnosed in penguins in Brazil for the first time.

DISCUSSION

In this study, *M. gallisepticum* was identified for the first time in Magellanic penguins that arrived on the shore of Rio de Janeiro state, Brazil. *M. sphenisci* has been isolated before in Connecticut, USA from jackass penguins (4), detected by PCR in the case Adela penguin feacal samples from Ross Sea Coast in Antarctica (1), and in Megellanic penguins in Brazil (7). *M. sphenisci* has been found into two penguins genera and three species, which implies this mycoplasma is host-related to penguins in general. Whether this *M. gallisepticum* is related to penguins and pathogenic to poultry needs to be clarified.

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THE USE OF THE REAL TIME POLYMERASE CHAIN REACTION (RT-PCR) TECHNIQUE FOR THE DETECTION OF VERTICALLY-TRANSMITTED PATHOGENS IN SPECIFIC PATHOGEN FREE CHICKEN FLOCKS

N. Ortega-Heinly[A,B] and T. Girshick[A]

[A]Charles River Laboratories Avian Vaccine Services, 67 Baxter Road, Storrs, CT 06268
[B]Department of Pathobiology and Veterinary Science, University of Connecticut, Storrs, CT

SUMMARY

Specific Pathogen Free (SPF) eggs are commonly used as the substrate for production of many vaccines. The Center for Veterinary Biologics (CVB) in the US, and the European Pharmacopoeia in Europe both have regulations on the qualifications for SPF eggs. VS Memo 800.65 and PhEur 5.2.2 describe a critical subset of avian pathogens that, if present in a flock, disqualifies the flock as SPF and unsuitable for use in vaccine manufacture. Testing for these avian pathogens relies almost exclusively on serologic methods to detect antibodies generated in response to exposure to these pathogens. The PhEur 5.2.2 notes

that at the end of the production period serologic testing of the flock must again be performed four wk after the last egg collection that was used for vaccine manufacture. However, there is a provision that allows for testing suitable sample materials within four wk of the final egg collection, for the presence of vertically-transmissible agents using validated nucleic acid amplification techniques as described in PhEur 2.6.21.

The Molecular Diagnostics Laboratory of Charles River Laboratories International developed and optimized the RT-PCR for the pathogens that are considered capable of vertical transmission as defined by the PhEur 5.2.2: avian adenovirus Group I, avian adenovirus Group III, avian encephalomyelitis virus, avian leukosis viruses (subgroups A, B, J), avian reovirus, avian reticuloendotheliosis virus, chicken anemia virus, *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, and *Salmonella* Pullorum. In this application the RT-PCR is used as a limit test (positive or negative results) for each pathogen. The limit of detection, specificity, and robustness were determined using cloacal swabs from SPF flocks that were spiked with each pathogen at different dilutions.

The results show that the RT-PCR was able to detect less than 47 infectious units of any of the organisms tested. Also, the primers were non-reactive to 200 negative cloacal swabs making the assay specific for each pathogen. In order to demonstrate robustness, a simple, intentional variable was used by not vortexing the samples after the addition of lysis buffer. Spiked samples remained positive even with the intentional change. Therefore, based on our validation, the sensitivity and specificity of these assays are adequate for the detection of infections in SPF flocks.

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ATYPICAL CHLAMYDIA IN POULTRY

E. Prukner-Radovčić[A], D. Horvatek Tomić[A], M. Lukač[A], and K. Laroucau[B]

[A]Faculty of Veterinary Medicine, University of Zagreb, Heinzelova 55, 10 000 Zagreb, Croatia
[B]Bacterial Zoonoses Unit, French Agency for Food, Environmental and Occupational Health Safety (Anses), Maisons-Alfort, France

SUMMARY

Chlamydia psittaci is the causative agent of avian chlamydiosis, and represents a significant problem in veterinary medicine all over the world (1). Infection in humans is also regularly reported, especially in those closely connected to infected birds and in persons of specific occupations (veterinarians, laboratory workers, employees in poultry slaughterhouses, zoo and pet shops workers, wildlife rehabilitators) (1, 4, 7, 8, 9, 12).

Some authors suggested that the spectrum of *Chlamydiaceae* spp. encountered in birds is not confined to a single species. Chlamydial infections in poultry are misdiagnosed due to symptoms similar to those caused by other pathogens, or very complex diagnosis which is also hazardous for laboratory personnel. New chlamydial agents with unknown zoonotic potential were recently detected from domestic poultry in Germany and France (5, 9). Progress of diagnostic methods has also resulted in findings of some new cases of atypical chlamydial infection in hens and turkey flocks in Croatia.

In different geographically part of Croatia, tracheal and fecal samples were taken from two hen flocks and one turkey flock, by using a *Chlamydiaceae*-specific real-time PCR assay targeting the 23S rRNA gene were found positive (3). Surprisingly, these PCR positive samples didn't react with any of the *C. psittaci* specific tool targeting the *ompA* gene as well as *C. abortus* and *C. pecorum* specific tools (11). Recently, novel *Chlamydophila* spp. strains have been isolated from poultry (12) and as a specific real-time PCR has been developed targeting the conserved 16S gene, the presence of novel *Chlamydia* sp. strain was also confirmed in turkeys and hens in Croatia.

Depending on the flock size and housing conditions, positive birds were treated with different antimicrobial agents (tetracycline or enrofloxacin) and biosecurity measures (quarantine, regular cleaning, and disinfection) were performed in all positive cases. After the treatment, the birds were examined until negative results were obtained.

CONCLUSIONS

Many species of birds may be intermittently infected with *Chlamydia*, without clinical signs, and shedding of this pathogen through feces or respiratory and conjunctival secretions, increases the risk of transmission of *Chlamydia* to other animals and to humans.

This report is our contribution to the statement that the spectrum of *Chlamydiaceae* spp. encountered in poultry is not confined to a single species, and further investigations are needed in an attempt to clarify the role of this microorganism in poultry and prevention of potential illness in humans.

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COMPARISON OF ANTIMICROBIAL RESISTANCE AMONG *E. COLI* STRAINS ISOLATED FROM YOUNG AND ADULT LAYING HENS

Leticia Cruz Lopez [A], Rigoberto Hernandez Castro [B], Maria Del Rosario Morales Espinosa [C], Armando Navarro Ocaña [C], Jesus Flores Larios [D], Cecilia Rosario Cortes [A]

[A]Departamento de Medicina y Zootecnia de Aves, FMVZ, UNAM, Circuito Exterior s/n Ciudad Universitaria, cp.04510 México, D.F. MEXICO
[B]Hospital General Dr. Manuel Gea Gonzalez
[C]Departemento de Salud Pública, Facultad de Medicina, Torre de investigación
[D]Grupo Estrada de la Torre, Tepatitlán of Morelos, Jal. rosario@unam.mx

INTRODUCTION

In recent years, poultry has become one example of production efficiency, mainly due to rapid life cycle of the poultry. To meet this demand, hens have been forced constantly, which causes them stress and predisposes them to infectious or noninfectious diseases. One of the problems that commonly affect laying hens is peritonitis, since it increases morbidity and mortality (1, 2). Although there is no published data in Mexico regarding the incidence of this problem, it is estimated that over 90% of the mortality that occurs commonly in commercial laying flocks are caused by bacterial infections associated to peritonitis.

Salpingitis, peritonitis, and salpingoperitonitis cause an acute mortality without previous clinical signs. At necropsy, generalized congestion can be observed and the presence of yellow material in the cavity. This disease occurs around peak (3). Several pathogens have been isolated from chickens with peritonitis, with *Escherichia coli* as the most frequent. Large economic losses in the poultry industry are related to this disease due to the high cost of treatments. For this reason, the correct diagnosis and medication are very important to solve the problem. The main measures to control the problems caused by *E. coli* in poultry is the use of some antibiotics, both prophylactic and therapeutically. This practice is so widespread, and exerts a selective pressure on bacteria that consequently, the incidence of isolation of strains resistant to multiple antimicrobial agents has increased (4).

MATERIALS AND METHODS

Two-hundred birds were recovered from the mortality during the first ten d of life from a laying hen flock. Samples from liver, lung, bone marrow and yolk sac were analyzed.

The second sampling was performed from the same flock around 24 to 28 wk. Bone marrow and abdominal cavity exudates were analyzed from hen carcasses associated to peritonitis. Both samples were isolated in TSA and McC. Once a pure culture was obtained identification was performed by biochemical tests. Antibiogram was performed according to the technique described by Bauer and Kirby (5) using 11 different antibiotics: tylosin (TL), enrofloxacin (ENR), florfenicol (FLC), fosfomycin (FSM), oxytetracycline (OXT), amoxicillin (AMX), doxycycline (DXT), sulphachlopirimidacin (SLF), chlortetraciline/tiamulin (CLT), gentamicin (GMT), and ceftiofur (CFT).

RESULTS

Two-hundred and seventeen and 228 strains were isolated from pullets and hens, respectively; from those 79.2% (n= 172) and 74.56% (n=174) were identified biochemically as *Escherichia coli*. The remaining 20.7% (45) and 25.44% (56) were identified as *Enterobacter* sp., *Klebsiella* sp., *Pseudomonas aeruginosa*, and *Proteus mirabilis.*

Microbial sensitivity showed close similarity among both groups; for example, resistance to TL, AMX, DXT and OXT increased from 80% (pullets) to 90% resistance in hens. A high level of multi-resistance was seen among pullet and hen strains. The most sensitive strains were resistant to one antibiotic in pullets, whereas in hens were resistant at least to

three antibiotics. In chicks, 41 strains (23.83%) were resistant to nine antibiotics, while 43.7% were resistant to eight antibiotics. Table 1 shows the antimicrobial sensitivity.

DISCUSSION

Colibacillosis is a disease that causes severe losses in the poultry industry worldwide. In the present study we analyzed samples from chicks in the first wk of age and of the same flock when it had the highest incidence of peritonitis during the peak.

Antibiotic resistance is linked to the virulence genes, an example *iss* gene (gene survival increase in the serum) in birds can be an indicator of its ability to cause disease and many genes associated with it. This gene has been located on a plasmid encoding resistance to tetracycline and ampicillin. By hybridizing, it has been revealed that this plasmid contains sequences homologous to the gene *tsh*, a gene associated with virulence of *E. coli*. In addition, this plasmid contains genes that encode resistance to antimicrobial agents, which may provide an advantage to *E. coli* (6).

We noticed that 75.3% strains were resistance to florphenicol in chicks and 37.7% resistance in hens; this may suggest that this antibiotic resistance gene can be for flor (7).

The use of antibiotics is widespread practice, it has exerted a selective pressure on bacteria, and consequently, the incidence has increased the isolation of resistant strains to multiple antimicrobial agents. This phenomenon has been associated with the emergence of efficient routes of spread, both horizontally and vertically, of mobile elements such as plasmids, transposons, and gene cassettes in integrons.

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Table 1. Microbial resistance among *E. coli* strains isolated from pullets and laying hens with yolk sac infection and peritonitis.

	Pullets	Hens	Pullets	Hens	Pullets	Hens
	Resistant		Intermediate		Sensitive	
	%	%	%	%	%	%
TL	99.4	100	0	0	0.6	0
ERF	37	55.3	3.5	8.8	59.4	35.9
FLF	37	75.3	1.17	0.58	61.7	24.11
FSF	5.2	23.52	0.58	0	94.1	76.5
OXT	84.7	95.3	1.17	3.52	14.1	1.2
AMX	96.4	100	3	0	0.6	0
DXT	80.5	95.3	2.3	1.2	17	3.5
SLF	71.7	98.3	13	0.58	15.3	1.2
CLT	78.2	84.11	7.6	14.11	14.1	1.7
GM	15.8	9.4	39.4	5.3	44.4	85.3
CFT	48.8	27.64	15.8	21.17	35.3	51.2

SOME APPLICATIONS OF QUANTITATIVE HISTOMORPHOMETRY TO POULTRY DIAGNOSTICS AND RESEARCH

F Wilson[A], A. Banda[A], D. Magee[A], S. Hubbard[A], K. Jones[A], L. Pace[A], E. Riley[B] , and P. Stayer[B]

[A]MVRDL & PRDL,
[B]MSU and Sanderson Farms

SUMMARY

Multiple studies using morphometric techniques have demonstrated differences that may be helpful to define normal growth patterns as well as aid in definitive diagnoses of various diseases. Quantification of bone marrow cellular elements present in the mid-shaft femur of broiler chickens and heavy fowl demonstrated age-associated changes in normal chicken populations. In addition, often extensive elevations in bone marrow cellularity were present in birds classified as septicemia-toxemia and with both severe localized or systemic inflammatory conditions. Dramatic elevations of bone marrow cellularity were documented for some, but not other viral diseases. Quantification of the marrow also demonstrated clear numerical differences between severe bone marrow hyperplasia occurring in response to infectious disease and those present in myeloid leukosis. Simple histomorphometric evaluations were also utilized to document quantitative changes in the glomeruli in broilers with inclusion body hepatitis associated glomerulonephritis, for the demonstration of age associated changes in the heart myocardium; tracheal effects of infectious laryngotracheitis vaccination; bursa changes with viral disease, and bone changes associated with “femoral head necrosis” and rickets.

BACKGROUND

Quantitative histomorphometric evaluations can provide quantitative results using simple, inexpensive

or even sometimes "free" methods. Morphometric studies are of value for poultry research and in particular allow for statistical comparisons of normal control and various disease or experimental groups. Quantitation of parameters allows for establishment of normal and pathological reference ranges. Numerical data facilitate the establishment of quantitative thresholds for expressing precise disease incidence.

Quantitative studies on bone marrow. Only a limited amount of quantitative information on avian bone marrow in normal and abnormal conditions is available in the scientific literature. We have developed a simple quantitative method for the evaluation of bone marrow in commercial chickens. Bone marrow was collected from the mid-shaft femur and five µm thick H &E stained sections were evaluated at 400-x magnification using a 1 mm^2 ruled ocular grid or reticule. The number of mature heterophils and large primitive cells present per grid area were enumerated for a total of ten fields for each sample. Mitotic figures and adipocytes were similarly enumerated for select studies.

A critical component to the elucidation of quantitative pathological changes in the bone marrow of poultry is documentation of normal composition including age-associated alterations. To this aim, we conducted studies on broiler chickens of various ages. The results demonstrated substantial reduction in hematopoietic elements occurring between one and 40 d of age.

Quantification of bone marrow in broiler chickens and heavy fowl demonstrated often extensive elevations in bone marrow cellularity in birds manifesting with septicemia-toxemia or with severe localized inflammatory conditions. Dramatic elevations occurring at high incidence in bone marrow cellularity were also documented for specific bacterial diseases (experimental intravenous injections of *E. coli* and spontaneous infections of *Onithobacterium rhinotracheale*), as well as some but not other viral diseases (marrow hyperplasia with inclusion body hepatitis but not with infectious laryngotracheitis). In addition, quantification of the marrow also demonstrated clear numerical differences between severe bone marrow hyperplasia occurring in response to infectious disease and those present in neoplastic myeloid leukosis.

A small pilot study was done to test the utility for bone marrow quantitation of the ImageJ software program (http://imagej.nih.gov/ij/download.html) for determination of optical density (pixel density) using selected cases of normal, hyperplastic, and myelod leukosis bone marrows. The study demonstrated, respectively, moderate and marked increase in optical density relative to the control for the hyperplastic and neoplastic marrows; suggesting that this method may provide a useful approach for documentation of quantitative pathological changes in the bone marrow.

Spleen histomorphometrics (measurement of percent follicular histiocytic area). Using the NIH Image J morphometric software program, the total area of interest was first measured, the area occupied by histiocytes within individual follicles was measured, and the percent follicular histiocytic area relative to the total area was calculated**.** A clear increase in histiocytic follicular area was demonstrated for birds with septicemia-toxemia relative to normal. In addition, an increased incidence for follicular histiocytic area expansion was observed in the small compared to the large carcass subgroups.

Kidney histomorphometric studies: broiler chicken inclusion body hepatitis. Broilers with viral inclusion body hepatitis were observed to exhibit a high incidence of membranoproliferative glomerulonephritis. Glomerular size was determined using the ImageJ NIH software program and the total cell counts of corresponding glomeruli were also determined using an ocular grid. The average glomerular area values for normal glomeruli in the sub-capsular cortical and central kidney regions respectively were 1,794 μm^2 and 5,304 μm^2. In contrast, glomerular measurements for kidneys exhibiting glomerulonephritis by routine histopathology, demonstrated average values for the two regions of 4,727 μm^2 and 11,063 μm^2. The average glomerular cell counts for the two regions in normals were 44 and 107 cells/glomeruli, while averages obtained for birds with glomerulonephritis were 90 and 193 cells/glomeruli. Studies by others demonstrated that proliferative glomerular lesions occur commonly in clinically normal market chickens manifesting with grossly swollen kidneys and the changes were also characterized using histomorphometric studies.

Histomorphometric studies on age-associated changes in the myocardium in broiler chickens. Studies were done to establish normal age-associated changes in the myocardium of broiler chickens using simple histomorphometric evaluations. The nuclear density of myocardial myofibers was determined for various age groups including aged birds appearing clinically normal or exhibiting transitory cyanosis. The numbers of nuclei per grid field at 400-x magnification for a total of ten fields was determined for each sample. The histomorphometric studies documented a progressive reduction in myocardial nuclear density with increasing age. A spectrum of histomorphometric changes for various parameters was appreciated within the aged roosters (nuclear density, nuclear size, and myofiber width) some of which might indicate possible early preclinical cardiomyopathy.

Bursa histomorphometric studies: comparison of microscopic methods for bursa histopathology

evaluation. The microscopic pathology of 600 bursa samples from four broiler farms was histologically evaluated and subsets of the samples were also evaluated using subjective and quantitative histomorphometric methods (subjective histopathology scoring, quantitative measurement of optical density, measurement of total & medulla follicular areas with calculation of cortical, area and measurement of average cortical width). One morphometric method employed was the measurement of optical density. In addition, calculation of cortex to medullary ratios using measurements of microscopic areas or cortical widths was performed. While the magnitude of differences were similar and extensive for both the cumulative pathology and percent cortex area (31% and 37% respectively), the percentage difference between least and most severe groups for optical density was dissimilar and of a much less magnitude. In addition while the group differences were highly significant for percent cortex area ($\leq$ 8.12E-06), they were of low significance ($\geq$ 0.04) for optical density.

Histomorphometric studies on trachea. The kinetics of the inflammatory responses of tracheas occurring with vaccine challenges or of unplanned natural infections with infectious laryngotracheitis virus (ILT) were investigated using simple quantitative histomorphometric approaches measuring cellular and geometric parameters. Vaccine groups were vaccinated with Trachivax. Histomorphometric measurements of mucosal area and average width utilized the ImageJ software program. A substantial pathological response following exposure to various doses and delivery methods of the modified live virus vaccine was observed. The magnitude of the responses to vaccination was typically far less than those observed for field cases of spontaneous ILT. The studies also demonstrated that while tracheal pathology occurring in response to vaccination procedures employed in the present study were on an average much milder than observed for field cases of spontaneous ILT, the maximum response for a few individual vaccinated birds did approach the spontaneous ILT level.

Histomorphometric bone studies: histological demonstration of a pathological or non-artifactual basis for epiphyseal separation induced during coxofemoral disarticulation in clinically normal poultry. The cause(s) of "femoral head necrosis", or separation of the epiphysis from the physis, occurring during routine necropsy procedures in poultry are controversial. Femoral head separation has been proposed to represent an artifact produced by disarticulation of the coxofemoral joint and not to reflect a true pathological process. We investigated the microscopic anatomy of femoral heads obtained from clinically normal 26-50 d old broilers, which either did or did not exhibit epiphyseal detachment during disarticulation of femurs. Our studies demonstrated a dramatic and statistically significant reduction in the chondrocyte density and a marked elevation in the occurrence of pyknotic nuclei along the surface of metaphyseal cartilage in detached samples relative to controls. The study also documented the occurrence of femoral head cartilage defects in clinically normal poultry that correlated with femoral head epiphysis separation occurring during disarticulation.

Newer bone histomorphometric studies. Our recent studies compared the ratio of the area measurements comprising hypertrophic and hyperplastic cartilage zones present in the physis (Hypertrophic /Hyperplastic Area Ratio). The ultimate goal is to develop reference tables for normal and abnormal states of endochondrial ossification at different ages in commercial chickens. The results for preliminary morphometric measurements demonstrated a significant elevation in either the ratio or percentage of the total physis cartilage occupied by hypertrophic cartilage in birds manifesting with rickets relative to normal bone.

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