Respiratory Diseases of Poultry

AAAP SYMPOSIUM 2001

July 15, 2001

Boston, Massachusetts

AVMA Annual Convention

AAAP SYMPOSIUM 2001

Respiratory Diseases of Poultry

July 15, 2001
Boston, Massachusetts

Symposium Goal: This symposium was designed to review the current clinical and research status of important respiratory diseases of poultry. Additional regulatory and international issues will be highlighted.

Symposium Co-Chairs:

Dr. Sherrill Davison
University of Pennsylvania
The New Bolton Center
Kennett Square, PA 19348

Dr. Frederic Hoerr
State of Alabama
Roberts Veterinary Diagnostic Lab
Auburn, AL 36831

Acknowledgement:

Thanks to the AAAP Respiratory Disease Committee members for guidance and direction in the effort to plan and execute this symposium.

AAAP SYMPOSIUM 2001

Alphabetical List of Speakers:

Davison, S.	University of Pennsylvania, Kennett Square, PA
Garcia, M.	University of Georgia, Athens, GA
Gelb, J.	University of Delaware, Newark, DE
Hoerr, F.	State of Alabama, Auburn, AL
Jackwood, M.	Ohio State University, Wooster, OH
Keeler, C.	University of Delaware, Newark, DE
King, J.	Southeast Poultry Research Lab, Athens, GA
Kleven, S.	University of Georgia, Athens, GA
Ley, D.	North Carolina State University, Raleigh, NC
Nagaraja, K.	University of Minnesota, St. Paul. MN
Seal, B.	Southeast Poultry Research Lab, Athens, GA
Shaw, D.	Pennsylvania State University, State College, PA
Smith, J.	Fieldale Farms Corporation, Baldwin, GA
Swayne, D.	Southeast Poultry Research Lab, Athens, GA

AAAP Symposium 2001

Respiratory Diseases of Poultry

July 15, 2001

Morning Session Moderator: Sherrill Davison

Infectious Bronchitis Virus:

8:00 AM Mark Jackwood - Tests for Infectious Bronchitis Virus – Advantages and Limitations of the Currently Used Techniques

8:15 AM Jack Gelb - S1 Sequence Analysis; A Tool for Understanding Infectious Bronchitis Virus Outbreaks

8:30 AM Sherrill Davison - Infectious Bronchitis in Pennsylvania

8:45 AM Fred Hoerr - The Current Status of the Detection and Typing of Infectious Bronchitis Virus

9:00 AM **Discussion**

Infectious Laryngotracheitis:

9:15 AM John Smith. - Current Field Status of Infectious Laryngotracheitis

9:30 AM Maricarmen Garcia. - Tracking Infectious Laryngotracheitis in the Field

9:45 AM Calvin Keeler - New Approaches in Infectious Laryngotracheitis Vaccine Development

10:00 AM **Discussion**

10:15 AM **Break**

Mycoplasma gallisepticum

10:45 AM David Ley - *Mycoplasma gallisepticum* in North Carolina: 1999 – 2000

11:00 AM Stanley Kleven - Some Perspectives on Mycoplasma Diagnosis and Control

11:30 AM **Discussion**
11:45 AM **Lunch Break**

Afternoon Session Moderator: Fred Hoerr

Avian Influenza:

1:30 PM David Swayne - National and International Avian Influenza Outbreaks and Strategies for Control

Newcastle Disease:

2:00 PM Jack King - Newcastle Disease Virus Pathotyping: The Current Emphasis in the ICPI

2:15 PM Bruce Seal - Molecular Evolution of Newcastle Disease Virus and the Application of Molecular Diagnostics

Pneumoviral Infection:

2:30 PM K. V. Nagaraja - Epidemiology of Avian Pneumovirus and Host Range

2:45 PM Daniel Shaw - Pathogenesis and Detection of Avian Pneumovirus Infection

3:00 PM **Discussion**

3:30 PM Adjourn

INFECTIOUS BRONCHITIS VIRUS

Diagnostic Tests for Infectious Bronchitis Virus: Advantages and Limitations of Currently Used Techniques

Mark W. Jackwood, Ph.D.
Department of Avian Medicine
University of Georgia
Athens, GA 30602

Infectious bronchitis is an acute upper respiratory tract disease in chickens. Clinical signs of the disease are coughing, sneezing, tracheal rales, gasping and nasal discharge. Mortality may occur in very young birds, whereas decreased weight gain and feed conversion usually occurs in older birds. A drop in egg production and egg quality are observed when layers are infected. It is extremely contagious and the clinical signs can become severe when secondary invaders such as *E. coli* complicate the disease (King *et al.*, 1991).

Infectious bronchitis virus (IBV) is classified as a coronavirus, and isolates of the virus have been grouped into different serotypes using antibodies. The serotype grouping of IBV isolates is important because it is well known that little or no cross protection between serotypes occurs. Thus, it is extremely important to identify the serotype of the virus causing disease so that birds can be properly vaccinated. The virus contains a single strand of RNA surrounded by a lipid sac, or envelope. On the surface of the virus particle are club shaped projections called spikes (S). Spikes consist of two-protein subunits designated S1 and S2. The S1 subunit, makes up the distal portion of the spike, and is anchored to the envelope by the S2 subunit. Neutralizing and serotype specific antibodies are directed against the S1 subunit (Cavanagh *et al.* 1988) and diagnostic procedures that identify the serotype of the virus involve either the spike protein or the genes that code for the spike. Complicating diagnosis is the natural ability of the virus to change genetically. When changes occur in the gene that codes for the spike protein, it can result in a new serotype of the virus (often referred to as a variant virus).

Serology. Diagnosis of infectious bronchitis can be based on the detection of serum antibodies against IBV using the enzyme linked immunosorbant assay (ELISA) test or the hemagglutination inhibition (HI) test. The ELISA test is commercially available, detects the presence of serum antibodies to IBV, and can be used to quickly test a large number of sera. The HI test is easy, inexpensive and can be used to detect serotype specific antibodies. However, cross-reactive antibodies can make it difficult to accurately identify the serotype of the virus. The HI test is conducted by treating the virus with neuraminidase followed by reacting it with antisera and chicken red blood cells (Gelb and Jackwood 1997, Ruano *et al.* 2000). Those tests have a place in an overall control program for IBV but are of limited value for determining the serotype of the virus responsible for the disease outbreak. Definitive diagnosis of IBV is based on virus isolation and serotype identification.

Traditional Serotype Identification. In order to interpret the results from molecular based diagnostic tests, one must first understand serotype grouping of viruses using the traditional virus-neutralization test. Traditionally virus isolation and cross virus-neutralization tests in embryonating eggs have been used. Virus isolation is conducted by inoculating the chorioallantoic sac (CAS) of 9 to 11 day old embryonating eggs (Gelb and Jackwood 1997). The eggs are incubated for 48 to 72 hours, and the virus is detected using antibodies conjugated to fluorescent tags and a microscope equipped with an ultra violet light source. In the virus-neutralization test, antisera against known serotypes of the virus are reacted with the unknown virus and antisera prepared against the unknown virus are reacted against known viruses of different serotypes (Gelb and Jackwood 1997). The titers are represented by the reciprocal of the highest dilution to neutralize the virus and are used to calculate a relatedness value,

which gives a measure of the similarity between two viruses (Archetti and Horsfall 1950). This is the gold standard for identifying different serotypes or variants of the virus but it is extremely labor intensive and it can take weeks or months to identify only a few isolates.

A Bridge Between Traditional and Molecular Diagnostics. Monoclonal antibodies (McAbs) can be used to determine the serotype of an IBV isolate, and have been prepared against several different serotypes of the virus. Several McAbs against IBV have been characterized and developed as diagnostic tools (Karaca *et al.* 1992). An indirect ELISA test using group specific, serotype-specific, and strain-specific McAbs was found to correlate well with the virus-neutralization test (Karaca *et al.* 1992). Although the panel of serotype-specific McAbs is small (Mass, Ark, Conn, and JMK), this technique offers an alternative means to identify the serotype of an isolated virus.

PCR Based Molecular Diagnostic tests. With the advent of molecular based diagnostic tests for IBV we now have the ability to rapidly identify any virus isolate. Two types of molecular based diagnostic tests for IBV are currently used. Both tests utilize the reverse transcriptase-polymerase chain reaction (RT-PCR) to amplify all or part of the S1 glycoprotein gene. The first is a type specific RT-PCR test (Keeler, *et al.* 1998) that utilizes specific PCR primers designed to identify the Arkansas, Connecticut, Massachusetts, DE072, JMK and California serotypes of IBV. Different sized amplicons, generated for each of the types, are identified by agarose gel electrophoresis (Figure 1). Primers for more than one type can be included in the same reaction. This decreases the number of tests conducted on each isolate and allows for more than one virus to be identified in the same sample.

The other molecular test currently used for identification is the RT-PCR/ restriction fragment length polymorphism (RFLP) test (Kwon *et al.* 1993 and Jackwood *et al.* 1997). That test uses a set of primers in the RT-PCR to amplify the entire S1 gene from all of the IBV types. Then, restriction enzymes, which cut DNA at specific sequences called recognition sites, are used to digest the amplified gene into different sized DNA fragments. Those fragments, which are visualized on an agarose gel, form a pattern of DNA bands unique for each type of the virus (Figure 2). The patterns can be used to identify all known types and variants of IBV. Like the type specific RT-PCR test, the RT PCR/RFLP test can be used to identify more than one IBV type in a single sample.

Sequence Based Molecular Diagnostic Tests. Nucleic acid sequencing, which determines the exact nucleotide sequence or genetic code for a gene, has been completely automated and can be used to identify IBV isolates. Automated sequencing or cycle sequencing is used to determine the sequence of an RT-PCR amplified S1 gene. That sequence data is then compared by computer analysis with the sequence of known IBV strains to specifically identify the type or to group it with viruses having a similar sequence. That data is generally presented in a phylogenetic tree that shows the percent relatedness between viruses (Figure 3). It can be used with sequence data on the entire S1 gene or with data from only the hypervariable regions located in the first third of that gene.

Advantages and Disadvantages of Molecular Tests. Advantages of molecular based identification tests over traditional tests for IBV are; 1) they can be used to identify the type of many isolates in a short period of time, 2) they are relatively easy to conduct, 3) they are extremely accurate, 4) in some cases, they can detect more than one type in a single sample, and 5) a newly identified isolate can be quickly compared to all of the previously isolated viruses. In addition, IBV killed by mixing allantoic fluid containing the virus with an equal volume of buffered phenol (pH 4.5), can also be identified. With the appropriate permits, phenol inactivated samples can be imported from outside the USA. Thus, IBV isolates from all over the world can be examined and compared.

Disadvantages of the tests include; 1) difficulties amplifying the viral genome because of contaminants that inhibit the reaction, a change in the genetic make up of the virus, poor quality samples, or reaction conditions that are not conducive to amplification and 2) expensive reagents and

equipment. Of those, the one that causes the greatest concern is when the reaction fails to amplify the viral genome because of a change in the sequence of the S1 gene. As a safeguard against that or any false negative result, molecular based tests utilize a universal set of PCR primers that amplify a highly conserved region of the viral genome.

The results of molecular identification tests must be taken with caution. Although molecular identification of IBV types correlates with the virus-neutralization test and with *in vivo* cross-protection assays, when a new previously unrecognized virus is found, virus-neutralization assays and or protection experiments must be conducted to verify that the previously unrecognized virus is indeed a new serotype. In addition, molecular identification tests do not elucidate the pathogenicity of an IBV isolate.

Summary. The ability to quickly identify the type of many IB viruses has significantly increased our understanding of this disease. For example, a given type of IBV, like the California virus tends to be endemic in a geographic region and evolves within that region. Sometimes a virus will evolve in one area then jump to a new region. This occurred in the late 1990's when the Delaware 072 virus jumped to the Southeastern USA. In addition, it appears that variant viruses continue to appear and that many different IBV variants are circulating in perfectly healthy chickens all the time. Finally, IBV will continue to change and evolve making it necessary to modify and adapt existing identification tests as well as develop new techniques for identification of IBV types.

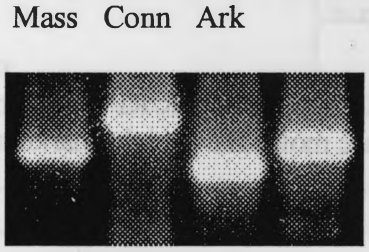

Figure 1. Serotype specific RT-PCR test. The serotypes of the PCR products are indicated on the figure. The size of the PCR products are as follows; Mass= 355bp, Ark= 308bp and Conn= 261bp.

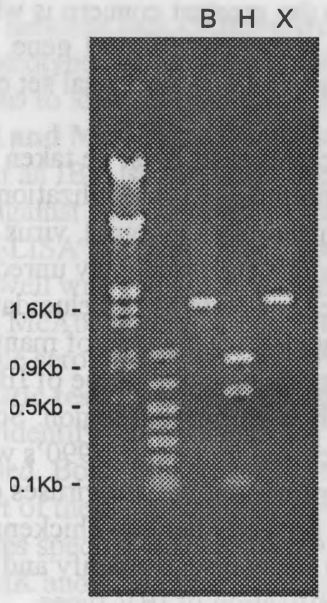

Figure 2. Serotype identification (DE 072) by RT-PCR/RFLP. Restriction enzymes are B= *Bst*YI, H= *Hae*III, and X= *Xcm*I. All other lanes are molecular weight markers.

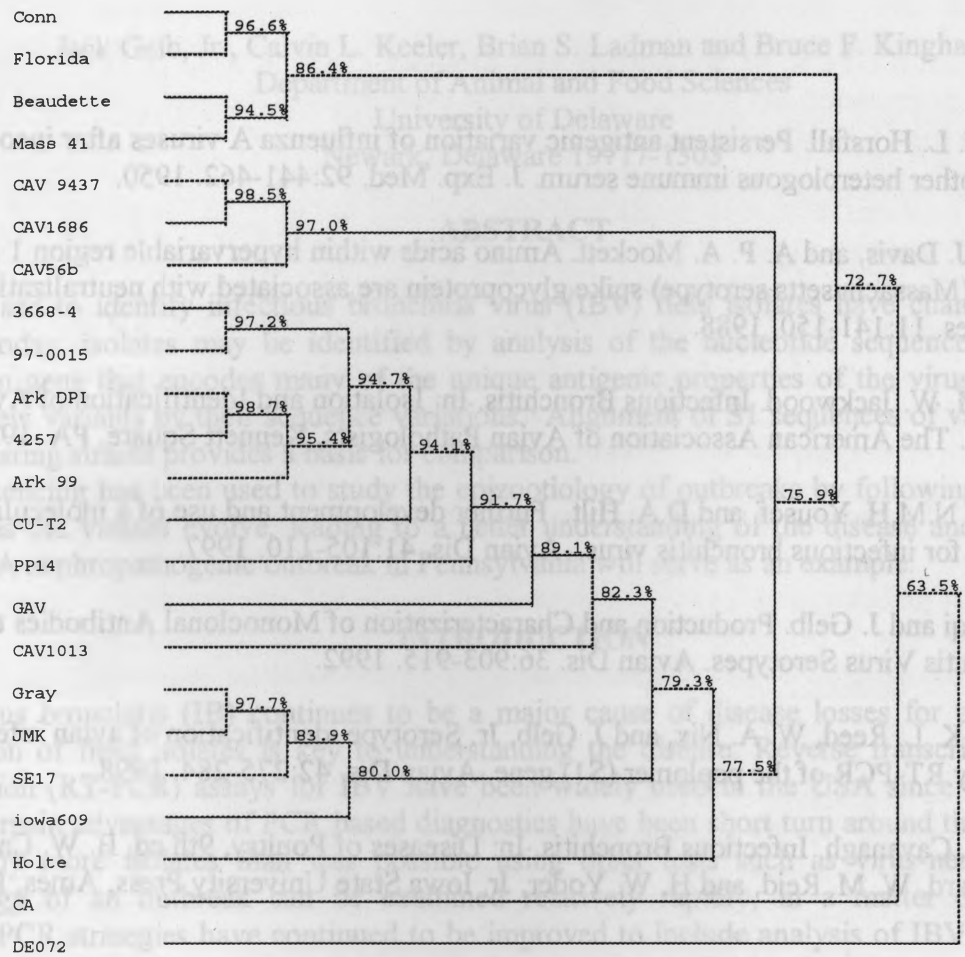

Figure 3. Phylogenetic analysis of the S1 amino acid sequences of various IBV types. The numbers represent percent similarity.

References

Archetti, I., and F. L. Horsfall. Persistent antigenic variation of influenza A viruses after incomplete neutralization in other heterologous immune serum. J. Exp. Med. 92:441-462. 1950.

Cavanagh, D., P. J. Davis, and A. P. A. Mockett. Amino acids within hypervariable region 1 of avian coronavirus IBV (Massachusetts serotype) spike glycoprotein are associated with neutralization epitopes. Virus Res. 11:141-150. 1988.

Gelb, J., Jr. and M. W. Jackwood. Infectious Bronchitis. In: Isolation and Identification of Avian Pathogens, 4th ed. The American Association of Avian Pathologists, Kennett Square, PA. 1997.

Jackwood, M.W., N.M.H. Yousef, and D.A. Hilt. Further development and use of a molecular serotype identification test for infectious bronchitis virus. Avian Dis. 41:105-110. 1997.

Karaca, K., S. Naqi and J. Gelb. Production and Characterization of Monoclonal Antibodies to Three Infectious Bronchitis Virus Serotypes. Avian Dis. 36:903-915. 1992.

Keeler, C. L., Jr., K. L. Reed, W. A. Nix, and J. Gelb, Jr. Serotype identification of avian infectious bronchitis virus by RT-PCR of the peplomer (S1) gene. Avian Dis. 42:275-284. 1998.

King, D. J. and D. Cavanagh. Infectious Bronchitis. In: Diseases of Poultry, 9th ed. B. W. Calnek, H. J. Barnes, C. W. Beard, W. M. Reid, and H. W. Yoder, Jr. Iowa State University Press, Ames, Iowa. pp.471-484. 1991.

Kwon, H. M., M. W. Jackwood, and J. Gelb, Jr. Differentiation of infectious bronchitis virus serotypes using polymerase chain reaction and restriction fragment length polymorphism analysis. Avian Dis. 37:194-202, 1993.

Ruano, M., J. El-Attrache, and P. Villegas. A rapid-plate hemagglutination assay for the detection of infectious bronchitis virus. Avian Dis. 44:99-104, 2000.

S1 SEQUENCE ANALYSIS; A TOOL FOR UNDERSTANDING IBV OUTBREAKS

Jack Gelb, Jr., Calvin L. Keeler, Brian S. Ladman and Bruce F. Kingham
Department of Animal and Food Sciences
University of Delaware
Newark, Delaware 19717-1303

ABSTRACT

Tools used to identify infectious bronchitis virus (IBV) field isolates have changed over the last decade. Today, isolates may be identified by analysis of the nucleotide sequence of the S1 spike glycoprotein gene that encodes many of the unique antigenic properties of the virus. S1 sequencing identifies new variants by their sequence variations. Alignment of S1 sequences of variant strains with those of existing strains provides a basis for comparison.

S1 sequencing has been used to study the epizootiology of outbreaks by following genetic changes over time as the viruses evolve, leading to a better understanding of the disease and possible control measures. A nephropathogenic outbreak in Pennsylvania will serve as an example.

INTRODUCTION

Infectious bronchitis (IB) continues to be a major cause of disease losses for poultry producers. Identification of field isolates is key to understanding the disease. Reverse transcription polymerase chain reaction (RT-PCR) assays for IBV have been widely used in the USA since the middle 1990s (7,9). The main advantages of PCR based diagnostics have been short turn around time and the ability to test many more isolates than was possible using other tests such as virus-neutralization. The epizootiology of an outbreak can be examined relatively rapidly, in a matter of several weeks. Moreover, PCR strategies have continued to be improved to include analysis of IBV genes to provide more comprehensive information on the genetic characteristics of field isolates (8).

The S1 subunit of the envelope spike (S) gene of IBV has been the major target for developing PCR diagnostics because S1 exhibits extensive genetic changes that translate into structural variations responsible for serotypic differences in IBV. The N-terminal subunit (S1) is responsible for attachment (1) and virus-neutralizing (VN) antibody induction (2). The C-terminal subunit (S2) anchors S1 to the viral envelope. The S1 subunit exhibits more sequence variability than S2 and researchers have identified sequences in S1 which have been designated as hypervariable region (HVR) 1 and HVR 2 (2,10). The sequences within the hypervariable regions have been used to distinguish serotype and subtype differences (3,11).

From 1997-2000, nephropathogenic IB (NIB) occurred in central and southeastern Pennsylvania in commercial poultry. Twenty-one IBV isolates were recovered from broilers, layer pullets, layers, and layer pullet breeders. Viruses were isolated at laboratories at University of Pennsylvania, New Bolton Center, Kennett Square and Pennsylvania State University, State College (12). In addition, one case of NIB was confirmed in a Maryland broiler flock on the Delmarva Peninsula (4). Diagnoses of NIB were made on the basis of clinical histories, gross and microscopic lesions (interstitial nephritis), IBV-specific immunohistochemistry of kidney specimens and virus isolation and S1 sequencing (5,12).

The IBV isolates were sent to our laboratory for confirmation and S1 sequencing. This paper will describe the sequencing approach used and the application of the findings to the NIB outbreak.

MATERIAL AND METHODS

RNA extraction, RT-PCR, S1 Sequencing and Analysis. Genomic RNA of IBV isolates obtained from NIB cases was extracted and RT-PCR and S1 gene cycle sequencing were performed as described (8). Degenerate primers CK4 and CK2, designed to amplify a wide variety of IBV genotypes, were used for PCR (7,8). PCR product sizes ranged from 603-631 nt depending on the strain of IBV. The PCR primers and their locations relative to the S1 gene are indicated in Fig. 1. S1 sequences of NIBV were aligned using the MegAlign v1.03© program of DNASTAR (Madison,WI) and compared to sequences obtained from the GenBank and EMBL databases (Fig. 2). MegAlign was used to provide percent S1 protein similarity values amongst the NIB isolates and selected IBV reference strains (6).

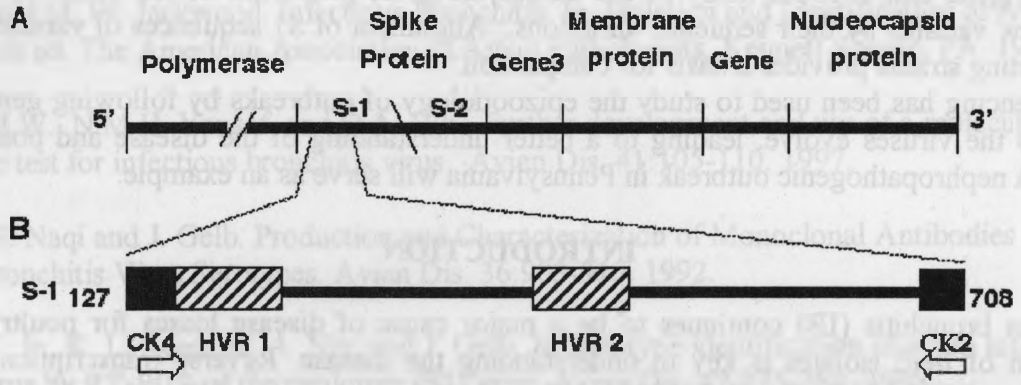

Fig. 1. IBV genome and S-1 gene. (A) Schematic representation of the single-stranded RNA genome of IBV. (B) The fragment of the IBV S-1 gene amplified by the degenerate primers CK4 and CK2 (black boxes). The hatched boxes indicate the location of the hypervariable (HVR) regions 1 and 2. Arrows denote the orientation of the CK4 and CK2 degenerate primers. (Modified from Kingham *et al.*, 2000).

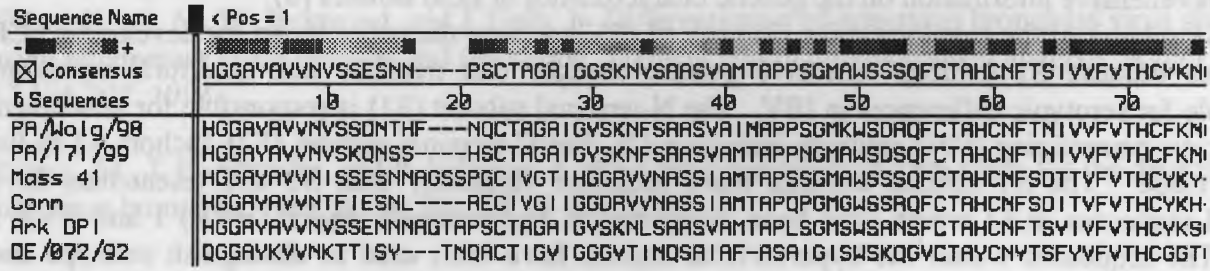

Fig. 2. Protein sequence alignment of the NIB isolates PA/Wolgemuth/98 and PA/171/99 and selected reference strains at the H(Q)GGAY (CK4) primer site. The sequence alignment was performed using DNAStar software (DNAStar, Inc.; MegAlign; Version 1.03, 1993) and the CLUSTAL method (Higgins and Clark, 1998).

RESULTS AND DISCUSSION

Two unique S1 genotypes, PA/Wolgemuth/98 and PA/171/99 were identified in poultry in Pennsylvania with NIB. The PA/Wolgemuth/98 and PA/171/99 genotypes were different from genotypes previously found in Pennsylvania (Fig. 3). Furthermore, the two unique genotypes were dissimilar to each other (Fig. 4). Nine isolates representing one genotype, typified by PA/Wolgemuth/98, were identified at the onset of the outbreak in August 1997 through November 1998. However, starting in January 1999 and for the next 18 months until July 2000, twelve isolates representing a different NIBV genotype, PA/171/99, were obtained. Only one PA/Wolgemuth/98

genotype isolate, (PA/3372/99) was recovered (August 1999) during the January 1999-July 2000 period when PA/171/98 genotype isolates were associated with cases of NIB. The replacement of the PA/Wolgemuth/98 genotype by the PA/171/99 genotype is interesting although the reason(s) is presently not understood.

NIBV isolate, MD/106/00, from broilers on Delmarva in February 2000 was found to a PA/171/99 genotype. Based on its high degree of S1 similarity to PA/171/99 (98%), gross and microscopic renal lesions consistent with NIB, and IBV-specific IHC findings, we strongly suspect the Maryland isolate to have originated from PA/171/99.

S1 sequence information was used to track the spread of NIBV. Two broiler cases submitted from successive flocks on the same farm produced isolates PA/5468/99 and PA/536/00. Comparison of the S1 sequences indicated that the viruses were identical (100% homology). Isolate PA/2617/99 was obtained in June 1999 from a case of NIB in commercial layer pullets. After the pullets were moved to a layer facility, the pullet house was cleaned, disinfected, and left vacant for eight weeks according to the owner. A replacement flock broke with NIB at 5 weeks of age and isolate PA/4661/99 was recovered. Sequencing of the two isolates yielded a 99% S1 similarity. The high degree of S1 similarity observed in the broiler and layer pullet outbreaks suggests that a nearly identical virus was responsible for subsequent outbreaks on the same farms.

In conclusion, S1 sequencing has proven to be a useful tool in understanding IBV outbreaks. In addition to identifying two unique genotypes, sequencing also provided a way of determining the causative genotype in an outbreak in Maryland and recurrent outbreaks on the same farm premises.

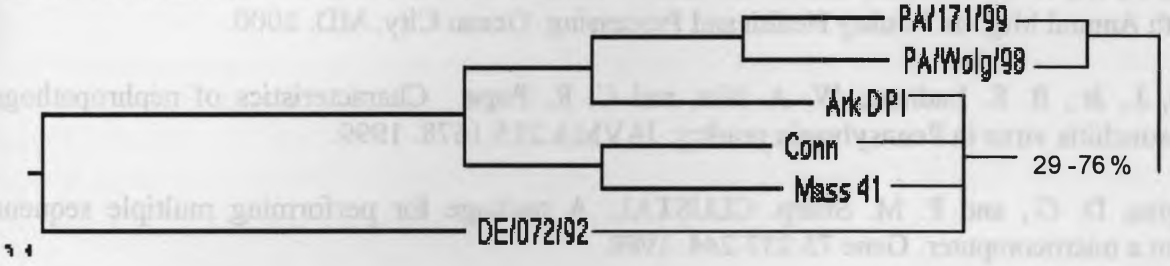

Fig 3. IBV S1 phylogenetic tree showing NIB genotypes, PA/Wolgemuth/98 and PA/171/99, and strains currently or previously found in Pennsylvania poultry.

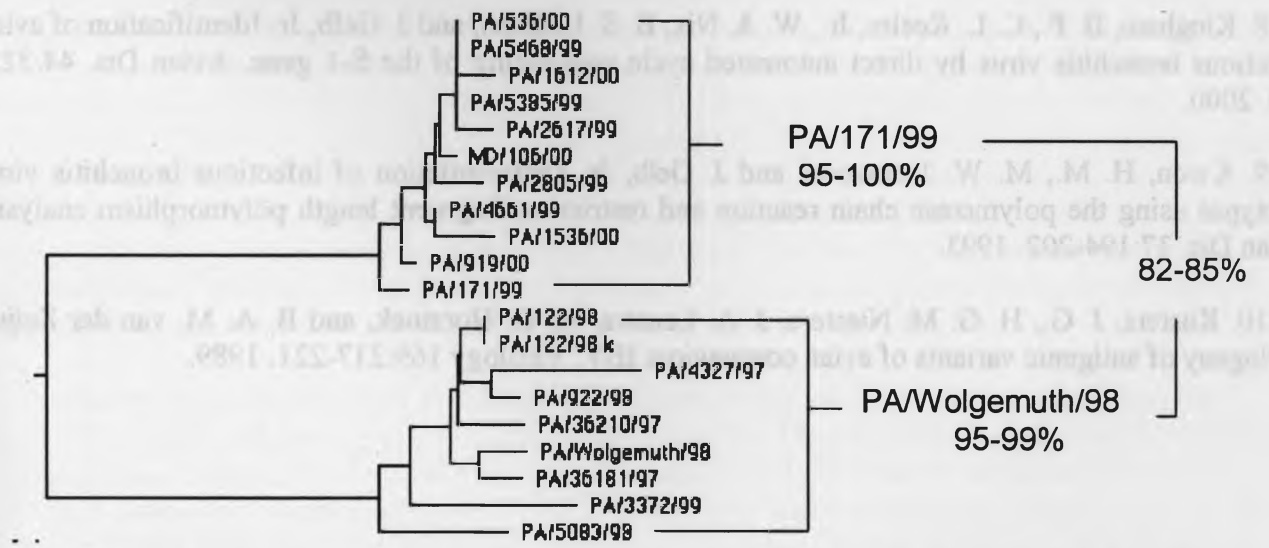

Fig 4. IBV S1 phylogenetic tree showing NIB isolates represented by the PA/Wolgemuth/98 and PA/171/99 genotypes.

REFERENCES

1. Cavanagh, D., and P. J. Davis. Coronavirus IBV: Removal of spike glycopolypeptide S1 by urea abolishes infectivity and haemagglutination but not attachment to cells. J. Gen. Virol. 67:1443-1448. 1986.

2. Cavanagh, D., P. J. Davis, and A. P. A. Mockett. Amino acids within hypervariable region I of avian coronavirus IBV (Massachusetts serotype) spike glycoprotein are associated with neutralization epitopes. Virus Res. 11:141-150. 1988.

3. Gelb, J., Jr., C. L. Keeler, Jr., W. A. Nix, J. K. Rosenberger, and S. S. Cloud. Antigenic and S-1 genomic characterization of the Delaware variant serotype of infectious bronchitis virus. Avian Dis. 41:661-669. 1997.

4. Gelb, J., Jr., B. S. Ladman, and C. L. Keeler. Studies on the control of infectious bronchitis. Thirty-fourth Annual Mtg. on Poultry Health and Processing. Ocean City, MD. 2000.

5. Gelb, J., Jr., B. S. Ladman, W. A. Nix, and C. R. Pope. Characteristics of nephropathogenic infectious bronchitis virus in Pennsylvania poultry. JAVMA 215:1678. 1999.

6. Higgins, D. G., and P. M. Sharp. CLUSTAL: A package for performing multiple sequence alignment on a microcomputer. Gene 73:237-244. 1988.

7. Keeler, C. L., Jr., K. L. Reed, W. A. Nix and J. Gelb, Jr. Serotype identification of avian infectious bronchitis virus by RT-PCR of the peplomer (S-1) gene. Avian Dis. 42:275-284. 1998.

8. Kingham, B. F., C. L. Keeler, Jr., W. A. Nix, B. S. Ladman, and J. Gelb, Jr. Identification of avian infectious bronchitis virus by direct automated cycle sequencing of the S-1 gene. Avian Dis. 44:325-335. 2000.

9. Kwon, H. M., M. W. Jackwood, and J. Gelb, Jr. Differentiation of infectious bronchitis virus serotypes using the polymerase chain reaction and restriction fragment length polymorphism analysis. Avian Dis. 37:194-202. 1993.

10. Kusters, J. G., H. G. M. Niesters, J. A. Lenstra, M. C. Horzinek, and B. A. M. van der Zeijst. Phylogeny of antigenic variants of avian coronavirus IBV. Virology 169:217-221. 1989.

11. Nix, W. A., D. S. Troeber, B. F. Kingham, C. L. Keeler, Jr. and J. Gelb, Jr. Emergence of subtype strains of the Arkansas serotype of infectious bronchitis virus in Delmarva broiler chickens. Avian Diseases. 44:568-581. 2000.

12. Ziegler, A. F., P. A. Dunn, J. Gelb, D. Weinstock, M. Castro, S. Davison. Nephropathogenic infectious bronchitis virus infections in Pennsylvania poultry. JAVMA 215:1689. 1999.

ACKNOWLEDGEMENTS

We thank the Laboratory of Avian Medicine, New Bolton Center, University of Pennsylvania, and Animal Diagnostic Laboratory, Pennsylvania State University for their collaboration. We also thank US Poultry and Egg Association and the United States-Israel Binational Agricultural Research and Development Fund for grants supporting this research.

INFECTIOUS BRONCHITIS IN PENNSYLVANIA

Sherrill Davison[A], Andre F. Ziegler[B], Jack Gelb, Jr.[C], Patricia A. Dunn[D], and Robert J. Eckroade[A]

New Bolton Center[A]
University of Pennsylvania
and
Lasher Laboratory[B]
University of Delaware
and
Dept of Animal and Food Sciences[C]
University of Delaware
and
The Animal Diagnostic Laboratory[D]
The Pennsylvania State University

ABSTRACT

Infectious bronchitis is a viral disease of chickens that primarily causes respiratory disease, but certain serotypes of the virus may produce renal disease. Two outbreaks of infectious bronchitis have occurred between 1994 and 2000 in Pennsylvania. The 1994 – 1995 outbreak involved respiratory disease in broilers caused by the Delaware 072 and Arkansas serotypes. The more recent outbreak involved a nephropathogenic strain in a wide variety of poultry.

INTRODUCTION

Respiratory infections caused by infectious bronchitis virus are common in broilers and layers in the United States and result in millions of dollars in lost income to poultry producers each year due to airsacculitis condemnations, mortality, increased medication costs, reduced egg production and shell quality problems. Between 1994 and 1995, in Pennsylvania, an outbreak of respiratory disease occurred in thirty-two broiler flocks (J. Jagne – unpublished data). These isolates were confirmed by RT-PCR at the University of Delaware as Delaware 072 and Arkansas (4). Clinical signs included sneezing, rales, lacrimation and cranial cellulitis. Mortality ranged from 3.4% to 23% and condemnations for airsacculitis ranged from 0.17% to 20%. This was the first evidence of these serotypes in Pennsylvania broilers. As a result of this outbreak, approval was granted by the Pennsylvania Department of Agriculture to use both the Delaware 072 and Arkansas commercial vaccines in Pennsylvania poultry. Subsequently, respiratory disease related to these two serotypes has diminished.

The nephropathogenic form of infectious bronchitis is also an important cause of disease and mortality in broilers and young pullets in other countries (1,7). The nephropathogenic form of infectious bronchitis is uncommon in U.S. poultry (5,8). Since August 1997, 28 cases of infectious bronchitis involving a particularly nephropathogenic strain of virus have been confirmed in Pennsylvania. A wide variety of poultry have been affected by this particular virus strain, including twenty broiler flocks, five

commercial layer pullet flocks, two commercial leghorn layer flocks and one layer breeder flock. Compared with other affected groups, morbidity and mortality appeared greatest in diseased broiler flocks. Characteristic gross lesions in broilers and pullets consisted of severe, diffuse renal swelling, with an increase in urate crystal retention in ureters and tubules. Affected kidneys were also bilaterally symmetrically enlarged, pale and several times normal volume. Gross necropsy findings in the affected layer and layer breeder flocks predominantly consisted of urolithiasis / visceral gout and moderate upper respiratory disease, respectively. Characteristic histopathological lesions of affected broilers or commercial pullets included a typically severe, diffuse lymphoplasmacytic interstitial nephritis. Immunohistochemical testing of renal tissues was confirmatory in many cases, with the greatest antigen staining present in the renal tubular epithelium.

The virus isolates from nephritis cases suspected as infectious bronchitis virus were screened at the Pennsylvania State University Animal Diagnostic Laboratory using monoclonal antibodies for Massachusetts, Connecticut, and Arkansas (3) and all were found to be unrelated to these serotypes. At the University of Delaware, isolates were then characterized by RT-PCR product cycle sequencing of a diagnostically relevant portion of the S1 gene responsible for encoding serotypic differences (6). Two new genotypes, PA/Wolgemuth/98 and PA/171/99, were identified as causes of the outbreaks.

Control of infectious bronchitis includes the use of modified live and / or killed vaccines. The currently available modified live infectious bronchitis vaccines do not protect chickens against these nephropathogenic strains (2). The use of an autogenous killed vaccine, in conjunction with or in place of the standard modified live infectious bronchitis vaccines, may result in better protection from challenge with the nephropathogenic strains. As the majority of clinical cases of nephropathogenic infectious bronchitis infection have been in younger birds, a strategy should be developed to vaccinate these birds prior to exposure to the virus. A commercial vaccine company has produced an autogenous killed vaccine from the PA/Wolgemuth/98 strain. The efficacy of this vaccine is being evaluated in both broilers and pullets.

It is unclear at this time how this virus was introduced and spread between flocks in Pennsylvania. Affected flocks are owned by various companies and are located in a variety of geographical areas. Since July 2000, no further cases of the nephropathogenic form of infectious bronchitis have been reported in Pennsylvania.

The economic impact of the nephropathogenic infectious bronchitis infection in Pennsylvania flocks varied. Factors such as bird type, age and severity of disease influence the cost associated with a particular infection. Overall, the average total cost associated with the disease ranged from $36 - $260 per thousand birds affected.

REFERENCES

1. Cumming, R. B. Studies on avian infectious bronchitis virus. 1. Distribution and survival of the virus in tissues of affected chickens and studies on the carrier status. Austr. Vet. Jour. 45: 305-308. 1969.

2. Gelb, J., Jr., B.S. Ladman, W.A. Nix and C. R. Pope. Characteristics of nephropathogenic infectious bronchitis virus in Pennsylvania poultry. JAVMA 215:1678. 1999.

3. Karaca, K., S. Naqi, and J. Gelb, Jr. Production and characterization of monoclonal antibodies to three infectious bronchitis virus serotypes. Avian Dis. 36:903-915. 1992.

4. Keeler, C. L., Jr., K. L. Reed, W. A. Nix, and J. Gelb, Jr. Serotype identification of avian infectious bronchitis virus by RT-PCR of the peplomer (S-1) gene. Avian Dis. 42:275-284. 1998.

5. Kinde, H., B. M. Daft, A. E. Castro, A. A. Bickford, J. Gelb, Jr., and B. Reynolds. Viral pathogenesis of a nephrotropic infectious bronchitis virus isolated from commercial pullets. Avian Dis. 35:415-421. 1991.

6. Kingham, B., J. Gelb, Jr., W. A. Nix, and C. L. Keeler. Direct automated sequencing of the avian infectious bronchitis virus S-1 gene amplified by RT-PCR: Use for serotype identification. Proc. 70th Northeastern Conference on Avian Diseases. University of Guelph, Ontario, Canada. June 10-12, 1998.

7. Meulemans, G., M. C. Cartier, M. Gonze, P. Petit, and M. Vanderbroeck. Incidence, charaterisation and prophylaxis of nephropathogenic avian infectious bronchitis viruses. Vet. Rec. 120:205-206. 1987.

8. Winterfield, R. W. and S. B. Hitchner. Etiology of a infectious nephritis-nephrosis syndrome of chickens. Am. J. Vet. Res. 23:1273-1278. 1962.

ACKNOWLEDGEMENTS

We thank the University of Delaware for their collaboration and the Pennsylvania Department of Agriculture, Animal Health Commission for a grant supporting this work.

The Current Status of the Detection and Typing of Infectious Bronchitis Virus

Frederic J. Hoerr
State of Alabama
Veterinary Diagnostic Laboratory
1001 Wire Road
Auburn, AL 36831

Infectious bronchitis (IB) is an acute contagious respiratory disease of chickens that is caused by infectious bronchitis virus (IBV), a Coronavirus (2). A nephropathogenic variant of IB occasionally occurs. In mature laying hens, decreases in egg production and shell quality are sometimes the chief clinical presentation during epornitics of infectious bronchitis. Coronaviruses genetically similar to IBV have recently been isolated and characterized from turkeys and pheasants, reviewed by Cavanagh (1).

The epidemiology of IB is influenced by IBV's ability to change its antigenic presentation by mutations in the spike (S) glycoproteins, especially the S1 subunit protein (2). The mutations chiefly occur in three hypervariable regions of S1, and can cause a change in serotype. This impacts the selection of attenuated live vaccines that are the primary means of controlling IB. Different serotypes exist wherever IBV is isolated from chickens throughout the world. There is some overlap in serotypes among geographic regions, but novel serotypes emerge within regions densely populated with chickens. New serotypes are usually detected when epornitics of IB occur in vaccinated chickens, and the new serotype is found to be different from the vaccine virus or viruses.

Primary isolation of IBV is usually done in specific-pathogen-free (SPF) chicken embryos inoculated by the chorioallantoic sac route, or by inoculation of tracheal ring cultures (3). Embryonic lesions provide presumptive evidence of IBV, which must be differentiated from adenovirus infection. Because embryonic lesions may not be initially apparent, serial passage may be required for visible embryo changes to develop as evidence of viral replication. Alternatively, allantoic fluids can be tested for IBV by antigen capture ELISA or RT-PCR. IBV replication in tracheal rings causes ciliostasis.

Antigenic typing of IBV can be done by *in vivo* cross-protection assays in chickens, and by virus neutralization, monoclonal antibody affinity, hemagglutination inhibition, and genotyping, however, there is no generally agreed upon international standard or reference typing laboratory (3). Cross-protection assays, or vaccine protection tests, using specifically immunized SPF chickens in groups of 10 or more, define the IBV protectotype, which generally reflects the more discriminating serotype definition. In an immunized host chicken, the neutralizing antibodies and other immunological responses will thwart infection by a challenge virus of the same (homologous) serotype as the vaccine. Clinical signs of IB do not develop, and challenge virus cannot be re-isolated from the trachea 5 days post challenge. In contrast, if the immunized chicken is challenged with a virus of a different serotype, clinical signs develop, and the challenge virus can be re-isolated from the trachea 5 days post challenge. If fewer than one-half of the challenged immunized chickens are protected, the challenge virus is determined to be antigenically different than the vaccine virus. These assays also provide information about the virulence of the challenge virus. The *in vivo* cross-protection assay is relatively time consuming and expensive to perform because it requires SPF chickens, special housing requirements to prevent cross contamination, and authorization for animal inoculation studies.

Serotype determination is based on the induction of virus neutralizing (VN) antibodies by the infecting IBV. A virus neutralizing assay using SPF chicken embryos, cell culture, or tracheal ring culture can provide typing information similar to a chicken cross protection assay. The test requires the

development of serotype-specific antisera in SPF chickens, including serum to the new IBV isolate, and may require the adaption of a new IBV in the laboratory culture system. Once the specific reagents are available and titrated, however, the assays provide relatively rapid serotype information about a new virus. Because of the need to generate antisera in SPF chickens, these assays have some of the same time and expense issues as the cross protection assays. Increasingly, chicken embryo inoculation studies are required to have the same regulatory review as chicken inoculations.

Serotype-specific monoclonal antibodies can rapidly identify field isolates as belonging to recognized serotypes. This assay involves antigen capture technology and immunofluorescence or immunohistochemical detection of the antigen-antibody complex. The development of the serotype specific monoclonal antibody is the chief constraint of this serotyping method. Once developed, however, the monoclonal antibodies can be produced and made available in perpetuity.

Hemagglutination inhibition also can provide serotype information. The hemagglutinating antigen is prepared from the field isolate and reacted against serum antibody to known serotypes.

Genotyping is increasingly used to characterize IBV isolates from diagnostic cases (4,5,6). This involves either the restriction fragment length polymorphism (RFLP) analysis based on an enzymatic digestion of an RT-PCR product, or the more specific sequencing of the DNA of an RT-PCR product. Both approaches use specifically amplified segments of the IBV genome, usually one encoding the hypervariable regions of the S1 gene. The standards involve correlating a reference IBV serotype with either a specific RFLP pattern or an S1 nucleotide sequence. For new IBV isolates, the latter is expressed as the percentage of homology with the reference serotype, either for the DNA sequence, or the amino acids deduced from the sequence. These assays can quickly provide detailed information for many IBV isolates. Genotyping analysis tends to focus on just a segment of the IBV genome, and usually to defined regions of S1, and does not fully characterize other segments of the genome that could potentially influence virus neutralization or protective immunity. As with any new diagnostic technology, these tests require personnel who are comfortable working with molecular techniques and interpreting nucleotide sequences, as well as appropriate facilities and equipment.

Recent experiences with IB in broilers in Georgia and Alabama highlight some current issues in IBV diagnostics. The isolation and characterization of a new IBV, now recognized as the Georgia 98 serotype, focused attention on the relative contributions of the various IBV typing tests. Over a two-year period this virus caused costly condemnations because of air sacculitis. It was first isolated from broilers in Georgia during November 1998 and was characterized by RT-PCR and RFLP in the laboratory of Dr. Mark Jackwood and colleagues at the University of Georgia (7). The RFLP pattern was similar but not identical to the Delaware serotype (DE072 reference strain). In Alabama, the virus was first isolated from broiler breeders in February 1999, and from broilers in March 1999. More than 100 isolates of this IBV from broilers and broiler breeders were made in Alabama through May 2000. Each isolate had the unique RFLP pattern that was similar but not identical to DE072 reference strain. S1 sequence analysis of nine Alabama isolates by Dr. Jack Gelb, Jr. at the University of Delaware, showed 97-100% homology among nine Alabama isolates, and 92-93% homology between the nine isolates and DE072, based on DNA sequence and deduced amino acids sequence. These findings were in general agreement with the analyses of the Georgia isolates by Lee, Hilt and Jackwood (7). This was suggestive of the new virus being, at least, closely related to and possibly a member of the Delaware serotype. Cross protection studies in SPF chickens and virus neutralization tests under the direction of Dr. Jackwood showed that it indeed qualified as a new serotype (7).

The situation facing producers was that a GA 98 vaccine was not available and would not be for possibly two more years. Many in Alabama and Georgia chose to use the Delaware serotype vaccine because it offered the possibility of partial protection. In Alabama, the IBV isolation data and field reports both supported the efficacy of the Delaware vaccine in controlling GA 98.

GA 98 IBV emerged at a time when molecular diagnostic procedures were fully employed in the region's diagnostic laboratories, and it was rapidly characterized. The genotyping tests (RFLP and nucleotide sequencing) suggested that it was similar to the Delaware serotype, but the significance of the differences was not known. The classical characterization studies in chickens and laboratory assays indicated that it fulfilled technical criteria as a new serotype. The field data showed that the Delaware vaccine, although only partially protective in the laboratory, was sufficient to improve the field situation. Thus, the GA 98 tested the paradigms of both the new genotypic and the classical assays for IBV typing, respectively. The experience with this virus was instructive in that molecular tests provided insight for the formulation of a presumptive control strategy. The classical tests were important in clarifying the assumptions based on the molecular characterizations. The production data showed that, although a new serotype-specific vaccine was not, nor would soon be available, strategic choices in vaccine selection could be made that were ultimately useful in controlling IB caused by the GA 98 virus.

In our experience with RT-PCR and RFLP diagnostics for IB, RFLP variants are relatively common. Most appear as just a few isolates in an area over several months. GA 98 was noteworthy because of the number of isolates, its persistence in a geographical area, and the economic significance of the disease. These are all features of the emergence of a new serotype of IBV.

In conclusion, molecular tests can identify and characterize isolates of IBV with reasonably reliable indication of serotype, and this capability will likely improve. Classical cross protection and serotyping assays still have a place in confirming the genotypic assumptions of serotype. Although serotype-specific IBV vaccines are desirable, the development issues for new vaccines require that currently approved vaccines be examined for application to emerging IBVs. The GA 98 experience affirmed the value of partial protection provided by a vaccine in a closely related serotype.

References

1. Cavanagh, D. A nomenclature for avian coronavirus isolates and the question of species status. Avian Pathology 30:109-115, 2001.

2. Cavanagh, D., and S.A. Naqi. Infectious bronchitis. In: Diseases of Poultry 10th ed. Ed. Calnek, B. W. Iowa State University Press, Ames, IA. pp. 51-526. 1997.

3. Gelb, J. Jr. and M.W. Jackwood. Infectious bronchitis. In: A Laboratory Manual for the Isolation and Identification of Avian Pathogens, 4th ed. Eds. Swayne, D.E., J.R. Glisson, M.W. Jackwood, J.E. Pearson, and W.M. Reed. American Association of Avian Pathologists, Kennett Square, PA. pp. 169-174, 1998.

4. Jackwood, M., W.H.M. Kwon, and D.A. Hilt. Infectious bronchitis virus detection in allantoic fluid using the polymerase chain reaction and a DNA probe. Avian Dis. 36:403-409. 1992.

5. Keeler, C.L., K.L. Reed, W.A. Nix, and J. Gelb, Jr. Serotype identification of avian infectious bronchitis virus (IBV) by RT-PCR of the peplomer (S-1) gene. Avian Dis. 42:275-284, 1998.

6. Kwon, W.H.M., M. Jackwood, and J. Gelb, Jr. Differentiation of infectious bronchitis virus serotypes using polymerase chain reaction and restriction fragment length polymorphism analysis. Avian Dis. 37:194-202. 1993.

7. Lee, C.W., D.A. Hilt, and M. Jackwood. Identification and analysis of the Georgia 98 serotype, a new serotype of infectious bronchitis virus. Avian Diseases 45:164-172, 2001.

Acknowledgements

The author thanks Lanqing Li and the staff of the Alabama Diagnostic Laboratories for their dedicated efforts. The collegial support and valuable suggestions offered by Jack Gelb, Jr. and Mark Jackwood are very much appreciated.

INFECTIOUS LARYNGOTRACHEITIS

The Current Field Status of Infectious Laryngotracheitis

John A. Smith DVM, MS, MAM
Fieldale Farms Corporation
P.O. Box 558
Baldwin, GA 30511

Overview

Infectious Laryngotracheitis (ILT) remains a major concern for the U.S. poultry industry. Currently, clinical ILT is a sporadic disease in most parts of the U.S., with locally widespread epornitics at irregular intervals, mainly in areas of concentrated production. These epornitics of ILT tend to occur where there are large populations of naïve, unvaccinated birds, i.e., in concentrated areas of broiler production.

Sporadic cases occur in all classes of birds, including hobby/show/game chickens, broilers, heavy breeders, and commercial leghorns. In the case of heavy breeders and leghorns, which are typically vaccinated against ILT, sporadic cases are often related to errors in vaccine application and to biosecurity failures. For example, commercial table egg producers may desire to avoid the expense associated with eyedrop administration of ILTV vaccine, and resort to mass application. These techniques may result in inadequate protection.[1] Since multiple-age layer complexes are common, an inadequately-vaccinated flock may be exposed to ILTV later, when a younger, vaccinated flock is moved into the complex and sheds the backpassaged vaccine virus, resulting in disease signs in the older flock. Cases in molted flocks that were not re-vaccinated, and the use of recently vaccinated "spiking males" in a poorly vaccinated, older breeder flock are other classic examples. Numerous scenarios involving the mixing of different populations with different vaccine programs are possible.

Current Incidence Patterns

Have epornitics of ILT increased in recent years? This question is difficult to answer due to lack of an accurate, long-term database. The AAAP published yearly disease incidence summaries by state through 1986.[2] (The last report, in 1987, contained only the data for the 4 reporting regions- NECAD, NCPDC, SCAD, and WPDC.) The pattern of ILT occurrence in selected states was examined between 1974 and 1986 (13 years), and compared to the incidence over the last 3 years, via personal communication with diagnosticians in those states.

The northeastern region (NECAD) showed a generally declining pattern from 1974-1986, but with substantial numbers of cases in all years (figure1). Maine had a large outbreak in 1974 (127 cases), which declined to low levels by 1977. Delaware and Maryland contributed the bulk of the cases throughout the period. Although the pattern on Delmarva also declined from the large 1974-76 outbreak, there were significant numbers of cases (>40) in all years except 1977, 1982, 1983, and 1986. Other states, most notably New Jersey and Pennsylvania, contributed sporadic cases or smaller scale outbreaks to the total, such as the 28 cases in Pennsylvania in 1985. Delmarva experienced large outbreaks in 1988-89 and 1994-95 that rivaled or exceeded those in 1974-76[3]. Since 1998, the Delmarva region has experienced a multi-year outbreak, with vaccination of broilers extending through February 2001. The 157 cases reported in 1999 compares to the roughly 150-200 cases per year in 1974-1976. Pennsylvania also experienced large outbreaks of virulent ILT in the late 1980's.

The north central region (NCPDC, figure 2) exhibited a more-or-less sporadic pattern between 1974 and 1986, with an outbreak in Iowa in 1982-1984. The western region (WPDC, figure 3) showed a similar pattern, with California contributing the majority of cases, and with peaks in 1974 and 1983. The situation in California for the last 3 years consists of sporadic outbreaks, mainly in layers.

In the southeast (SCAD, figure 4), the pattern presents more yearly fluctuation, and a generally increasing trend. Georgia and Alabama have contributed most of these cases, with North Carolina helping out in 1986. Georgia reported >50 cases in 1976, 1979, 1980, 1983, and 1985, and Alabama had $\geq$ 48 cases in 1979, 1980, and 1982. This pattern has continued unabated in the late 1990's. Georgia experienced a larger outbreak in 1994-96 than any of those recorded in 1974-1986, and other outbreaks have been contained by widespread vaccination in 1998-99, 1999-2000, and 2001. Alabama has likewise experienced several large outbreaks in 1998-99, 1999-2000, and 2001.

Among 15 broiler company veterinarians surveyed, 3 felt the incidence was increasing, 3 felt it was not, 2 were unsure, and 2 expressed the opinion that ILT was not increasing, but was concentrating or becoming "seasonally endemic" in concentrated areas. Without accurate, long–term data, it is difficult to assess whether the incidence of ILT is changing. It would appear that the disease indeed is following the dense concentrations of broilers, with the Delmarva and southeastern regions leading the way.

Current Vaccination Practices

A survey was conducted of 15 broiler company veterinarians and 3 layer veterinarians, soliciting information on vaccination practices and opinions on the origin, means of spread, and control of ILT outbreaks. The 15 broiler veterinarians work for 10 of the 12 largest integrators, accounting for approximately 70 % of broiler placements. None of the 15 routinely vaccinates broilers for ILT, although 2 routinely vaccinate certain classes of birds in certain areas. When broilers must be vaccinated (as in an outbreak situation), almost all respondents use a full dose of a chick embryo origin (CEO) vaccine via the drinking water, usually at about 14 days of age. Some will vaccinate as late as 21 days, and some cautioned against later vaccination due to excessive reaction. One respondent does spray ILTV vaccine, and one gives a reduced dose. In their breeder populations, 11 of 14 indicate that all breeder pullets are vaccinated, 2 indicated that most are vaccinated but that some locations do not vaccinate, and one does not vaccinate breeder pullets. Most breeder pullets receive a full dose of tissue culture origin (TCO) vaccine via eyedrop at 10-12 weeks of age. One of the 14 that do vaccinate pullets for ILT uses CEO vaccine in one location, and 2 use CEO vaccine exclusively.

Most layer breeders are vaccinated, and an estimated 80-90% of layers are vaccinated. Layers usually are vaccinated around 10 weeks of age, and programs vary. Those vaccinated via eyedrop are given a full dose of either TCO or CEO vaccine. Those vaccinated via spray or drinking water usually are given CEO vaccine, often at 2X dose. In summary, the majority of long-lived birds are vaccinated for ILT. While TCO vaccines appear most popular, there is considerable use of CEO vaccine.

Origins, spread, and control of outbreaks

Most respondents agreed with the statement that the majority of sporadic outbreaks in breeders and layers could be attributed to lapses in vaccination programs and biosecurity. A variety of hypotheses were advanced about the origin of broiler epornitics. High on the list is the use of CEO ILTV vaccines in the area, with several mentioning leghorns specifically. Backyard birds were also frequently suspected as the source. Interestingly, 4 respondents advanced the hypothesis that ILTV may be subclinically endemic or latent in the broiler population itself, and unknown factors lead to the

emergence of clinical epornitics from this source. Recent findings of occult cases via PCR techniques lend some creedence to this hypothesis.[4]

The spread of epornitics is almost universally attributed to lapses in biosecurity and spread via people, litter, equipment, vehicles, and along live haul routes. Several felt that wind may contribute. Likewise, there was nearly universal agreement on the use of zone vaccination, biosecurity measures, and traffic control to control epornitics. Several stressed the importance of cooperation among operators in designing zones, vaccinating all susceptible birds, and coordinating vaccination start and stop dates.

Current molecular evidence indeed does indicate that most strains isolated from clinical cases bear very close resemblance to the current vaccines[4], and almost all outbreaks are officially recognized as being due to escaped vaccine. Two interesting observations from the survey also provide circumstantial support for the escaped CEO vaccine hypothesis. Vaccination for ILT in any class of poultry has been uncommon in Mississippi for many years. The state of Texas allows the use of only the TCO ILTV vaccine. The one broiler integrator that reported no ILT vaccination of breeders was in Texas. There has been no clinical ILT in Mississippi since 1982, and only sporadic cases in Texas for many years, and those cases are suspected to arise from unauthorized CEO vaccine use.

Conclusions

Whether the incidence is increasing, static, or "concentrating", ILT remains a perplexing problem, especially in concentrated areas of broiler production. And, whether the source is from escaped CEO vaccine or from latent or subclinical viruses circulating in broiler populations, it is clear that better control measures are needed. The CEO vaccines are effective and readily available. However, they create harsh reactions, extract performance and condemnation penalties, interfere with existing IBV and NDV vaccination programs, and are capable of reverting to virulence, producing carriers, shedding virus, and spreading to unintended populations.[5] The TCO vaccine, while avoiding many of the disadvantages of CEO vaccines, is less immunogenic, is not amenable to mass application, and since it is available from only a single source, is more susceptible to supply interruptions. The ideal ILTV vaccine would prevent infection and shedding, while not reverting to virulence, shedding virus, or spreading itself. It would be mild, economical, readily available, and amenable to mass application. Since ILTV infects only chickens, a vaccine such as this should make eradication of the disease a feasible and worthwhile objective.

Acknowledgements: The following persons contributed the data used in this presentation: Drs. Calvin E. Anthony, Bernard J. Beckman, Bruce R. Charlton, Samuel P. Christenberry, Travis A. Cigainero, Lousie Dufour-Zavala, Adrian T. Garritty, Luis Gomez, Julie D. Helm, William W. Hewat, Frederic J. Hoerr, George T. Holder, Benjamin C. Johnson, Kenton S. Kreager, Danny Magee, Stephen McCarter, Kenneth Opengart, Richard A. Phillips, Patrick Pilkington, Marshall R. Putnam, Charles S. Roney, Mariano Salem.

References

1. Fulton, R. M., D. L. Schrader, and M. Will. Effect of route of vaccination on the prevention of infectious laryngotracheitis in commercial egg-laying chickens. Avian Dis. 44:8-16, 2000.

2. American Association of Avian Pathologists. Summary of Disease Reports.
   a. 1974. Avian Dis. 19:839-882, 1975.
   b. 1975. Avian Dis. 20:777-821, 1976
   c. 1976. Avian Dis. 22:211-255, 1978

d. 1977. Avian Dis. 23:241-277, 1979
e. 1978. Avian Dis. 24:532-568, 1980
f. 1979. Avian Dis. 25:555-591, 1981
g. 1980. Avian Dis. 26:640-680, 1982
h. 1981. Avian Dis. 27:860-900, 1983
i. 1982. Avian Dis. 28:816-843, 1984
j. 1983. Avian Dis. 29:258-294, 1985
k. 1984. Avian Dis. 29:1281-1317, 1985
l. 1985. Avian Dis. 30:856-892, 1986
m. 1986. Avian Dis. 31:926-978, 1987

3. Odor, E. M., M. Salem, J. K. Rosenberger, C. Pope, M. Murphy, and S. S. Cloud. Laryngotracheitis: The Delmarva experience. Proceedings, 30th National Meeting on Poultry Health and Processing. Pp71-74, 1995.

4. Garcia, M. and J. R. Glisson, personal communication.

5. Guy, J. S., H. J. Barnes, and L. Smith. Increased virulence of modified-live infectious laryngotracheitis vaccine virus following bird-to-bird passage. Avian Dis. 35:348-355, 1991.

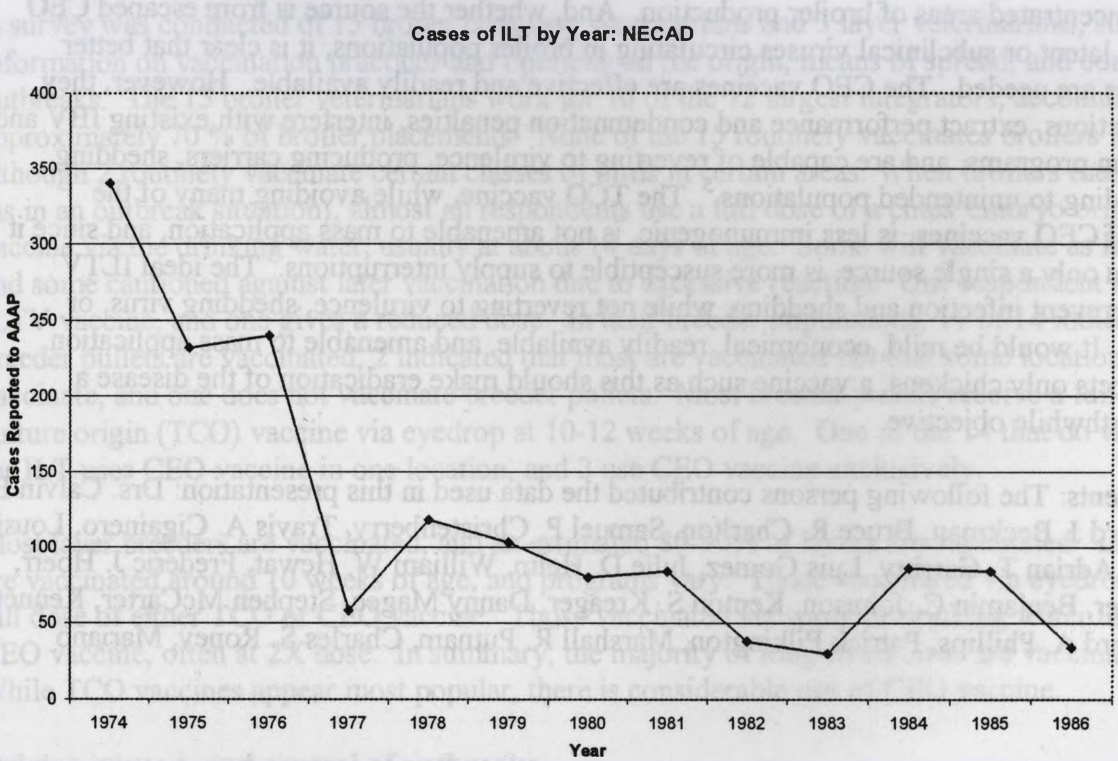

Figure 1. Cases of ILT reported to AAAP from the northeastern region, 1974-1986

d. 1977. Avian Dis. 23:241-277, 1979
e. 1978. Avian Dis. 24:532-568, 1980
f. 1979. Avian Dis. 25:555-591, 1981
g. 1980. Avian Dis. 26:640-680, 1982
h. 1981. Avian Dis. 27:860-900, 1983
i. 1982. Avian Dis. 28:816-843, 1984
j. 1983. Avian Dis. 29:258-294, 1985
k. 1984. Avian Dis. 29:1281-1317, 1985
l. 1985. Avian Dis. 30:856-892, 1986
m. 1986. Avian Dis. 31:926-978, 1987

3. Odor, E. M., M. Salem, J. K. Rosenberger, C. Pope, M. Murphy, and S. S. Cloud. Laryngotracheitis: The Delmarva experience. Proceedings, 30th National Meeting on Poultry Health and Processing. Pp71-74, 1995.

4. Garcia, M. and J. R. Glisson, personal communication.

5. Guy, J. S., H. J. Barnes, and L. Smith. Increased virulence of modified-live infectious laryngotracheitis vaccine virus following bird-to-bird passage. Avian Dis. 35:348-355, 1991.

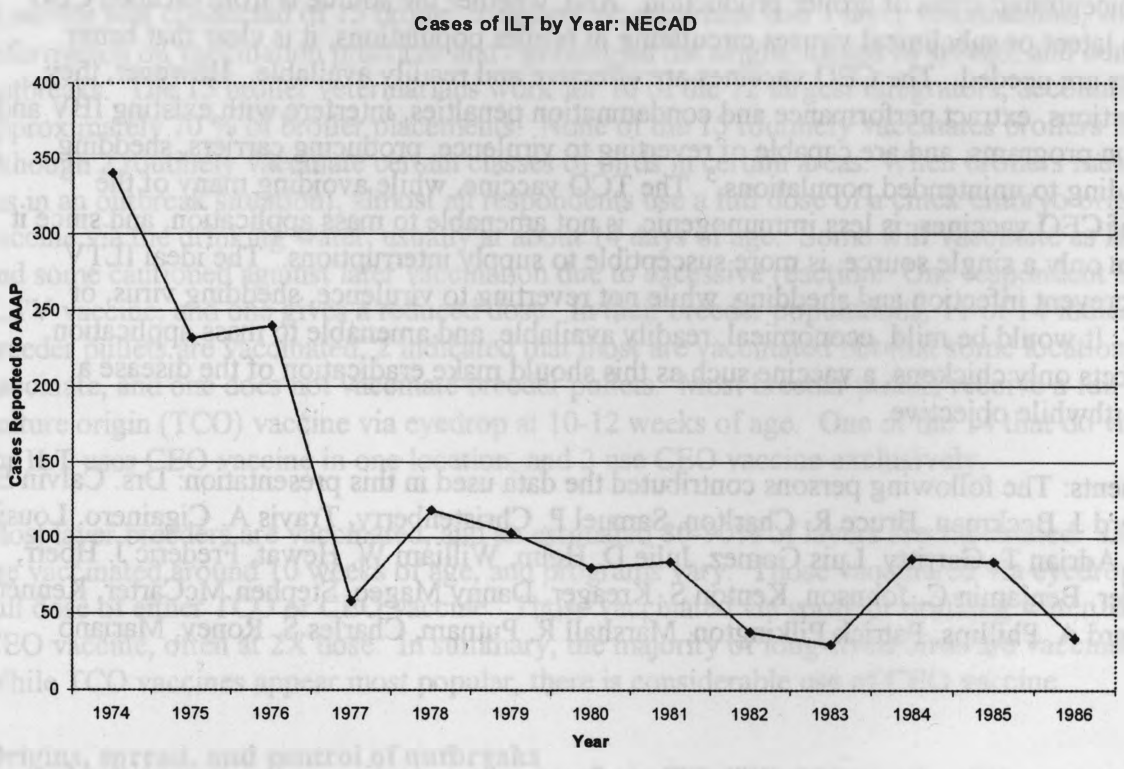

Figure 1. Cases of ILT reported to AAAP from the northeastern region, 1974-1986

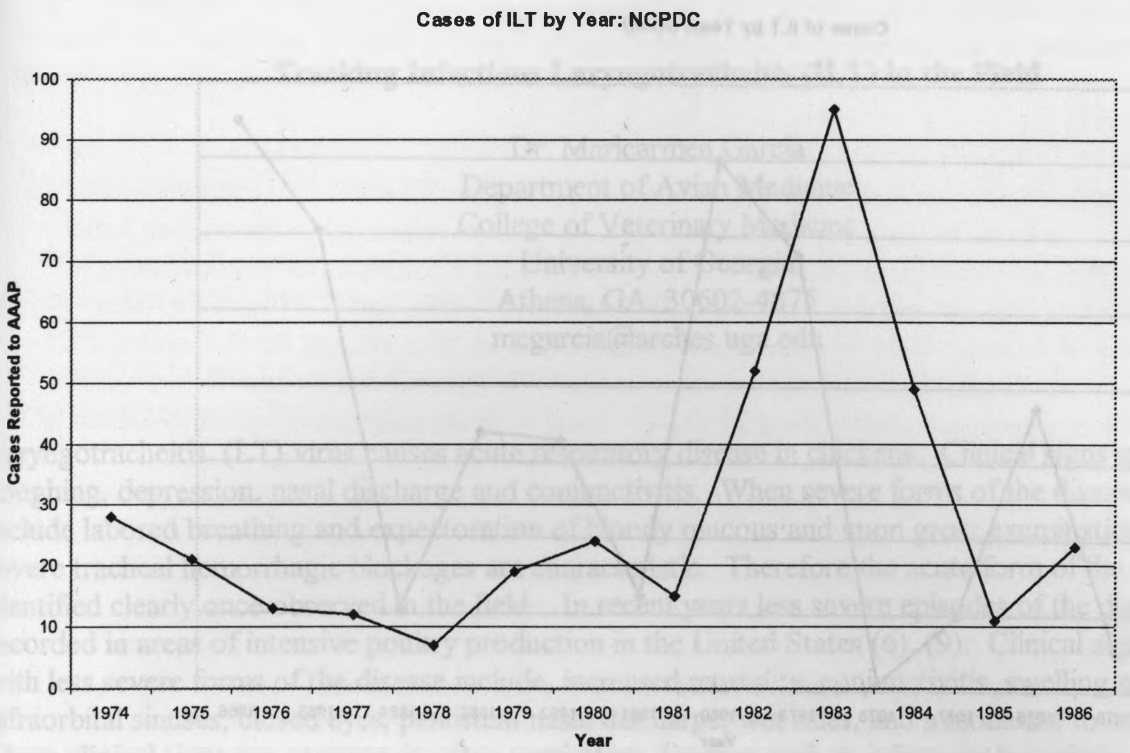

Figure 2. Cases of ILT reported to AAAP from the north central region, 1974-1986

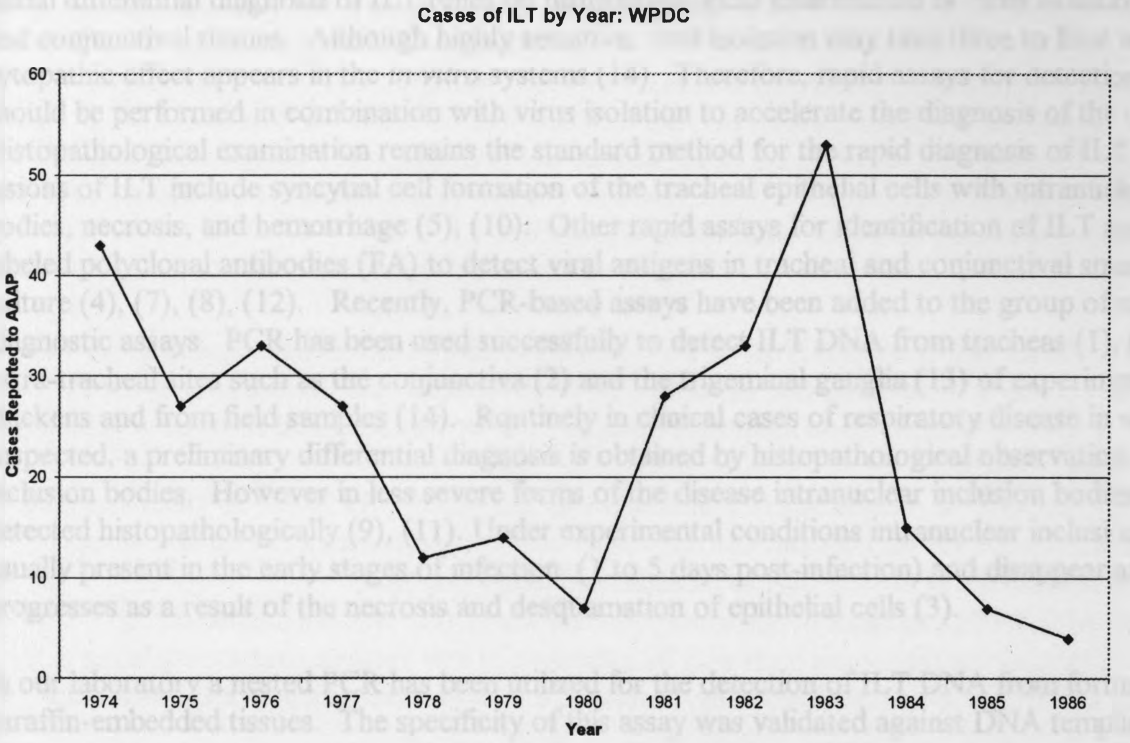

Figure 3. Cases of ILT reported to AAAP from the western region, 1974-1986

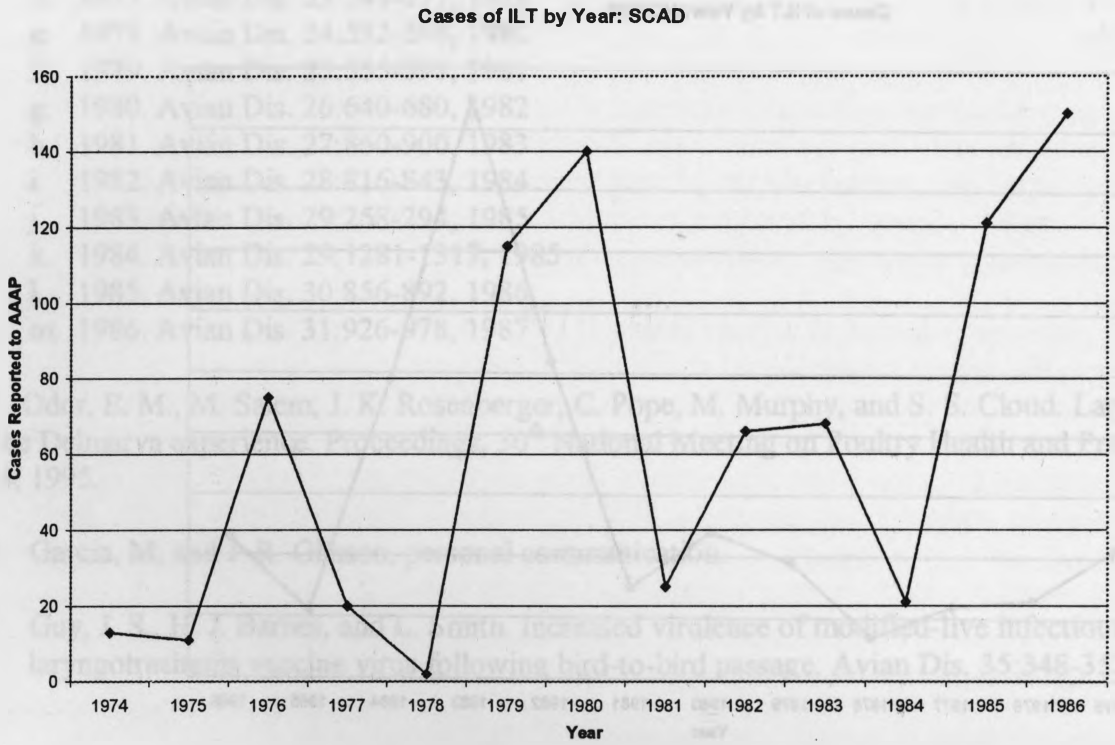

Figure 4. Cases of ILT reported to AAAP from the southern region, 1974-1986

Tracking Infectious Laryngotracheitis (ILT) in the Field

Dr. Maricarmen García
Department of Avian Medicine
College of Veterinary Medicine
University of Georgia
Athens, GA. 30602-4875
mcgarcia@arches.uga.edu

Laryngotracheitis (LT) virus causes acute respiratory disease in chickens. Clinical signs include gasping, coughing, depression, nasal discharge and conjunctivitis. When severe forms of the disease occur signs include labored breathing and expectoration of bloody mucous and upon gross examination of the trachea severe tracheal hemorrhagic blockages are characteristic. Therefore the acute form of the disease can be identified clearly once observed in the field. In recent years less severe episodes of the disease have been recorded in areas of intensive poultry production in the United States (6), (9). Clinical signs associated with less severe forms of the disease include, increased mortality, conjunctivitis, swelling of the infraorbital sinuses, closed eyes, persistent nasal discharge, wet rales, and a moderate to severe tracheitis. These clinical signs are common in other respiratory diseases such as infectious bronchitis, newcastle disease (NDV), and *Mycoplasma galisepticum* (MG) among others (3).

Initial differential diagnosis of ILT relies on histopathological examination or virus isolation from tracheal and conjunctival tissues. Although highly sensitive, viral isolation may take three to four weeks before cytopathic effect appears in the *in vitro* systems (14). Therefore, rapid assays for detection of the virus should be performed in combination with virus isolation to accelerate the diagnosis of the disease. Histopathological examination remains the standard method for the rapid diagnosis of ILT. Characteristic lesions of ILT include syncytial cell formation of the tracheal epithelial cells with intranuclear inclusion bodies, necrosis, and hemorrhage (5), (10). Other rapid assays for identification of ILT are fluorescent-labeled polyclonal antibodies (FA) to detect viral antigens in tracheal and conjunctival smears or tissue culture (4), (7), (8), (12). Recently, PCR-based assays have been added to the group of rapid ILT diagnostic assays. PCR has been used successfully to detect ILT DNA from tracheas (1), (2) and from extra-tracheal sites such as the conjunctiva (2) and the trigeminal ganglia (13) of experimentally infected chickens and from field samples (14). Routinely in clinical cases of respiratory disease in which ILT is suspected, a preliminary differential diagnosis is obtained by histopathological observation of intranuclear inclusion bodies. However in less severe forms of the disease intranuclear inclusion bodies may not been detected histopathologically (9), (11). Under experimental conditions intranuclear inclusion bodies are usually present in the early stages of infection (1 to 5 days post-infection) and disappear as infection progresses as a result of the necrosis and desquamation of epithelial cells (3).

In our laboratory a nested PCR has been utilized for the detection of ILT DNA from formalin-fixed paraffin-embedded tissues. The specificity of this assay was validated against DNA templates of ILT, MG, MS, Marek's Disease Virus (MDV), herpesvirus of turkeys (HVT), fowl pox virus (FPV), chicken anemia virus (CAV), and avian adenovirus serotypes 1, 4, 5, 8 and 10. In addition, primer specificity was tested against DNA from tracheas of clinically normal birds and from uninfected embryo cells. Only ILT DNA was amplified producing the expected PCR product of 296 bp in size. To further validate the application of the assay 34 clinical cases of respiratory illness, submitted between January of 1999 and

February 2000, were chosen for ILT nested PCR analysis based on the initial clinical observations. After initial clinical examination, ILT infection was suspected in 11 of the 34 cases. In addition to PCR most of the cases were tested by direct fluorescent antibody test (DFAT) and virus isolation for the presence of NDV, IBV and ILT.

At post-mortem examination necrotizing laryngitis and hemorrhagic tracheitis were observed in the 11 ILT-suspect cases. In some of these cases formation of a diphtheric membrane in the trachea lumen was observed. After clinical examination tracheal, conjunctival samples were taken for histopathological examination, direct fluorescent antibody test (DFAT) against NDV, IBV and ILTV and for virus isolation. Sections of formalin-fixed paraffin-embedded tissues were also utilized for ILT nested-PCR analysis. Results from diagnostic assays are shown in Table 1. Intranuclear inclusion bodies were detected upon histopathological examination for the 11 cases. ILT antigen was detected by DFAT and virus isolation in 9 of the 11 cases, and ILT DNA was detected by PCR in 10 of the 11 cases.
For the remaining 23 cases of respiratory disease post-mortem examination presented different degrees of tracheitis and airsacculitis. Results from diagnostic assays for this group are summarized in Table 2. Upon histopathological examination subacute to chronic tracheitis with epithelial hyperplasia, mild lymphocytic infiltration, and loss of cilia were the most common lesions reported. For purposes of this study, these lesions will be designated as nonspecific tracheitis (NST) (Table 2). ILT DNA was detected by nested PCR in 7 samples where typical ILT inclusions were not observed. A single step PCR targeted to a different gene was utilized to re-test the seven nested PCR positive cases, 2 of the 7 cases were positive with the single step PCR indicating the higher detection sensitivity of a nested amplification. Virus isolation was attempted for 6 of these 7 NST/PCR positive samples. Adenovirus was isolated from 3 samples; IBV and NDV were isolated from 2 samples, whereas ILT was not isolated from any of the samples. Seventeen of the 23 samples diagnosed as NST by histopathology analysis were negative for ILT DNA by nested PCR. Virus isolation was accomplished for 15 of these 17 NST/ PCR negative samples. Adenovirus was isolated from 7 samples; IBV was isolated from 6 samples; NDV was isolated from one sample; no viral isolation was recorded for 4 of the 17 NST/ PCR negative samples. Recently less severe forms of the disease has been diagnosed using a combination of nested-PCR, histopathological examination of tracheas and embryo chorioallantoic membranes (CAM), and virus isolation. The history on these cases will be discussed.

Conclusions:

- A nested PCR for detection of ILT DNA from formalin-fixed, paraffin-embedded sections was developed. It was demonstrated that the nested PCR was specific and did not amplify DNA from other avian viruses, or from uninfected chicken cells, or from tracheal samples of clinically normal birds.
- Based on initial clinical observation ILT infection was suspected in 12 of the 35 cases analyzed. The high correlation (91%) between histopathology analysis and nested PCR on ILT suspect cases validate the use of nested PCR as an additional diagnostic tool.
- Detection of ILT DNA in cases of respiratory disease where ILT was not suspected or confirmed by classical diagnostic tools may be indicative of low virulent strains circulating in the field that are difficult to isolate. Secondly, nested PCR may be detecting low-level persistent infections, or detection of latently infected birds, or both.
- The presence of ILT DNA in cases of mild respiratory illness raise questions on the role that ILT plays in the respiratory process, and suggest that low-level persistent infection may be more prevalent in the field than previously considered. Efforts to isolate ILT from PCR positive birds are in progress to further studies the pathogenicity and tissue tropism of these isolates.

References:

1. Abbas, F., J. R. Andreasen, Jr., and M. W. Jackwood. Development of a polymerase chain reaction and a nonradioactive DNA probe for infectious laryngotracheitis virus. Avian Dis 40:56-62. 1996.

2. Alexander, H. S., and E. Nagy. Polymerase chain reaction to detect infectious laryngotracheitis virus in conjunctival swabs from experimentally infected chickens. Avian Dis 41:646-653. 1997.

3. Bagust, T. J., and J. S. Guy. Laryngotracheitis. In: Diseases of Poultry, 10th ed. B. W. Calnek, ed. Iowa State University Press, Ames, Iowa. pp. 527-539. 1997.

4. Braune, M. O., and R. F. Gentry. Standardization of the fluorescent antibody technique for the detection of avian respiratory viruses. Avian Dis. 9:535-545. 1965.

5. Cover, M. S., and W. J. Benton. The biological variation of infectious laryngotracheitis virus. Avian Dis. 2:375-383. 1958.

6. Davison, S., R. J. Eckroade, and K. Miller. 1988. Laryngotracheitis - The Pennsylvania Experience, p. 14-19. Proc. 23rd Nat. Meet. Poultry Health and Condemnations, Ocean City, Maryland.

7. Goodwin, M. A., M. A. Smeltzer, J. Brown, R. S. Resurreccion, and T. G. Dickson. Comparison of histopathology to the direct immunofluorescent antibody test for the diagnosis of infectious laryngotracheitis in chickens. Avian Dis 35:389-391. 1991.

8. Ide, P. R. Sensitivity and specificity of the fluorescent antibody technique for detection of infectious laryngotracheitis virus. Can J Comp Med 42:54-62. 1978.

9. Linares, J. A., A. A. Bickford, G. L. Cooper, B. R. Charlton, and P. R. Woolcock. An outbreak of infectious laryngotracheitis in California broilers. Avian Dis 38:188-192. 1994.

10. Pirozok, R. P., C. F. Helmbolt, and E. L. Jungherr. A rapid histological technique for the diagnosis of infectious avian laryngotracheitis. J. Am Vet. Med. Assoc. 103:406-407. 1957.

11. Russell, R. G. Respiratory tract lesions from infectious laryngotracheitis virus of low virulence. Vet Pathol 20:360-369. 1983.

12. Wilks, C. R., and V. G. Kogan. An immunofluorescence diagnostic test for avian infectious laryngotracheitis. Aust Vet J 55:385-388. 1979.

13. Williams, R. A., M. Bennett, J. M. Bradbury, R. M. Gaskell, R. C. Jones, and F. T. Jordan. Demonstration of sites of latency of infectious laryngotracheitis virus using the polymerase chain reaction. J Gen Virol 73:2415-2420. 1992.

14. Williams, R. A., C. E. Savage, and R. C. Jones. A comparison of direct electron microscopy, virus isolataion and a DNA amplification method for the detection of avian infectious laryngotracheitis virus in fiels material. Avian Pathology 23:709-720. 1994.

Table 1. Summary of diagnostic results on clinically clear ILT cases

Clinical Samples	Histopathology	DFAT[2]	Virus Isolation[3]	ILT Nested PCR
1	ILT[1]	ILT	ILT	+
2	ILT	ILT	-	+
3	ILT	-	-	+
4	ILT	ILT	ILT	-
5	ILT	ILT, IBV	ILT	+
6	ILT	ILT	ILT	+
7	ILT	ILT	ILT	+
8	ILT	ILT	ILT	+
9	ILT	ILT, IBV	ILT	+
10	ILT	ILT	ILT	+
11	ILT	ILT	ILT	+

[1]Syncytial cells and nuclear inclusion bodies observed; [2]Direct fluorescent antibody test (DFAT) against IBV NDV and ILT; [3]IBV, NDV and ILT were detected in tissue culture by DFAT

Table 2. Summary diagnostic results on cases respiratory disease

Clinical Samples	Histopathology	DFAT[2]	Virus Isolation[3]	ILT Nested PCR
1	NST[1]	nd	IBV	-
2	NST	nd	ADE, IBV	-
3	NST	-	-	+
4	NST	nd	ADE	-
5	NST	nd	nd	-
6	NST	-	nd	+
7	NST	-	ADE	+
8	NST	-	-	+
9	NST	-	ADE	-
10	NST	-	-	-
11	NST	IBV	-	-
12	NST	NDV	nd	-
13	NST	NDV	nd	-
14	NST	-	-	-
15	NST	-	ADE,IBV	+
16	NST	-	ADE,IBV	-
17	NST	-	ADE,IBV	-
18	NST	-	ADE,IBV	-
19	NST	-	ADE,NDV	+
20	NST	-	-	-
21	NST	nd	NDV,IBV	-
22	NST	-	ADE	-
23	NST	-	-	-
24	NST	NDV	-	+

[1]NST-nonspecific tracheitis; [2]DFAT-Direct fluorescent antibody test against IBV NDV and ILT, [3] IBV, NDV and ILT were detected in tissue culture by DFAT

NEW APPROACHES IN ILTV VACCINE DEVELOPMENT

Calvin L. Keeler, Jr., Ph.D.
Professor
Department of Animal and Food Sciences
University of Delaware
Newark, Delaware 19717-1303
ckeeler@udel.edu

ABSTRACT

Infectious laryngotracheitis (ILT) causes an acute upper respiratory infection in chickens. Vaccination for ILT has generally been used only in areas where the disease is endemic, since current vaccines are themselves mildly pathogenic, with a resulting economic "cost". There is justifiable concern over the negative performance (growth, mortality, feed conversion) associated with current ILT vaccines. After reviewing the benefits and pitfalls associated with the use of the current generation of live attenuated ILTV vaccines, this talk will focus on the status of recombinant ILTV vaccine development and on new approaches to ILTV vaccination, including genetic vaccination technologies.

OVERVIEW

Infectious laryngotracheitis virus (ILT) was first described in 1925 (May and Tittlser, 1925). In its acute form, ILT is characterized by signs of respiratory distress in birds, accompanied by gasping and expectoration of bloody exudates (Bagust and Guy, 1997). In addition, the mucous membranes of the trachea become swollen and hemorrhagic. The epizootic form of the disease spreads rapidly and although severe forms of the disease cause high morbidity (90-100%), mortality generally averages 10-20%. The etiological agent for ILT is infectious laryngotracheitis virus (ILTV), an alpha herpesvirus. Like other herpesviruses, ILTV can also establish a latent carrier state in recovered animals. Virus shed after the latent period is another source of virus capable of causing disease in susceptible birds. Fortunately, ILT is a slowly spreading, controllable disease, which does not normally exhibit the mortality and condemnation statistics associated with some other avian respiratory disease agents.

ILTV infections are usually limited to the upper respiratory tract and viremia is rarely observed. Furthermore, the humoral immune response, including secretory and maternal antibodies, and levels of neutralizing antibodies do not correlate well with protection. Instead, protection seems to be mediated primarily by the cellular immune response. These points are important to consider in the development of a vaccination strategy. Any effective vaccine will have to elicit an effective mucosal, cell-mediated, protective immune response.

Vaccination for ILT has generally been used only in areas where the disease is endemic, since vaccination can result in the occurrence of long-term "carrier" birds due to the virus' ability to enter a latent state in the sensory ganglia. Furthermore, current vaccines are themselves mildly pathogenic, with a resulting economic "cost". There is justifiable concern over the negative performance (growth, mortality, feed conversion) associated with current ILT vaccines. Some investigators (Guy *et al.*, 1991) have reported that modified live ILT vaccines increase in

virulence by mutation during bird-to-bird passage in the field. This has led to an additional reluctance to vaccinate for ILT unless a region is faced with an active outbreak of the disease. Other groups contend that vaccine strains of ILTV are genetically stable (Keeler *et al.*, 1993). However, by spreading into flocks that may contain birds of different ages and with a different immune status, the incompletely attenuated modified live vaccine strains of ILTV may manifest a clinically more severe disease.

TRADITIONAL LIVE ATTENUATED ILT VACCINES

Traditionally there have been two sources of live attenuated ILT vaccines. Vaccines attenuated by multiple passages in embryonating eggs (CEO) are highly effective. However, in many cases their use can result in lower performance and higher condemnation rates. Broilers are generally vaccinated with CEO vaccine by drinking water only in the face of an outbreak. Furthermore, CEO-derived vaccine strains of ILTV are generally indistinguishable from true field isolates of ILTV, providing diagnosticians and regulators with additional challenges. ILT vaccines generated by multiple passages in tissue culture (TCO) generally offer less protection as they are more highly attenuated and less immunogenic. TCO vaccines are commonly used in layer breeders and layers.

As researchers we are faced with the task of developing new generations of ILT vaccines with the correct balance between pathogenicity and immunogenicity. An ILT vaccine would also ideally not establish latency, or at least be limited in its ability to shed. Furthermore, it should be easily distinguishable from other strains of the virus and be shown experimentally to be genetically stable. Besides these criteria it also needs to be relatively cheap and easy to produce and administer.

RECOMBINANT SUBUNIT VACCINES

There are many excellent examples in the literature of the use of fowlpox virus and Marek's disease virus as vectors for the insertion of genes from avian pathogens. In these cases, a gene encoding an immunogenic protein is inserted into a region of the host genome which is nonessential for the host's replication. Although ILTV genes have also been inserted into these vectors, most of this data has not been published. Typically, these efforts have involved either the ILTV glycoprotein B or glycoprotein D genes (Keeler *et al.*, 1992). In both cases these are virally-encoded structural glycoproteins which are located on the viral envelope and the surface of infected cells and are required for viral attachment. There have been extensive studies of the immunogenic and protective properties of the herpes simplex virus (HSV) homologs of these proteins, which suggest that the ILTV proteins would be effective immunogens. The ILTV genes for these two proteins have been inserted into fowlpox or Marek's disease virus vector systems. The recombinant viruses produce proteins which are immunogenic and elicit a protective immune response. These viruses have been the focus of recent licensing and commercialization efforts.

LIVE ATTENUATED RECOMBINANT ILT VACCINES

Traditionally, recombinant vaccines for the poultry industry have been constructed by selecting for relatively rare *in vivo* homologous recombination events. The classic model systems for avian vaccine development again have been fowlpox virus and Marek's disease virus. The technique depends on introducing a DNA fragment into tissue culture cells by transfection and then co-transfecting the culture with viral DNA or infecting the culture with virus. This technique can be used to mutate a viral gene by replacing it with a foreign, or marker, gene. Although the technique has been extensively used for a number of herpesviruses, it has proven to be difficult to adapt for use with ILTV. The difficulties stem from the limited host range of ILTV. Besides being limited to infecting chickens *in vivo*, ILTV will also only grow on primary chicken embryo or chick kidney (CK) cells or chicken embryonic liver cells (CEL) *in vitro*. Some strains of ILTV have been adapted to grow on chicken hepatocyte cell lines (LMH). Unfortunately, primary cells grown in culture do not have the robust replicative machinery needed to ensure that a recombination event will occur and the adaptation of ILTV to continuous cell lines may introduce uncharacterized mutations in the virus.

Despite these technical hurdles, a number of groups have successfully constructed defined ILTV mutants (Guo *et al.*, 1994, Okamura *et al.*, 1994, Schnitzlein *et al.*, 1995). The first target of these efforts was invariably the thymidine kinase gene. The protein encoded by this gene is involved in DNA metabolism and viral pathogenicity. For various reasons these initial live attenuated strains of ILTV have not been suitable commercial vaccine candidates. However, another generation of viral mutants is being produced and evaluated.

NOVEL VACCINE APPROACHES

Genetic immunization has been shown in a number of animal models to induce protective immunity to infectious diseases (Lewis and Babiuk, 1999). DNA vaccines can be relatively quick and easy to generate. Plasmid DNA is not infectious and it doesn't replicate. Furthermore, plasmid DNA is stable and can be stored under conditions that would destroy a live virus. In addition, plasmid DNA can be administered by a variety of methods, including the potential of *in ovo* administration. As evidenced by the papers at this meeting, there is considerable interest in the application of this technology to avian health issues. The first ILTV DNA vaccination experiments were reported in 1995 (Keeler *et al.*, 1995). Birds vaccinated intramuscularly with DNA encoding glycoprotein B were found to have levels of protection comparable to those vaccinated with traditional live attenuated ILTV vaccines. Enhancement of DNA vaccine efficacy and the development of a practical cost-effective application of this technology will be required before its acceptance by the poultry industry.

Recently, two novel methods for constructing viral mutants have been developed in mammalian systems. In cosmid cloning, a viral genome is cloned as 4-6 large overlapping subgenomic fragments into *Escherichia coli* bacterial cells. Recombination/mutational events can then be effected in a much more efficient manner in the milieu of a bacterial system. The overlapping fragments can then be introduced back into avian cells where they recombine to produce a

complete infectious genome. In BAC (bacterial artificial chromosome) cloning, a portion of the *E. coli* F plasmid is introduced into the viral genome. This confers the ability to maintain the entire viral genome as a bacterial plasmid, where manipulations can be more easily performed. This intact genomic "plasmid" DNA can then be used to generate virus when transfected into avian cells. In both systems, the primary genetic manipulations occur in bacteria instead of in tissue culture and all of the progeny virus contain the desired insertion/deletion of genetic material. Within the past year both technologies have been successfully adapted for constructing Marek's disease virus mutants (Schumacher *et al.*, 2000) and laboratories are working to develop these same techniques for ILTV.

CONCLUSIONS

ILT is a serious upper respiratory disease of poultry. Although current live attenuated ILTV vaccines are effective, new vaccines which protect birds without economic and health consequences would be embraced by the poultry industry. Although still in the developmental phase, recombinant ILTV vaccines should be available for commercial use within the next five years.

REFERENCES

Bagust, T. J., and J. S. Guy. Laryngotracheitis. In: Diseases of Poultry, 10th ed. B.W. Calnek, H.J. Barnes, C.W. Beard, L.R. McDougals and Y.M. Saif eds. Iowa State University Press, Ames, Iowa. pp. 527-539. 1997.

Guo, P., E. Schulz, B. Maloney and E. Welniak. 1994. Construction of recombinant avian infectious laryngotracheitis virus expressing the β-galactosidase gene and DNA sequencing of the insertion region. Virology. 202:771-781.

Guy, J.S., H.J. Barnes and L.G. Smith. 1991. Increased virulence of modified-live infectious laryngotracheitis vaccine virus following bird-to-bird passage. Avian Dis. 35:348-355.

Keeler, C.L., Jr., J.W. Hazel, J.E. Hastings and J.K. Rosenberger. 1993. Restriction endonuclease analysis of Delmarva field isolates of infectious laryngotracheitis virus. Avian Dis. 37:418-426.

Keeler, C., Jr., D. Poulsen, H. Robinson, J. Santoro and D. Thureen. (1995) Immunization of chickens with gene (DNA) vaccines. 132nd Annual Meeting of the AVMA. Pittsburgh, PA. p.143.

Keeler,C.L., Jr., J.K. Rosenberger, S.S. Cloud, D.J. Poulsen, K. Nazerian and A. Yanagida. (1992) A recombinant fowlpox virus protects chickens against challenge by infectious laryngotracheitis virus (ILTV). 129th Annual Meeting of the AVMA. Boston, MA. p.137.

Lewis, P.J. and L.A. Babiuk. 1999. DNA vaccines: a review. Adv. Virus Res. 54:129-188.

May, H.G. and R.P. Tittsler. 1925. Tracheo-laryngitis in poultry. J. Am. Vet. Med. Assoc. 67:229-231.

Okamura, H., M. Sakaguchi, T. Honda, A. Taneno, K. Matsuo and X. Yamata. 1994. Construction of recombinant infectious laryngotracheitis virus expressing the gene of *E. coli* within the thymidine kinase gene. J. Vet. Med. Sci. 56:799-801.

Schnitzlein, W.M., R. Winans, S. Ellsworth and D.N. Tripathy. 1995. Generation of thymidine kinase-deficient mutants of infectious laryngotracheitis virus. Virology. 209:304-314.

Schumacher, D., B.K. Tischer, W. Fuchs and N. Osterrieder. 2000. Reconstitution of Marek's disease virus serotype 1 (MDV-1) from DNA cloned as a bacterial artificial chromosome and characterization of a glycoprotein B-negative MDV-1 mutant. J. Virol. 74:11088-11098.

MYCOPLASMA GALLISEPTICUM

Mycoplasma gallisepticum in North Carolina: 1999-2000 (and beyond)

David H. Ley, Jean-Pierre Vaillancourt, and Algis Martinez

Department of Farm Animal Health and Resource Management
College of Veterinary Medicine, North Carolina State University, Raleigh, NC 27606

ABSTRACT

In 1999-2000, North Carolina experienced unprecedented outbreaks of *Mycoplasma gallisepticum* (MG) in commercial poultry flocks. Our efforts focused on MG isolation and strain identification; and epidemiology consisting of field investigations, a case control study, and reporting. Random amplification of polymorphic DNA (RAPD), a PCR-based method of DNA fingerprinting, was used for MG strain identification. By October 2000, we had identified four different 'RAPD types' among 71 field isolates, and found that their fingerprints were different from MG vaccine strains (F, ts11 and 6/85) and the 'House finch' strain. RAPD type A was isolated from a single backyard flock and multiple houses of a nearby turkey breeder farm. Type B was isolated from a broiler breeder farm, but before the infection was recognized progeny were moved to a site near other poultry farms in a neighboring county. Type B became widespread and was involved in major foci of infections in eastern and western North Carolina. Type E was isolated from a single broiler breeder farm in the northwestern part of the state. RAPD type F was identified in northeastern North Carolina and involved a cluster of farms consisting of a broiler breeder flock and multiple flocks of progeny. Evidence suggests that inadequate monitoring of some broiler breeder flocks, the movements and interactions of people, and lack or lapses of biosecurity were major factors contributing to this epidemic of MG in North Carolina. Since January 2001, MG has been isolated from only one broiler breeder and two commercial turkey farms. Each of these isolates was a new and unique RAPD type, which suggests that they were introduced to the poultry farms from separate external sources (evidence suggests backyard flocks), and there was no evidence of transmission to or from other commercial poultry. RAPD fingerprinting of MG field isolates enabled us to learn more about the epidemiology of the outbreaks and validated the utility of strain identification.

INTRODUCTION

Mycoplasma spp. are important respiratory and systemic pathogens of poultry causing substantial economic losses worldwide. Mycoplasmas are cell wall-less bacteria that are small in size and also have minimal genomes. The functional result of these features means that these organisms do not survive for long outside a host, and they are nutritionally fastidious and may be difficult to isolate but do grow on cell-free media. Definitive diagnosis relies on 1) isolation of mycoplasmas in culture media and identification using species-specific polyclonal antisera in immunofluorescence or growth inhibition tests, or 2) species-specific polymerase chain reaction (PCR) tests. However, these tests are not capable of recognizing the marked intraspecies (strain) genotypic (DNA sequence) and phenotypic (e.g. antigenic, pathogenic, immunogenic) variation among avian mycoplasma field isolates. The ability to readily differentiate avian mycoplasma field isolates from one another is key to 1) improving diagnostics, 2) outbreak and epidemiological investigations, and 3) recognizing and understanding virulence factors.

Random amplification of polymorphic DNA (RAPD) is a PCR-based method of DNA fingerprinting that results in amplification of 'anonymous' stretches of DNA with one (or sometimes more) short arbitrary primers and subsequent visualization of the amplification products by agarose gel electrophoresis resulting in characteristic banding patterns. Organism or samples with the same DNA will have the same banding pattern; if they are different their banding patterns will differ. RAPD banding patterns may be thought of as analogous to barcodes or fingerprints. Compared to other currently available methods of avian mycoplasma strain identification, RAPD is fast, relatively simple to perform and cost effective. However, there are disadvantages and limitations to RAPD fingerprinting that must be considered. Starting material for the test requires a pure culture of the mycoplasma isolate. RAPD tests are known to have problems with reproducibility because they are sensitive to alterations in PCR conditions. Interpretation of RAPD banding patterns can also be challenging due to the aforementioned problem of reproducibility, and the possibility of co-migrating bands. The 'challenges' of reproducibility and interpretation can usually be overcome by using one or more additional primer sets to confirm apparent relationships or resolve ambiguous results.

MG OUTBREAKS

Beginning in October 1999, North Carolina experienced unprecedented outbreaks of *Mycoplasma gallisepticum* (MG) in commercial poultry flocks. By October 2000, the epidemic involved breeder and meat flocks of chickens and turkeys resulting in 104 farms quarantined (18 broiler breeder, 40 broiler, 8 turkey breeder, 40 commercial turkey) and 11 backyard flocks. Investigations of this epidemic involved the North Carolina State University College of Veterinary Medicine (Poultry Health Management faculty in the Department of Farm Animal Health and Resource Management) and College of Agriculture and Life Sciences (Cooperative Extension Service, Department of Poultry Science), North Carolina Department of Agriculture, National Poultry Improvement Plan, and commercial poultry integrators. NCSU-CVM efforts have focused on 1) MG isolation, and strain identification by DNA fingerprinting, and 2) epidemiology consisting of field investigations, a case control study, and reporting (supported in part by grants from the USDA Fund for Rural America, and US Poultry and Egg Association).

A primary objective was to perform RAPD analyses on MG isolates from affected flocks in North Carolina. Of 91 mycoplasma cultures that we examined in 1999-2000, 69% were pure MG, 23% contained MG and MS, and 7% contained MG and some other *Mycoplasma* species. Of 71 isolates that we fingerprinted by RAPD we identified four different 'RAPD types': approximately 13% were type A, 83% type B, 1% type E, and 3% type F. We also found that these RAPD types or field strains had fingerprints that were different than the MG vaccine strains (F, ts11 and 6/85) and the 'House finch' strain, which indicates that these strains were not involved in this epidemic.

The ability to assign RAPD type or strain identities to MG-positive flocks enabled us to learn more about the epidemiology of the outbreaks. For example, a large cluster of MG-positive flocks in eastern North Carolina involved RAPD types A and B. RAPD type A was isolated from a single backyard flock and multiple houses of a nearby turkey breeder farm and nowhere else. Type B was isolated from a broiler breeder farm, but before the infection was recognized progeny were moved to a site near other poultry farms in a neighboring county. Unfortunately, type B became widespread and was involved in major foci of infections in eastern and western North Carolina. RAPD type E has only been isolated from a single broiler breeder farm in the northwestern part of the state and the suspected origin was a nearby backyard flock. MG RAPD type F was identified in northeastern North Carolina and involved a cluster of farms consisting of a broiler breeder flock and multiple flocks of progeny.

CONCLUSIONS

Although investigations are still in progress, current evidence suggests that inadequate monitoring of some broiler breeder flocks, the movements and interactions of people, and lack or lapses of biosecurity were major factors contributing to this epidemic of MG in North Carolina.

Through May of 2001, MG has been isolated from only one broiler breeder and two commercial turkey farms. Each of these isolates was a new and unique RAPD type, which suggests that they were introduced to the poultry farms from separate external sources. In two of these cases there were nearby backyard flocks that tested seropositive for MG. There was no evidence of transmission of these RADP types to or from other commercial poultry, so these appear to be isolated and separate outbreaks.

The successful application of RAPD fingerprinting to MG field isolates has validated the utility of strain identification. Future developments and improvements in strain identification technology can be anticipated and may include, 1) the use of computerized DNA fingerprint analysis and database system, and/or 2) new methodologies for molecular typing. Computer-assisted DNA analysis systems can be used to correct, process and analyze gels in order to compare banding patterns, and are particularly effective in epidemiological studies. The power of a computerized DNA analysis system resides in its capacity to compare every new strain with all previously analyzed strains, and to develop large databases for comparison. Additionally, there is the prospect that new molecular typing methodologies will be developed for avian mycoplasma strain identification that improve upon RAPD's requirement for growth and a pure culture, and problems of reproducibility and interpretation.

SELECTED REFERENCES

Artiushin S and FC Minion. Arbitrary primed PCR analysis of Mycoplasma hyopneumoniae field isolates demonstrates genetic heterogeneity. *Int. J. Syst. Bacteriol.* 46: 324-328. 1996.

Caetano-Anollés G, BJ Bassamand and PM Gresshoff. DNA amplification fingerprinting using very short arbitrary oligonucleotide primers. *Bio/Technology,* 9:553-557. 1991.

Caetano-Anollés G, BJ Bassam and PM Gresshoff. DNA amplification fingerprinting: a strategy for genome analysis, *Plant Mol. Biol. Rep.,* 9:294-307. 1991.

Caetano-Anollés G, BJ Bassam and PM Gresshoff. DNA fingerprinting - MAAPing out a RAPD redefinition. *Bio/Technology,* 10:937. 1992.

Charlton BR, et al. Complementary randomly amplified polymorphic DNA (RAPD) analysis patterns and primer sets to differentiate Mycoplasma gallisepticum strains. *J. Vet. Diagn. Invest.* 11:158-161. 1999.

Fan HH. SH Kleven and MW Jackwood. Application of polymerase chain reaction with arbitrary primers to strain identification of Mycoplasma gallisepticum. *Avian Dis.*, 39: 729-735. 1995.

Fan HH, SH Kleven and MW Jackwood. Studies of intraspecies heterogeneity of mycoplasma synoviae, M. meleagridis, and M. iowae with arbitrarily primed polymerase chain reaction. *Avian Dis.*, 39:766-77. 1995.

Geary SJ, MH Forsyth, S. Aboul Saoud, G Wang, DE Berg and CM Berg. Mycoplasma gallisepticum strain differentiation by arbitrary primer PCR (RAPD) fingerprinting. *Mol. Cell. Probes*, 8:311-316. 1994.

Jeffreys AJ, V Wilson and SL Thein. Individual-specific 'fingerprints' of human DNA, *Nature (London),* 316:76-79. 1985.

Ley DH, JE Berkhoff and JM McLaren. Mycoplasma gallisepticum isolated from house finches (Carpodacus mexicanus) with conjunctivitis. *Avian Dis.*, 40:480-483. 1996.

Ley DH, JE Berkhoff and S Levisohn. Molecular epidemiologic investigations of Mycoplasma gallisepticum conjunctivitis in songbirds by random amplified polymorphic DNA analyses. *Emerg. Infec. Dis.*, 3:375-380. 1997.

Lowe AJ, O Hanotte and Lguarino. Standardisation of molecular genetic techniques for the characterisation of germplasm collections: The case for Random Amplified Polymorphic DNA (RAPD). *Plant Genetic Resources Newsletter* IPGRI/FAO. 1996.

Lynch M. The similarity index and DNA fingerprinting, *Mol. Biol. Evol.*, 7:478-484. 1990.

Rawadi G, B Lemercier and D Roulland-Dussoix. Application of an arbitrary-primed polymerase chain reaction to mycoplasma identification and typing within the Mycoplasma mycoides cluster. *J. Applied Bacteriol.*, 78:586-592. 1995.

Vos P, R Hogers, M Bleeker, M Reijans, T van de Lee, M Hornes, A Frijters, J Pot, J Peleman, M Kuiper and M Zabeau. AFLP: a new technique for DNA fingerprinting, *Nucl. Acids Res* ., 23:4407-4414. 1995.

Welsh J and M McClelland. Fingerprinting genomes using PCR with arbitrary primers, *Nucl. Adics Res.*, 18:7213-7218. 1990.

Williams JGK, AR Kubelik, KJ Livak, JA Rafalski and SV Tingey. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers, *Nucl. Acids Res.*, 18:6231-6235. 1990.

Zietkiewicz, E., Rafalski, A. and Labuda, D., (1994) Genome fingerprinting by simple sequence repeats (SSR)-anchored PCR amplification, *Genomics*, 20:176-183.

Some Perspectives on Mycoplasma Diagnosis and Control

S. H. Kleven
University of Georgia
Department of Avian Medicine
Athens, Georgia 30602-4875

Efforts in the United States to control *Mycoplasma gallisepticum* (MG) began in the 1960's, primarily as a response to high condemnations from airsacculitis after the initiation of USDA post mortem inspection of poultry. Somewhat later, *M. synoviae* (MS) and *M. meleagridis* (MM) were added to the program. Since then, significant progress has been made in controlling Mycoplasma infections in turkey and chicken breeding stocks. Voluntary MG control programs in the U. S. are administered under the National Poultry Improvement Plan; testing provisions and protocols are provided in their official publication (1). The majority of poultry production in the U. S. is mycoplasma-free; however, MG and MS infection are common in commercial egg production flocks. Unfortunately, in spite of increased efforts at control, outbreaks continue to occur.

There have been changes which have resulted in an evolving situation in MG control, both in the United States and world-wide. These include changes in the poultry industry itself, improved detection methods, better understanding of the agent and its pathogenesis, and improved control methods.

Changes in the of the Poultry Industry Which Affect Mycoplasma Control

In most modern poultry producing areas of the world, the emphasis on the control of Mycoplasma infections has been centered around maintenance of Mycoplasma-free breeding stock and keeping parent and production flocks free of infection by utilizing single-age, all-in all-out farms with good biosecurity. In many parts of the world, this has been very successful, and the majority of broiler, turkey and egg production is free of infection. In contrast, areas with less-developed poultry industries tend to have high levels of contamination with MG and MS; this poses special problems for companies attempting to institute modern production methods.

With the rapid growth of poultry production world-wide, there has been concentration of large numbers of birds into small areas, leading to increased risk of exposure to pathogenic Mycoplasmas. In some areas, poultry production is so concentrated that from an epidemiological point of view, it is almost like a very large multi-age farm. Also, general improvements in disease control have sometimes resulted in decreased efforts in biosecurity, thus enhancing the possibilities for the spread of Mycoplasma infections.

There has been a tendency to drift away from all-in all-out production and to concentrate production on multi-age sites. This has been especially true for commercial egg production – the majority of egg production in the U.S. is now on multi-age sites, and this trend is developing around the world. Such multi-age production sites are mostly MS-positive, and many are also MG positive (21), even though grandparent and parent stocks are generally MG and MS-free.

In many locations, multi-age management of broiler breeders or broilers may occur. In turkey production, multi-stage production farms, on which 2 or even 3 different ages are maintained, are becoming quite common.

Therefore, in spite of sometimes heroic efforts at biosecurity and improved understanding of the survival of Mycoplasmas outside the host, Mycoplasma outbreaks continue to occur.

Improvements in Detection Methods

The basis for control programs has centered around serological methods such as agglutination and hemagglutination-inhibition, with reactors often confirmed by isolation of the organism. More recently, commercial ELISA kits have become available (IDEXX Laboratories, Westbrook, Maine, USA; Kirkegaard and Perry Laboratories, Gaithersburg, Maryland, USA) and are becoming widely used. Such kits have excellent sensitivity and specificity, but non-specific reactions may still occur. Potential improvements in ELISA specificity may result from the utilization of highly purified antigens, or the use of a blocking ELISA utilizing a specific monoclonal antibody.

MG strains of low virulence typically produce a poor antibody response, and isolation from clinical specimens may be difficult (26). This may be especially true if the antigenic makeup of the MG strain involved is not a good match with the strains used to produce test antigens. Variability in strains and clinical responses have also been noted for MS. We have encountered situations where flocks have exhibited a low-level serological response with a low percentage of PCR reactions. Such flocks have been culture negative. It has been possible to transfer such reactivity by placing SPF chickens in contact with the principals. These observations suggest that there may be atypical strains which have been undetectable with traditional diagnostic methods.

Polymerase chain reaction represents a rapid and sensitive alternative to traditional culture methods, which require specialized media and reagents and are time consuming. At least one company (IDEXX Laboratories, Westbrook, Maine, USA) produces commercial PCR kits, which are being widely used. A PCR procedure developed by Dr. Lauerman at Auburn University (15) is also in wide use.

Such improvements in serological methods and rapid detection by PCR have done much to facilitate the rapid and accurate diagnosis of MG infection.

Variability Among and Within Strains of *M. gallisepticum* and *M. synoviae*

MG and MS strains are known to vary in pathogenicity and antigenicity (11, 13). Variability in pathogenicity among strains of MG has been recognized for some time (26). Significant antigenic variability among MG strains also exists (13), which could affect the sensitivity of serological tests, depending on the strain infecting the flock and the strain used to prepare antigen. There are also significant differences in virulence among strains of MS. Recently, a strain of MS was encountered in turkeys which did not induce an antibody response even though birds were culture positive in the upper respiratory tract (14). Although this has not been well studied, this is also likely true for MM and *M. iowae* strains.

Restriction Length Polymorphism (RFLP) of whole-cell DNA has been shown to be useful for differentiating MG strains (13). However, the RFLP procedure is time-consuming and laborious, making identification of specific strains a tedious procedure. More recently, Random Amplified Polymorphic DNA (RAPD) has been developed for identifying specific strains (6, 7, 17). This procedure is very simple and rapid, and has provided a routine procedure for the rapid identification of MG strains. This has proven to be very useful for epidemiological studies and

for identification of specific MG strains in field outbreaks. More recently, we have utilized a PCR for the PvpA (19), mgc1 (gapA) (8, 10) and lipoprotein (22) genes, followed by RFLP or sequencing of the PCR product to identify specific MG strains. Using this method we have been able to more closely pinpoint the identity of field and vaccine strains.

Studies utilizing Western blots and monoclonal antibodies have shown a high degree of variability in expression of surface antigens among strains of MG; many of these proteins are variably expressed (2, 20). This has led to a large effort in characterizing the variable expression of surface antigens have shown that phase variation also occurs in vivo. Similar variability of surface antigen expression has now also been shown to occur among strains of MS. For example, clones of MS which are hemagglutinin negative are less virulent than clones which are hemagglutinin positive. The significance of such variability in the expression of surface antigens is not well understood; however, it seems logical that it would play a role in pathogenesis, serological responses, and evasion of the immune system of the host.

***M. gallisepticum* Vaccination**

With the advent of multi-age commercial layer complexes, control by vaccination became desirable.

The first commercially available MG vaccines were oil-emulsion bacterins (9). Bacterins protect well against airsacculitis and egg production losses, but provide little protection against colonization by field strains of MG, thus providing little value in eradication programs. Major disadvantages of bacterins are the need for 2 doses for optimal protection and the cost of administration.

Live MG vaccines include F strain (3), which has been available for some time through several manufacturers, strain 6/85 from Intervet America, Millsboro, Delaware (4, 5), and strain ts-11, developed and widely used in Australia and licensed in the U.S. by Select Laboratories, Gainesville, Georgia (24, 25).

F strain exhibits moderate virulence in chickens (it is virulent for turkeys), colonizes the upper respiratory tract efficiently, spreads relatively slowly from flock to flock, and offers protection against losses in egg production. It provides excellent protection against colonization by challenge strains, and displaces the wild-type field strains present in multi-age commercial egg operations. Unfortunately, F strain has been implicated in field infections in commercial turkeys (16).

Strains 6/85 and ts-11 offer significant advantages over F strain. They both offer protection against challenge, but are avirulent and have very limited potential to spread from bird to bird (18), thus presenting less risk to neighboring poultry flocks. F strain has better ability to displace challenge strains in pen trial studies than does 6/85 or ts-11 (12), but field experience in a commercial layer operation suggests that strain ts-11 may be able to displace F strain in multi-age commercial layers . After ts-11 vaccination was discontinued, the flock has remained MG-free (23). Similar data with the 6/85 strain is not available, but there are complexes which have used 6/85 which now are seronegative, suggesting that displacement of wild type strains is also possible with 6/85. If the wild-type strains are highly virulent, it may be necessary to vaccinate with F strain for 1 or more production cycles and then switch over to either 6/85 or ts-11.

One major concern about live MG vaccines is safety. There have been numerous instances of clinical respiratory disease caused by "escaped" F strain vaccine; this strain should probably not be used if there is potential danger of spread to turkeys, even though it is the most efficacious strain in chickens. The 6/85 strain has been shown to be very safe in chickens, and

has never been detected in unvaccinated chicken flocks, to the knowledge of this author. However, there have been several instances of isolation of 6/85-like MG strains from turkeys showing clinical disease. In some cases there was a history of vaccination of nearby chickens or turkeys. The ts-11 strain has been detected on at least two occasions in unvaccinated chicken flocks. In both instances there is a history of possible use of contaminated vaccination equipment and in one of the instances, subsequent spread to neighboring broiler breeders. We also know that ts-11 can spread from vaccinated spike males to unvaccinated breeder females. These experiences suggest that even though the newer vaccines are very safe, they do have the potential for spread, and their safety should be very carefully before a decision is made to vaccinate. An important rule for consideration in the use of these vaccines, is that high titered vaccine should be used and administered properly in order to give the vaccine strain little opportunity to spread from bird to bird. Table 1 summarizes the author's experience with the characteristics of the various vaccine strains.

MG vaccines have had less use in turkeys. The F strain is too pathogenic for consideration in turkeys, but 6/85 or ts-11 strains may have potential use under very limited circumstances. In one vaccination trial conducted by us, administration of 6/85 or ts-11 did not result in respiratory signs or lesions in turkeys. There was little or no measurable resistance against airsacculitis after heavy aerosol challenge, but there was some protection detected against lesions in the upper respiratory tract. The ts-11 strain appears to have limited ability to infect turkeys.

Field experiences utilizing live vaccines have been very favorable in commercial layers, and field experiences in multi-age broiler breeders has also been favorable. These experiences suggest that live vaccines may be viable tools for the eradication of MG infection on multi-age commercial poultry farms.

There has been relatively little work on MS vaccines. There has been one MS bacterin licensed in the U.S., but it apparently has had little field use. A temperature sensitive MS strain has been licensed for use in Australia, and is widely used there. It has been licensed in Mexico and some other countries, but is not available in the U.S.

References

1. Anonymous, *National Poultry Improvement Plan and Auxiliary Provision.* Vol. APHIS-91-55-054. 2000, Washington, DC: USDA.

2. Boguslavsky, S., D. Menaker, I. Lysnyansky, T. Liu, S. Levisohn, R. Rosengarten, M. Garcia, and D. Yogev. Molecular characterization of the Mycoplasma gallisepticum pvpA gene which encodes a putative variable cytadhesin protein. Infect. Immun. 68:3956-3964. 2000.

3. Carpenter, T. E., E. T. Mallinson, K. F. Miller, R. F. Gentry, and L. D. Schwartz. Vaccination with F-strain Mycoplasma gallisepticum to reduce production losses in layer chickens. Avian Dis. 25:404-409. 1981.

4. Evans, R. D., and Y. S. Hafez. Evaluation of a Mycoplasma gallisepticum strain exhibiting reduced virulence for prevention and control of poultry mycoplasmosis. Avian Dis. 36:197-201. 1992.

5. Evans, R. D., Y. S. Hafez, and C. S. Schreurs. Demonstration of the genetic stability of a Mycoplasma gallisepticum strain following in vivo passage. Avian Dis. 36:554-560. 1992.

6. Fan, H. H., S. H. Kleven, and M. W. Jackwood. Application of polymerase chain reaction with arbitrary primers to strain identification of Mycoplasma gallisepticum. Avian Dis. 39:729-735. 1995.

7. Geary, S. J., M. H. Forsyth, S. A. Saoud, G. Wang, D. E. Berg, and C. M. Berg. Mycoplasma gallisepticum strain differentiation by arbitrary primer PCR (RAPD) fingerprinting. Molec. Cell. Probes. 8:311-316. 1994.

8. Goh, M. S., T. S. Gorton, M. H. Forsyth, K. E. Troy, and S. J. Geary. Molecular and biochemical analysis of a 105 kDa Mycoplasma gallisepticum cytadhesin (GapA). Microbiol. 144:2971-2978. 1998.

9. Hildebrand, D. G., D. E. Page, and J. R. Berg. Mycoplasma gallisepticum (MG) — laboratory and field studies evaluating the safety and efficacy of an inactivated MG bacterin. Avian Dis. 27:792-802. 1983.

10. Keeler Jr., C. L., L. L. Hnatow, P. L. Whetzel, and J. E. Dohms. Cloning and characterization of a putative cytadhesin gene (mgc1) from Mycoplasma gallisepticum. Infect. Immun. 64:1541-1547. 1996.

11. Kleven, S. H., O. J. Fletcher, and R. B. Davis. Variation of pathogenicity of isolates of Mycoplasma synoviae with respect to development of airsacculitis and synovitis in broilers. Am. J. Vet. Res. 163:1196-1196. 1973.

12. Kleven, S. H., M. I. Khan, and R. Yamamoto. Fingerprinting of Mycoplasma gallisepticum strains isolated from multiple-age layers vaccinated with live F strain. Avian Dis. 34:984-990. 1990.

13. Kleven, S. H., C. J. Morrow, and K. G. Whithear. Comparison of Mycoplasma gallisepticum strains by hemagglutination-inhibition and restriction endonuclease analysis. Avian Dis. 32:731-741. 1988.

14. Kleven, S. H., G. N. Rowland, and M. C. Kumar. Poor serological response to upper respiratory infection with Mycoplasma synoviae in turkeys. Avian Dis. In press. 2001.

15. Lauerman, L. H. Mycoplasma PCR Assays. In: Nucleic Amplification Assays for Diagnosis of Animal Diseases, ed. L. H. Lauerman, eds. American Association of Veterinary Laboratory Diagnosticians, Auburn, AL. pp. 41-52. 1998.

16. Ley, D. H., A. P. Avakian, and J. E. Berkhoff. Clinical Mycoplasma gallisepticum infection in multiplier breeder and meat turkeys caused by F Strain: Identification by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, restriction endonuclease analysis, and the polymerase chain reaction. Avian Dis. 37:854-862. 1993.

17. Ley, D. H., J. E. Berkhoff, and S. Levisohn. Molecular epidemiological investigations of Mycoplasma gallisepticum conjunctivitis in songbirds by random amplified polymorphic DNA analysis. Emerging Inf. Dis. 3:375-380. 1997.

18. Ley, D. H., J. M. McLaren, A. M. Miles, H. J. Barnes, S. H. Miller, and G. Franz. Transmissibility of live Mycoplasma gallisepticum vaccine strains ts-11 and 6/85 from vaccinated layer pullets to sentinel poultry. Avian Dis. 41:187-194. 1997.

19. Liu, T., M. Garcia, S. Levisohn, D. Yogev, and S. H. Kleven. Molecular Variability of the Adhesin-Encoding Gene pvpA among Mycoplasma gallisepticum Strains and Its Application in Diagnosis. J Clin Microbiol. 39:1882-1888. 2001.

20. Markham, P. F., M. D. Glew, J. E. Sykes, T. R. Bowden, T. D. Pollocks, G. F. Browning, K. G. Whithear, and I. D. Walker. The organisation of the multigene family which encodes the major cell-surface protein, pMGA, of Mycoplasma gallisepticum. FEMS Microbiol. Lett. 352:347-352. 1994.

21. Mohammed, H. O., T. E. Carpenter, and R. Yamamoto. Evaluation of factors associated with infection of commercial layers with Mycoplasma gallisepticum and Mycoplasma synoviae. Avian Dis. 31:470-476. 1987.

22. Nascimento, E. R., R. Yamamoto, K. R. Herrick, and R. C. Tait. Polymerase chain reaction for detection of Mycoplasma gallisepticum. Avian Dis. 35:62-69. 1991.

23. Turner, K. S., and S. H. Kleven. Eradication of live F strain *Mycoplasma gallisepticum* vaccine using live ts-11 on a multiage commercial layer farm. Avian Dis. 42:404-407. 1998.

24. Whithear, K. G., Soeripto, K. E. Harrigan, and E. Ghiocas. Immunogenicity of a temperature sensitive mutant Mycoplasma gallisepticum vaccine. Aust Vet J. 67:168-174. 1990.

25. Whithear, K. G., Soeripto, K. E. Harrigan, and E. Ghiocas. Safety of temperature sensitive mutant Mycoplasma gallisepticum vaccine. Aust Vet J. 67:159-165. 1990.

26. Yoder Jr., H. W. A historical account of the diagnosis and characterization of strains of Mycoplasma gallisepticum of low virulence. Avian Dis. 30:510-518. 1986.

Table 1. Comparison of live MG vaccine strains.

	F Strain	6/85	Ts-11
Form	Lyophilized	Lyophilized	Frozen
Route	Various	Spray	Eye Drop
Virulence	Moderate	None	None
Persistence	Excellent	???	Good
Antibody	Moderate	None	Weak
Transmission	Moderate	Poor	Poor
Displacement	Excellent	???	Good

AVIAN INFLUENZA

NATIONAL AND INTERNATIONAL AVIAN INFLUENZA OUTBREAKS AND STRATEGIES FOR CONTROL

David E. Swayne

U.S. Department of Agriculture, Agricultural Research Service,
Southeast Poultry Research Laboratory, 934 College Station Road, Athens, GA 30605

ABSTRACT

Avian influenza (AI) is a viral disease of poultry and has impacted international trade in poultry and poultry products. During 1999-2000, highly pathogenic (HP) AI (H7N1) occurred in Italy following mutation of a mildly pathogenic (MP) AI virus. This H7N1 HPAI virus was eradicated in April 2000, but the H7N1 MPAI virus has re-emerged in poultry of northern Italy during August 2000. Other outbreaks of MPAI have been ongoing in Mexico (H5N2); Guatemala (H5N2); El Salvador (H5N2), U.S. Live-Poultry Markets (H7N2); and the Middle East, Pakistan, China and Hong Kong (H9N2). An H5N1 HPAI virus has been detected sporadically in geese in Hong Kong wholesale market and recently was isolated in chickens from 10 markets in association with high mortality. Control of individual outbreaks has varied by country and incident. The HPAI (H7N1) in Italy was eliminated by a depopulation program, but control of the current MPAI outbreak is being attempted by use of an inactivated whole virus vaccine (H7N3) in turkeys and layers, and a controlled marketing program. Non-vaccinated sentinel birds are being used in surveillance. Because of events in Italy, the European Union Scientific Committee on Animal Health and Animal Welfare has recommended redefinition of avian influenza for regulatory action to include all H5 and H7 avian influenza viruses.

INTRODUCTION

Avian influenza (AI) is a viral disease of poultry caused by type A Orthomyxovirus and has impact on international trade in poultry and poultry products. Two forms of AI exist: 1) mildly pathogenic, and 2) highly pathogenic. Reports of highly pathogenic avian influenza (HPAI) are compiled and listed on the Office International des Epizooties (OIE) website, http://www.oie.int/, and in the bimonthly OIE Bulletin, but such reporting is mandatory for member nations. Mildly pathogenic (MP) AI is not on list A or B and thus are not reported to OIE. This report compiles information from OIE, recent scientific literature and credible personal sources on avian influenza in the world. Much of the information is fragmentary and incomplete, but this report represents the best information available.

H7N1 MP AND HPAI VIRUSES IN ITALY

An outbreak of H7N1 MPAI appeared in turkeys of northern Italy in March 1999. By mid-December, the AI virus affected at least 199 farms, mostly meat turkeys, but also turkey breeders, broiler breeders, layers, broilers, and guinea fowl. The problem was primarily respiratory disease with accompanying secondary pathogens or egg production drops. Control strategies used in the outbreak included increased surveillance, quarantine of infected flocks, controlled slaughter and enhanced biosecurity measures. Vaccine was not allowed. Unfortunately, the MPAI virus mutated to become HPAI virus with the first HP case appearing in turkeys on 17 December 1999 (3-5). By the last outbreak of HPAI on 5 April 2000, 13,732,912 birds were involved in 413 flocks and an additional 3-4 million were

depopulated as a pre-emptive action. Birds affected included 8,118,929 layers; 2,692,917 meat turkeys; 1,625,628 broilers; 743,319 broiler breeders; 260,340 quail, ducks and pheasants; 247,379 guinea fowl, 42,276 turkey breeders, 387 ostrich and 1,737 backyard poultry. Following repopulation, an outbreak of velogenic Newcastle disease (vND) occurred in the same geographic region during the summer 2000. In August 2000, H7N1 MPAI virus re-emerged in meat turkeys in northern Italy (southern part of Verona province).

Emergency vaccination has been undertaken as part of the AI control strategy and will be allowed from mid-November 2000 to May 2002 in meat turkeys and table-egg layers in a restricted zone south of Verona (6). In addition, authorization for vaccine use may be obtained for meat-type guinea fowls, capon farms and cockerel farms (1). The vaccine does not contain the homologous H7N1 MPAI virus, but an inactivated H7N3 HPAI virus (A/CK/Pakistan/95/H7N3) (6). The reason for such a selection is the usage of a natural "marker" vaccine, or termed a DIVA (Differentiating Infected from Vaccinated Animals) vaccine. The presence of the H7 antigen in the vaccine provides protection against clinical signs and will reduce virus shedding. Screening for antibodies to N1 and N3 would differentiate vaccinated from field exposed birds and diagnostic ELISA tests are being developed for detection of N1 and N3 antibodies. Currently, a minimum of 100 non-vaccinated sentinels are being used per farm or 50 per house. Ten sentinels are serologically tested each month, and if positive for AI antibodies on agar gel precipitin test (AGP), 10 sentinels are sacrificed and samples taken for virus isolation. Table eggs and meat from vaccinated flocks must be consumed in Italy and cannot be exported to other European Union countries.

Since August 2000, 71 flocks of H7N1 MPAI virus infected birds have been identified. However, among the 210 vaccinated flocks only 1 has had evidence of infection, as determined by serology and virus isolation.

H5N2 MPAI VIRUSES IN CENTRAL AMERICA

The H5N2 MPAI virus which was first detected in the fall of 1993 continues to circulate in some commercial poultry flocks in Mexico. The number of infected farms has declined since 1995 and no reports of HPAI have been made since June 1995. Control efforts have focused on vaccination with inactivated H5N2 AI vaccines and a recombinant fowlpox vaccine containing an H5 hemagglutinin gene insert. Between January 1995 and July 2000, total vaccine use has been 1.3 billion doses of inactivated vaccine and 400 million doses of recombinant fowlpox vaccine.

There have been no evidence for new introductions of MPAI (H5N2) from wild birds, but MPAI (H5N2) has been perpetuated in domestic and confined poultry. An H5N2 MPAI virus appeared in poultry of Guatemala in April 2000 and El Salvador in April 2001. In Guatemala, MPAI virus infections were detected in chickens on 25 commercial premises. Chickens on these premises were depopulated, but the total numbers are unknown. Vaccination with the inactivated H5N2 AI vaccine obtained from Mexico has been allowed, but not the use of the fowlpox recombinant vaccine. Comparison of the hemagglutinin gene from MPAI viruses of Guatemala and El Salvador with other H5 AI viruses has shown a close relationship with the MPAI H5N2 isolated from chickens in Chiapas, Mexico in 1997.

MPAI VIRUSES IN UNITED STATES LIVE-BIRD MARKETS

Surveillance for AI viruses in various poultry species of the Live-Bird Markets (LBM) by the Departments of Agriculture in New York and New Jersey continues with the assistance of National Veterinary Services Laboratory (NVSL), Ames, Iowa. Since 1994, H7N2 MPAI viruses have been the predominate subtype isolated, but other less frequent subtypes of MPAI viruses isolated included H1N1, H2N3, H3N2, H3N8, H4N2, H4N6, H5N2, H6N4, H6N6, H7N3, H9N2, H10N7 and a few others (11,12). During a 1998-99 survey, the distribution of infected birds varied, but 40-62% of the retail markets had infected birds while only 17-20% of the delivery trucks had infected birds and it was uncommon to find infected birds at the wholesale markets. Currently, 40% of the markets are positive in New York and New Jersey, but incidence in chickens on delivery trucks is unknown (T.J. Myers, personal communication).

These H7N2 MPAI viruses are of particular concern, especially in view of the potential of H7N2 MPAI viruses to mutate to HP as occurred in Italy during 1999. In 1994, the amino acid sequence of the hemagglutinin proteolytic cleavage site of H7N2 viruses from the LBM was ....PENPKTR/G.... The presence of two basic amino acids at -1 (arginine) and -3 (lysine) positions are typical of H7 MPAI viruses. In 1998, the cleavage site changed with substitution of a lysine at the -5 position (...PEKPKPR/G...) and this virus has become the predominate lineage in 1999 and 2000. Unfortunately, the substitution of only 1 additional basic amino acid will give a cleavage site compatible with a HPAI virus. Therefore, elimination of H7N2 MPAI viruses from the LBM is a necessity to protect the commercial poultry industries.

A testing, certification, and cleaning and disinfection program over the past 2 years has not reduced the incidence of H7N2 AI in the LBM system. A State-USDA Cooperative Control Program has been developed and will include the following action items: 1) conduct an epidemiology study in early summer 2001 to determine distribution and frequency of infected birds in the LBM system, 2) establish emergency interim state rules or quarantine orders, and 3) institute a simultaneous, regional closure of LBM system to break the infection cycle during the fall 2001. The latter item will involve a three day pre-announced closure of all wholesale and retail markets in New Jersey, New York and Philadelphia;, cleaning and disinfection of premises and equipment; an education program; repopulation with AI-free birds; surveillance of birds for AI virus and financial compensation to market owners for the three day closure (T.J. Myers, personal communication). The program is not an indemnification program since the state governments and USDA are not seizing and depopulating birds. T.J. Myers of Animal and Plant Health Inspection Service provides coordination of the Cooperative Program.

Rapid diagnosis is a necessity in the decision making process for repopulation with birds and re-opening the markets. Traditional techniques of virus isolation take 1-6 weeks to get answers about active virus infections. In response, D.L. Suarez and M.L. Perdue of the Agricultural Research Service (ARS), Southeast Poultry Research Laboratory (SEPRL) have developed a real-time PCR test to detect AI viral genes in oral and cloacal swabs. The test uses a probe to the matrix gene of Type A influenza virus and has high sensitivity and specificity on laboratory specimens. This probe when used in a prototype portable light cycler PCR machine gives a diagnosis in 40 minutes. This test will be evaluated and validated during the epidemiology study of early summer 2001. D. Senne of the National Veterinary Services Laboratories (NVSL) will provide virus isolation services and D.L. Suarez of SEPRL will provide real-time PCR testing in the field.

H9N2 MPAI VIRUSES IN ASIA

The H9N2 MPAI viruses have been reported to cause morbidity and mortality in Asian countries, primarily the Middle East and in Pakistan. In many cases, the clinical signs and mortality have been the result of secondary bacterial and/or viral pathogens. Infections with H9N2 AI viruses without mortality have been reported in China and Hong Kong. Sequence data of the H9 and N2 gene of H9N2 AI viruses from Saudi Arabia, Iran, Pakistan and Hong Kong by Veterinary Laboratories Agency (D. Alexander, United Kingdom) and SEPRL, respectively, have shown they are all closely related and of the same virus lineage (2). H9N2 AI viruses were first reported in China in the mid-1990's and spread to the Middle East and Pakistan in the late 1990's. In the Middle East and China, the recent outbreaks of vND has complicated the diagnosis and control of MPAI viruses.

Iran has reported avian influenza (H9N2) causing severe problems especially in areas of intensive poultry production and in the presence of unhygienic conditions. The outbreak began in 1997 and is ongoing. Cost estimates for 1998 alone were $11 million for 20 million meat chickens affected. Several breeder operations were also involved and these flocks had to be depopulated. Over the past 3 years, avian influenza viruses (H9N2) have also been isolated from chickens in Saudi Arabia. Mortality and morbidity has varied, but it is high when accompanied by secondary pathogens such as vND or *Mycoplasma gallisepticum*. Avian influenza has not been reported in Syria, Jordan or Lebanon. During 2000, there was widespread serological evidence of H9N2 infection of chickens in the LBM of Hong Kong, but not associated with disease. MPAI viruses of H9N2 subtype were isolated from swabs collected from birds in retail markets.

H5N1 HPAI VIRUSES IN HONG KONG

In 1999, H5N1 AI viruses were isolated from fecal material under cages in the Western Wholesale Poultry Market, where geese and ducks were housed. This market only houses waterfowl and all birds entering the market are slaughtered on site. Based on studies at SEPRL, the 1999 H5N1 viruses have the same hemagglutinin gene as the 1997 H5N1 AI viruses, but internal genes were from separate lineage (7). The internal genes of 1999 H5N1 viruses were most similar to those circulating in domestic geese in south China during 1996 (A/GS/Guangdong/1/96 [H5N1]) (7). These 1999 H5N1 viruses were highly pathogenic for chickens in experimental studies (7). During 2000, four H5N1 AI viruses were isolated from geese or swabs from goose cages in the wholesale market, and were HP for chickens. These viruses were similar to the 1999 Hong Kong H5N1 and 1996 A/GS/Guangdong/1/96 (H5N1) HPAI virus (D. Suarez, personal communication). No isolates were made from chickens in the wholesale or retail markets.

From December 2000 to April 2001, 29 H5N1 AI viruses were isolated from ducks and geese in the Western Wholesale Poultry Market (L. Sims, personal communication). The internal genes of these viruses differed from A/GS/Guangdong/1/96 and were a different lineage to the 1997 HP avian influenza viruses (L. Sims and K. Shortridge, personnal communication). An H5N1 AI virus was isolated from a chicken retail market in February. Increased mortality or clinical signs in birds were not reported. This virus was very similar to A/GS/Guangdong/1/96 (H5N1) and was not re-isolated despite repeated sampling of the market in the following 6 weeks. In mid-May (the writing of this report), 10 different retail markets had chickens with confirmed infections by H5N1 HPAI virus and three of these retail

markets had chickens with high mortality rates. However, H5N1 infections of chickens was not demonstrated on Hong Kong farms. On 21 May, all goose/duck and chicken wholesale and chicken retail markets were closed, and the birds destroyed (http://www.info.gov.hk/gia/general/200105/18/0518293.html, http://www.who.int/disease-outbreak-news/n2001/may/18may2001.html). The retail markets will remain closed for about four weeks. Live chickens, quail and pigeons on local farms ready for market will be depopulated within the next two weeks and will total near one million birds. Due to closure of markets, Importation of chickens from mainland China has stopped. No human cases of influenza A(H5N1) virus have been detected. The strains isolated from these retail markets are genetically similar to the viruses isolated from ducks and geese in December 2000.

OTHER AI ISSUES: EU DEFINITION

Currently, federal regulatory action is undertaken with HPAI and not MPAI. HPAI is defined as those viruses that kill 6, 7 or 8 of 8 inoculated susceptible chickens, or H5 and H7 AI viruses having a cleavage site with multiple basic amino acids as reported for previous HPAI viruses, or AI viruses that produce cytopathic effect in cell culture without exogenous trypsin. However, the EU is considering a change in definition to include all H5 and H7 AI viruses along with HPAI H5 and H7 AI viruses as requiring regulatory action (8). This is in response to the outbreak in Italy during 1999 and 2000 when a MPAI (H7N1) mutated and became a HPAI. They are also proposing the option of using vaccines with future outbreaks of H5 and H7 AI. Below is a summarization of the European Union Scientific Committee Recommendations as reported in the specific website http://europa.eu.int/comm/food/fs/sc/scah/out45_en.pdf.

1. "Avian influenza" means an infection of birds caused by any influenza A virus which has an intravenous pathogenicity index in six-week-old chickens greater than 1.2 or any infection with influenza A viruses of H5 or H7 subtype. However, in making this recommendation the Committee was concerned at the current lack of knowledge on the prevalence of LPAI viruses of H5 and H7 subtypes in poultry populations. It would seem a wise precaution that before the recommendation is implemented serological surveys of poultry populations in Member States should be undertaken to determine this prevalence and the likely economic impact that would be involved.

2. Throughout the EU there is a marked lack of surveillance for avian influenza, particularly in free-living birds, and yet routine surveillance could give an early warning of the prevalence of viruses of H5 or H7 subtype in the locality of domestic birds. Member States should put in place routine surveillance systems for the detection of influenza viruses in free-living birds.

3. Vaccination against influenza A viruses of H5 and H7 subtype should not normally be allowed. The possible use of emergency vaccination... should be retained. ....., it considers that there is a potential for a greater role of vaccination in the control of avian influenza, that could be realized by the development of novel marker vaccines

4. In order to improve the efficacy of emergency vaccination as an aid to avian influenza control the Commission is urged to support the development of novel marker vaccines.

5. "Poultry" are all birds that are reared or kept in captivity for: the production of meat or eggs for consumption, the production of other commercial products, for restocking supplies of game or for breeding these four categories of birds.

6. The Committee, recognizing that there is at present no adequate in vitro alternative, agreed to the continued inclusion of an in vivo test for virus virulence ..., but with some reluctance. The Commission is urged to encourage and support further research into the development of in vitro tests aimed at replacing the use of birds in virulence tests for avian influenza.

Changes in the definition of AI as proposed above could have a negative effect on future exports U.S. poultry products to European countries. Furthermore, should such a definition be adopted by the OIE, the impact on U.S. poultry exports could be significantly affected to other countries. The USA needs to be proactive in examining this issue and making recommendations through appropriate channels to international organizations.

ACKNOWLEDGMENTS

The authors thank Ilaria Capua, David Suarez, Thomas J. Myers, Dennis Senne, Max Brugh, Les Sims, Dennis Alexander, Michael Schwartz and Juan Garcia Garcia for providing information through personal communications. Some of the information was presented at the National Poultry Health and Processing Meeting in Ocean City, Maryland, October 18-20, 2000 (10), and the 104 annual meeting of the USAHA in Birmingham, Alabama, October 22-26, 2000 (9).

REFERENCES

1. Anonymous. Emergency vaccination programme against low pathogenic avian influenza (LPAI) in Verona region. 1-26. 2000.

2. Banks, J., E.C. Speidel, P.A. Harris, and D.J. Alexander. Phylogenetic analysis of influenza A viruses of H9 haemagglutinin subtype. Avian Pathol. 29:353-360. 2000.

3. Capua, I., F. Mutinelli, S. Marangon, and D.J. Alexander. H7N1 avian influenza in Italy (1999 to 2000) in intensively reared chickens and turkeys. Avian Pathol. 29:537-543. 2000.

4. Capua, I. The 1999 avian influenza (H7N1) epidemic in Italy. Proc West Poult Dis Conf 20-24. 2000.

5. Capua, I. and S. Marangon. The avian influenza epidemic in Italy, 1999-2000: a review. Avian Pathol. 29:289-294. 2000.

6. Capua, I., S. Marangon, P.M. Dalla, and U. Santucci. Vaccination for avian influenza in Italy. Vet.Rec. 147:7512000.

7. Cauthen, A.N., D.E. Swayne, S. Schultz-Cherry, M.L. Perdue, and D.L. Suarez. Continued circulation in China of highly pathogenic avian influenza viruses encoding the hemagglutinin gene associated with the 1997 H5N1 outbreak in poultry and humans. J.Virol. 74:6592-6599. 2000.

8. European Commission. The definition of avian influenza and the use of vaccination against avian influenza. Sanco/B3/AH/R17/2000:1-35. 2000.

9. Swayne, D.E. and D.L. Suarez. Update on international incidences and outbreaks of avian influenza 1999-2000. In: Proceedings of the 104th annual meeting of the United States Animal Health Association, U.S. Animal Health Association, Richmond, Virginia. pp. 600-604. 2000.

10. Swayne, D.E., D.L. Suarez, and D.J. King. Avian influenza (AI) and velogenic Newcastle disease (VND) update. In: Proceedings of 35th National Meeting on Poultry Health and Processing, Delmarva Poultry Industry, Georgetown, Delaware. pp. 37-45. 2000.

11. USAHA. Report of the Committee on Transmissible Diseases of Poultry and Other Avian Species. In: Proceedings of the 103rd Annual Meeting of the U.S. Animal Health Association, U.S. Animal Health Association, Richmond, Virginia. pp. 527-588. 1999.

12. USAHA. Report of the Committee on Transmissible Diseases of Poultry and Other Avian Species. In: Proceedings of the 104th Annual Meeting of the U.S. Animal Health Association, U.S. Animal Health Association, Richmond, Virginia. pp. 559-632. 2000.

NEWCASTLE DISEASE

NEWCASTLE DISEASE VIRUS PATHOTYPING: THE CURRENT EMPHASIS ON THE ICPI

Daniel J. King

Southeast Poultry Research Laboratory, USDA, Agricultural Research Service
Athens, GA

ABSTRACT

Chicken inoculation has been utilized since the 1950s to differentiate virulence among isolates of avian paramyxovirus-serotype 1 (APMV-1), a term synonymous with Newcastle disease virus (NDV). Three tests, the intracerebral pathogenicity index (ICPI), intravenous pathogenicity index (IVPI), and intracloacal inoculation have been employed to make that differentiation across the full spectrum of NDV virulence and clinical forms. Characterization of isolates as low (lentogenic), moderate (mesogenic), or high virulence (velogenic) were accomplished with that test battery. Because there is an international trend to separate APMV-1 isolates into two virulence groups, the lentogens and a more virulent group comprised of previous mesogenic and velogenic viruses, the role of the ICPI as a differential test has become emphasized. During 1999, that new emphasis was codified when member countries of the Office International des Epizooties (OIE) approved a new definition of ND. ND is now defined by OIE as an infection with an APMV-1 of virulence measured by an ICPI of 0.7 or greater, a definition that expands those NDV isolates that present a risk to poultry beyond the previous velogenic classification.

OVERVIEW

The manifestations of Newcastle disease (ND) may vary from high morbidity and mortality as first described in 1926, to subclinical infections reported in the 1960s. The clinical signs observed vary with the virulence of the infecting Newcastle disease virus (NDV) strain, the avian species infected, and the predilection of the infecting virus for the respiratory, digestive, and/or nervous systems. Although NDV is readily isolated from infected birds, the determination of virulence of those isolates requires further laboratory evaluation. This is because the immune status and susceptibility of different natural hosts may modify the clinical picture from that observed in susceptible chickens inoculated with field isolates. Therefore, the inoculation of susceptible chickens became the standard biological method for virulence differentiation, the pathotyping, of isolates of APMV-1 (Alexander, 1997, 1998).

The procedures for biological evaluation were initially developed to differentiate NDV vaccine strains of low and moderate virulence (Hanson and Brandly, 1955). The strains were typed according to their growth rate as measured by the mean time to death (MDT) of 10-day-old embryonated chicken eggs inoculated with a minimum lethal dose and the neuropathogenicity by intracerebral inoculation of day-old chicks. Results of the MDT of NDV strains that varied from low to high virulence provided the basis for creation of the terms that have become a fixture of the NDV literature. Lentogenic or slowly growing strains, like B1 and La Sota, were of low pathogenicity for chickens and took 90 hours or more to kill embryos, mesogenic strains, like Roakin and MK 107, were of low to high pathogenicity and took 60 to 90 hours to kill embryos, and velogenic strains were rapidly growing and killed embryos in 40 to 60 hours. The latter strains were of moderate to high pathogenicity. Further differentiation of the lentogenic strains from the mesogens and velogens was provided by determination of an intracerebral pathogenicity index (ICPI) in day-old chicks, but an additional test, the intravenous pathogenicity index (IVPI), was needed to differentiate the mesogens from velogens such as Texas GB. The indigenous velogenic NDV strains were potential contaminants of the mesogenic wing-web vaccines in use at that time. In the initial IVPI tests, parenteral inoculation of six-week old chickens by intramuscular or

intravenous routes yielded similar results. Clinical signs evident following inoculation were scored and an IVPI was calculated to differentiate mesogens from velogens (Rosenwald et al., 1959).

A further pathotype differentiation was required during the 1971 viscerotropic velogenic ND (VVND) outbreak in California. At that time neurotropic velogenic ND (NVND) was still indigenous in the U. S. Demonstration of hemorrhagic lesions in the intestinal tract of chickens infected with VVNDV field isolates is required to differentiate those exotic ND viruses from the NVNDV strains that produced mortality with no hemorrhagic lesions. Hanson et al. (1973) reported that chickens inoculated by swabbing the vent with VVNDV field isolates consistently produced VVND lesions not produced by inoculations administered by other routes or by other NDV strains inoculated by vent swab, an inoculation method now known as the intracloacal inoculation pathogenicity test (Alexander, 1998).

Although all of the biological evaluation procedures described above are still used in APMV-1 research, the ICPI has become the most widely used procedure for APMV-1 virulence assessment worldwide. Why has this occurred? There are several reasons that have contributed to that change that will be discussed briefly here. First is the evolving definition of ND and the recognition of a virulence marker that can be identified by nucleotide sequence analysis. Second is the history of outbreaks attributed to the acquisition of virulence by viruses originally of low or moderate virulence and third is the failure to recover viruses more virulent than lentogenic vaccine strains from poultry in countries such as the U. S. where ND is effectively controlled.

The current U. S. definition of ND as an acute, rapidly spreading and usually fatal viral disease of birds and poultry (9CFR53.1, 2001) was similar to the Office International des Epizooties (OIE) definition prior to the approval of a new OIE ND definition in 1999. ND is now defined by OIE as an infection of birds caused by a virus of APMV-1 that meets one of the following criteria for virulence: a) the virus has an ICPI in day-old chicks (*Gallus gallus*) of 0.7 or greater. or b) Multiple basic amino acids have been demonstrated in the virus at the fusion protein cleavage site (OIE Official Acts, 1999). Details of the fusion cleavage site as a molecular determinant of virulence are given by Seal (2001) and examples of virus characterization by ICPI and amino acid sequence at the fusion protein cleavage site are given in Table 1. Notable in the new OIE definition is the change from defining ND as a severe disease, typical of a velogenic NDV infection, to ND being an infection with viruses of mesogenic or velogenic pathotype. A critical issue for poultry exporting countries like the U. S. is that the OIE International Animal Health Code has not been revised to provide criteria for applying the new ND definition for the purposes of international trade.

The basis for the differentiation of lentogens from mesogens and velogens by the ICPI wasn't initially evident, but the importance of differences in the fusion protein became evident with further research. Nagai et al. (1976) defined the host range of the different pathotypes when he and his co-workers demonstrated that the number of cell types that would replicate lentogenic NDV strains was restricted, a restriction not observed with replication of mesogenic and velogenic strains. The observed restricted host range of lentogens was demonstrated to be a function of the virus surface glycoproteins, primarily the fusion protein, and could be reversed when trypsin was added to the growth medium of non-permissive cells infected with lentogenic strains. Later, nucleotide sequence analysis identified the basis for the host restriction is the amino acid sequence at the fusion protein cleavage activation site – mesogens and velogens have a similar sequence that differs from the amino acid sequence in lentogens (Collins et al., 1994; Seal et al., 1995; See also Table 1). Confirmation of the importance of the fusion cleavage for NDV virulence was recently reported by Peeters et al. (1999). In their studies they compared the virulence of a molecularly cloned La Sota strain and the same strain changed only by inserting the consensus fusion cleavage site sequence of virulent NDV strains. The ICPI of the virus with the virulent insert was 1.28 in contrast to an ICPI of 0.00 for the original La Sota strain without the insert. Therefore, the insert of a virulent sequence into the fusion protein gene had changed the virus from a lentogenic to a mesogenic pathotype.

An increased concern with NDV isolates of moderate virulence began with the ND outbreak in chickens in England during 1984. APMV-1 isolates from pigeons, also called pigeon paramyxovirus type 1 (PPMV-1), have presented some notable pathotyping difficulties. They often have MDT values greater than 90 hours, a lentogenic characteristic, and ICPIs greater than 1.0, a mesogenic trait (Pearson et al., 1987). It can be seen in Table 1 that some of the pigeon isolates with ICPIs low enough to be in the upper lentogenic range have amino acid sequences typical of virulent viruses. When these viruses are passaged in chickens, some acquire higher virulence and some don't (Collins, et al., 1994). PPMV-1 with ICPIs lower than those in Table 1 and with virulent fusion cleavage sites have recently been reported in Europe (D. J Alexander, I. Capua, Personal communication, 2000). The concern is that some of these isolates that appear to be of low or moderate virulence may acquire high virulence as occurred in an outbreak in chickens in Great Britain during 1984 that originated from viruses infecting pigeons (Alexander et al., 1985).

Although most recent outbreaks around the world have been due to viruses with properties of high or moderate virulence, there are recent outbreaks of ND that have been attributed to viruses that apparently evolved from low virulence strains. These include outbreaks in Ireland (1990) and in Australia (1998 – 2000)(Alexander, 2001; Kirkland, 2000). Virulence acquisition in each case was associated with changes in the fusion protein cleavage activation site of the viruses from one of low virulence to the virulent form. The frequency of similar events through the history of Newcastle disease is unknown. The reported occurrences of a mutation of a low virulence strain to high virulence are rare given the opportunities that do exist from the widespread use of the lentogenic vaccines, and the continued exposure of poultry to low virulence field isolates (King and Seal, 1998).

Pathotyping of APMV-1 isolates from U. S. chickens and turkeys has identified no viruses with an ICPI greater than 0.5 and most have ICPIs <0.31. There is always the concern that severe respiratory reactions are due to more virulent APMV-1 isolates; however, when recent isolates were characterized there was no evidence that the isolates were more virulent than the NDV vaccine strains utilized to control those infections (King and Seal, 1998). Similar findings are reported from other countries where infections with mesogenic and velogenic virus infections are not found in poultry. There have been no reported NDV isolates of mesogenic or velogenic pathotype from chickens and turkeys with the exception of the turkeys in North Dakota that became infected with a virulent virus transmitted from a cormorant die-off during 1992 (King, 1996).

CONCLUSIONS

The clinical forms of ND reported in birds remain unchanged from early descriptions of the disease, but some evolutionary changes in NDV are now recognized as the source of those problems. Of the biological tests developed in the 1950s to differentiate the virulence of APMV-1 isolates, the ICPI is used more widely than the others today because it basically separates those isolates into two groups – those of low virulence and those of moderate to high virulence. We have always been concerned with viruses at the high end of the virulence spectrum; however, I believe it is equally important that we pursue more extensive characterization of the diverse isolates of APMV-1 of low to moderate virulence. That is the only way that we can be cognizant of any mutations that are occurring and any increased risk to poultry of viruses that are evolving in poultry and in non-poultry species that have epidemiological connections to poultry.

REFERENCES

Alexander, D. J. (1997). Newcastle disease and other avian *Paramyxoviridae* infections. In B.W. Calnek (Ed.), *Diseases of poultry* (10th edn, pp. 541-569). Ames: Iowa State University Press.

Alexander, D. J. (1998). Newcastle disease virus and other Avian Paramyxoviruses. In D. E. Swayne (Ed.), *A Laboratory Manual for the Isolation and Identification of Avian Pathogens* (4th edn. pp. 156-163). Kennett Square, PA: The American Association of Avian Pathologists.

Alexander, D. J. (2001). Newcastle Disease. *British Poultry Science*, 42:5-22.

Alexander, D. J., Wilson, G. W. C., Russell, P. H., Lister, S. A. & Parsons, G. (1985). Newcastle Disease Outbreaks in Fowl in Great Britain During 1984. *The Veterinary Record*, 117:429-434.

Code of Federal Regulations (9CFR53.1) (2001). Title 9 – Animals and Animal Products, Volume 1, Chapt. 1 – APHIS, USDA, Part 53 – Foot and Mouth Disease, Pleuropneumonia, Rinderpest, and Certain other Communicable Diseases of Livestock or Poultry, Sect. 53.1 – Definitions, p. 160.

Collins, M. S., Strong, I., & Alexander, D. J. (1994). Evaluation of the Molecular Basis of Pathogenicity of the Variant Newcastle Disease Viruses Termed "Pigeon PMV-1 Viruses". *Archives of Virology*. 134(3-4), 403-411.

Hanson, R P. & Brandly, C. A. (1955). Identification of Vaccine Strains of Newcastle Disease Virus. *Science*, 122:156-157.

Hanson, R. P., Spalatin, J., & Jacobsen, G. S. (1973). The Viscerotropic Pathotype of Newcastle Disease Virus. *Avian Diseases*, 17:354-361.

King, D. J. (1996). Influence of Chicken Breed on Pathogenicity Evaluation of Velogenic Neurotropic Newcastle Disease Virus Isolates from Cormorants and Turkeys. *Avian Diseases*, 40:210-217.

King, D.J. & Seal, B.S. (1998). Biological and molecular characterization of Newcastle disease virus (NDV) field isolates with comparisons to reference NDV strains. *Avian Diseases*, 42, 507-516.

Kirkland, P. D. (2000). Virulent Newcastle Disease Virus in Australia: In Through the Back Door. *Australian Veterinary Journal*, 78:331-333.

Nagai, Y., Klenk, H.-D., & Rott, R. (1976). Proteolytic Cleavage of the Viral Glycoproteins and Its Significance for the Virulence of Newcastle Disease Virus. *Virology*, 72:494-508.

OIE Official Acts. (1999). Resolution No. XIII.- Newcastle disease. OIE Bulletin. 111 (3):266-267.

Pearson, J. E., Senne, D. A., Alexander, D. J., Taylor, W. D., Peterson, L. A., & Russell, P. H. (1987). Characterization of Newcastle Disease Virus (Avian paramyxovirus-1) Isolated from Pigeons. *Avian Diseases*, 31:105-111.

Peeters, B. P. H., de Leeuw, O. S., Koch, G., & Gielkens, A. L. J. (1999). Rescue of Newcastle Disease Virus from Cloned cDNA: Evidence that Cleavability of Fusion Protein is a Major Determinant for Virulence. *Journal of Virology*, 73:5001-5009.

Rosenwald, A. S., Hanson, R. P., & Brandly, C. A. (1959). Studies on Pathogenic Newcastle Disease Virus Contaminants in Newcastle Disease Wing-Web Vaccines. *American Journal of Veterinary Research*, 20:946-953.

Seal, B. S. (2001). Molecular Evolution of Newcastle Disease Virus and Application of Molecular Diagnostics. Proceedings of Respiratory Diseases Symposium, AAAP, Boston.

Seal, B. S., King, D. J., & Bennett, J. D. (1995). Characterization of Newcastle disease virus isolates by reverse transcription PCR coupled to direct nucleotide sequencing and development of sequence database for pathotype prediction and molecular epidemiological analysis. *Journal of Clinical Microbiology* 33(10), 2624-2630.

Table 1. The virulence of different NDV strains based on ICPI and molecular characterization of the sequence at the fusion protein cleavage site as defined by 1999 OIE standards.

Pathotype/Origin - NDV Strain	ICPI[A]	Amino Acid Sequence At Fusion Protein Cleavage Site						Potential Virulence Based on Amino Acid Sequence
		112[B]	113	114	115	116	117	
Lentogen - La Sota, B1, VG-GA	<0.7	G[C]	**R**	Q	G	**R**	L	Low virulence
Mesogen - Roakin	1.6	**R**	**R**	Q	**K**	**R**	F	Virulent
Velogen - CA 1083 (VVNDV)	1.8	**R**	**R**	Q	**K**	**R**	F	Virulent
Velogen - Texas GB (NVNDV)	1.7	**R**	**R**	Q	**K**	**R**	F	Virulent
Pigeon - (Collins et al., 1994)	0.66-1.36	**R**	**R**	Q	**K**	**R**	F	Virulent
Pigeon - (Collins et al., 1994)	1.16-1.80	G	**R**	Q	**K**	**R**	F	Virulent

[A] ICPI = Intracerebral pathogenicity index.

[B] Amino acid order in sequence. Dashed line between 116 & 117 represents the cleavage site.

[C] Amino acid symbols: F = phenylalanine; G = glycine; **K = lysine**; L = leucine; Q = glutamine; **R = arginine**; Basic amino acids are in bold.

Molecular Evolution of Newcastle Disease Virus and the Application of Molecular Diagnostics

Bruce S. Seal, PhD

Southeast Poultry Research Laboratory, Agricultural Research Service, USDA
934 College Station Road, Athens, GA 30605 USA

ABSTRACT

Highly virulent Newcastle disease virus (NDV) isolates are List A pathogens, and it is compulsory that reports of its isolation be made to the Office of International Epizootes (OIE). The principal molecular determinant for NDV virulence is the fusion protein cleavage site amino acid sequence. Determination of the fusion protein cleavage site amino acid sequence is now an alternative along with biological assessments for NDV virulence characterization. Degenerate oligonucleotide primers have been used to reliably amplify nucleotide sequences that encode the fusion protein cleavage activation site utilizing NDV genomic RNA as a template. Results of phylogenetic analyses among NDV isolates demonstrates there are two clades of these viruses. Virulent NDV recovered during disease outbreaks since the 1970's, are members of a phylogenetically distinct clade that exponentially expanded separate from current vaccine viruses B1 or La Sota and standard challenge strains such as Texas/GB. Viral genomic information surrounding the fusion protein cleavage site coding sequence was used to develop a heteroduplex mobility assay (HMA) to aid in further identification of molecular markers as predictors of NDV virulence. The HMA was exploited to distinguish vaccine-like viruses from other isolates potentially virulent for chickens. These techniques will help improve international harmonization of veterinary biologics as set forth by the OIE and the Veterinary International Cooperation on Harmonization of Technical Requirements of Veterinary Medicinal Products.

OVERVIEW

Newcastle disease virus (NDV) is a member of the *Paramyxoviridae* family that has been designated avian paramyxovirus-1 (APMV-1) as there are nine APMV serotypes. Formerly the type virus of the paramyxovirus subfamily, NDV has since been recently categorized as a rubulavirus (Alexander, 1997). The enveloped virus has a negative sense, single-stranded RNA genome of 15,186 nucleotides (Peeters et al., 1999) which codes for a nucleoprotein (N), phosphoprotein (P), matrix (M) protein, fusion (F) protein , hemagglutinin-neuraminidase (HN) protein and RNA directed RNA polymerase (L) protein (Alexander, 1997). Based on genome structure (Peeters et al., 1999), phosphoprotein gene usage (Locke et al., 2000) and phylogenetic analysis of protein predicted amino acid sequences among viruses of the *Paramyxoviridae* it has been suggested that avian paramyxoviruses have their own subfamily (Seal et al., 2000; 2001).

Outbreaks of Newcastle disease were first reported among poultry in Java, Indonesia and England during 1926. It is a worldwide problem and all orders of birds have been reported to be capable of infection with NDV (Alexander, 1997). Isolates of NDV may be categorized into three

main pathotypes depending on severity of disease following chicken inoculation (Alexander, 1997; 1998). Lentogenic isolates are of low virulence and cause mild respiratory or enteric infections. Viruses of intermediate virulence that cause primarily respiratory disease are termed mesogenic, while virulent viruses that cause high mortality are termed velogenic. Velogenic NDV can be classified as neurotropic or viscerotropic based on clinical manifestation (Alexander, 1997). Virulent NDV isolates are List A pathogens, and it is compulsory that reports of its isolation be made to the Office of International Epizootes (OIE; www.oie.int).

Traditional biological pathotyping of NDV field isolates is determined by embryo and chicken inoculation (Alexander, 1998). The mean death time in eggs (MDT) and intracerebral pathogenicity index (ICPI) differentiates low virulent lentogens from mesogens of intermediate virulence and highly virulent velogens. The intravenous pathogenicity index (IVPI) differentiates mesogens from velogens and intra-cloacal inoculation is used to differentiate viscerotropic velogens from neurotropic velogens (Alexander, 1997; 1998). Antigenic differences occur among strains (Schloer, 1974) and monoclonal antibodies (Alexander, 1987; Russel and Alexander, 1983) have been used to identify at least 13 antigenic NDV groups (Alexander, 1997). Most isolates within a group are of a similar pathotype, but the results do not provide a reliable alternative to conventional live-animal pathotyping. The hemagglutination-inhibition (HI) test is the most widely used conventional serological method for detecting antibodies to NDV (Alexander, 1997). However, virus neutralization, neuraminidase inhibition, hemolysis inhibition, and ELISA have also been used to identify NDV or antibodies to the virus (Alexander, 1998; Jestin et al., 1989; Wilson et al., 1984).

The principal molecular determinant for NDV pathogenicity is reported to be the F protein cleavage site amino acid sequence (Glickman et al., 1988; Nagai et al., 1976) and the ability of various cellular proteases to cleave the F protein of different pathotypes (Gotoh et al., 1992; Ogasawara et al., 1992). Dibasic amino acids surrounding glutamine at position 114 are present in the F protein cleavage site of mesogenic or velogenic strains, while lentogenic NDV isolates lack this motif (Glickman et al., 1988; Nagai et al., 1976). Presence of dibasic amino acids in the F protein cleavage sequence allows for systemic spread of velogenic NDV, whereas with the exception of cardiac muscle, replication of lentogenic NDV is primarily in mucosal surfaces of the host (Brown et al., 1999). This is a major factor in differentiating velogenic and mesogenic NDV from lentogenic NDV isolates in cell culture. All NDV isolates will replicate in chicken embryo kidney cells (King, 1993), presumably due to the presence of a required protease (Ogasawara et al., 1992). However, lentogens must have proteases added to cell cultures for replication in avian fibroblasts or mammalian cell types whereas mesogenic and velogenic NDV do not have this requirement (Glickman et al., 1988; Nagai et al., 1976).

Reverse transcription coupled to polymerase chain reaction (RT-PCR) has been used by several investigators to amplify F gene sequences of many NDV isolates obtained worldwide (Alexander et al., 1999; Collins et al., 1993; Jestin and Jestin, 1991; Lomniczi et al., 1998; Yang et al., 1997; Werner et al., 1999). Amplification products were analyzed by gel electrophoresis before and after digestion with restriction enzymes giving somewhat inconsistent results (Jestin and Jestin, 1991). Collins et al. (1993) amplified nucleotide sequences coding for the F protein cleavage activation site and the amino acid sequence was deduced from nucleotide sequences of the RT-PCR product. Hybridization of PCR fragments with fluorogenic probes specific for NDV pathoypes were developed to estimate the virulence of isolates using a modified TaqMan procedure (Aldous, 2001). However, in the preceding cases several primer sets were needed to hybridize and amplify sequences from a variety of strains.

At the Southeast Poultry Research Laboratory (SEPRL, ARS, USDA) we have utilized degenerate oligonucleotide primers to amplify nucleotide sequences from portions of the F and M protein genes of NDV genomic RNA to be used diagnostically (Seal et al., 1995; 1998). Sequence

differences can be detected surrounding the F protein cleavage site and the M protein nuclear localization signal. These primers continue to be used in single-tube RT-PCR with NDV genomic RNA as a template. Modifications of the technique include use of "long-read" polymerases to amplify the 3' region of the M gene and the 5' region of the F gene as a single product. This has been followed by direct nucleotide sequencing of the amplified products to routinely characterize NDV isolates. Sequence analysis of NDV isolates has continued to include the other proteins encoded by the viral genome (Locke et al., 2000; Seal et al., 2000; 2001).

The idea that multiple lineages of NDV circulate simultaneously was first reported by Toyoda et al. (1989) and Sakaguchi et al. (1989). Sequence analysis of the HN gene from NDV isolates resulted in three distinct groups based upon variation in the HN protein length. Our analyses (Seal et al., 1998; 2000) further supports the hypothesis of multiple circulating lineages (Alexander et al., 1998; Lomniczi et al., 1998). However only two major clades are evident following analyses of the NDV genes and predicted amino acid sequence information (Fig 1). Our grouping of (TX)GB/48, BeaudetteC/59, Roakin/48, Kimber/47, B1/48 and LaSota/46 agrees with the previous classification of Sakaguchi et al. (1989) reported with some of the same isolates. In contrast to Sakaguchi et al. (1989), the topology of our phylogenetic trees do not strongly support the hypothesis of three co-circulating clades of NDV isolates. Our analyses have indicated that in the second clade, isolates D26/78, QV4/66 and Ulster/64 formed a related branch, compared to the virulent viruses in this cluster. The only clade that contained virulent isolates obtained in the last ten years was the upper branch of a second clade, which also contained isolates involved in the 1972 and 1992 U.S. Newcastle disease outbreaks (Seal et al., 1995, 1998). Interestingly, Herts/33 and AV/32 were positioned as possible progenitors in the M (Seal et al., 2000) and P (Locke et al., 2000) gene phylogenetic trees.

Phylogenetic trees generated following alignment of NDV genomic sequences were subjected to population analyses utilizing lineages through time plots (Raumbaut et al., 1997). Isolates of NDV arising since the 1970's belong to a population of viruses wherein genetic heterogeneity has expanded at an exponential rate (Seal et al., 2000). The virus causing the early 1970's outbreak of velogenic viscertropcic NDV was epidemiologically linked to a pet bird (Utterback and Schwartz, 1973). Consequently, these viruses may have their origins in free-living birds, are present worldwide, and continue to circulate causing disease in poultry. The NDV lineage composed of virulent isolates obtained in the U.S. prior to 1970 appears to no longer exist among free-living birds or commercial poultry. However, "vaccine-like" viruses are common in the U.S. and continue to circulate among commercial poultry (Marin et al., 1996).

Specific regions of NDV proteins have increased variability that may be reflected by a majority of nonsynonymous nucleotide changes in those respective areas of the genome. However, there is an overall predominance of synonymous nucleotide changes in the NDV genome suggesting that the virus has undergone purifying selection, resulting in disadvantageous mutations being eliminated from the viral population. The increased genetic heterogeneity among virulent NDV isolates currently circulating (Seal et al., 1999; 2000) is not surprising considering evolution of viruses with RNA genomes (Domingo et al., 1998).

Recently, viral genomic information surrounding the fusion protein cleavage site coding sequence was used to develop a heteroduplex mobility assay (HMA) to aid in further identification of molecular markers as predictors of NDV virulence (Berinstein et al., 2001). The HMA (Delwart, 1993) can be employed by hybridizing amplification products following RT-PCR to detect differences in nucleotide sequences among viral isolates by gel analysis (Fig. 2). Using common vaccine strains as a reference, we were able to distinguish virulent viruses among NDV isolates that correlated with phylogenetic analysis of the nucleotide sequence. This technique was also used to examine NDV isolates not previously characterized. We were able to distinguish vaccine-like

viruses from other isolates potentially virulent for chickens. This was true even for virulent NDV in Australia that mutated from a low-virulence virus (Westbury, 2001) with only two pont mutations in the F cleavage site (Berinstein et al., 2001).

The constant evolution of NDV and the previously undetected emergence of virulent virus from low virulence strains in Australia emphasize the importance for continued monitoring of this pathogen. The techniques described will help improve international harmonization of veterinary biologics as set forth by the OIE and the Veterinary International Cooperation on Harmonization of Technical Requirements of Veterinary Medicinal Products (Espeseth and Chapek, 1998). Ultimately, techniques such as the HMA could be used for rapid identification of potentially virulent NDV that continue to threaten commercial poultry worldwide.

CONCLUSIONS

* Degenerate oligonucleotide primers have been developed to amplify nucleotide sequences from the genomes of divergent NDV isolates.

* Utilizing phylogenetic analysis following alignment of nucleotide or predicted amino acid sequences mildly virulent vaccine NDV isolates can be separated from highly virulent viruses.

*There are apparently two major groups of NDV isolates. One group includes vaccine viruses and velogenic NDV isolated prior to the 1970's. A second phylogenetically distinct population of virulent viruses has expanded exponentially with regard to genetic diversity since the 1970's.

*Based on complete genome sequence, gene usage and predicted amino acid sequences NDV has evolved independently of their mammalian counterparts and deserve a distinct genus designation among the subfamily *Paramyxovirinae* in the family *Paramyxoviridae.*

*The mutation of low-virulence NDV to highly virulent virus in Australia contrary to current dogma emphasizes the need for continued monitoring of NDV and diagnostics improvement.

*Techniques such as the heteroduplex mobility assay (HMA) have also been exploited to differentiate highly virulent viruses from vaccine strains of NDV.

REFERENCES

Aldous, E.W., M..S. Collins, A. McGoldrick and D.J. Alexander. 2001. Rapid pathotyping of Newcastle disease virus (NDV) using fluorogenic probes in a PCR assay. *Vet. Microbiol.* 80:201-212.

Alexander, D.J. 1997. Newcastle disease and other avian Paramyxoviridae infections. pp. 541-570. *in* Calnek, B.W., H.J. Barnes, C.W. Beard, W.M. Reid, and H.W. Yoder, Jr. eds. Diseases of Poultry. 10th ed. Iowa State University Press. Ames, Iowa.

Alexander, D.J. 1998. Newcastle disease and other avian paramyxoviruses. Chapt. 30, pp. 156-163. *In:* Swayne. D.E., J.R. Glisson, M.W. Jackwood, Pearson, J. E., W.M Reed (ed.), A Laboratory Manual for the Isolation and Identification of Avian Pathogens 4th ed. American Association of Avian Pathologists, Inc. Kennett Square, Pennsylvania.

Alexander D.J., J. Banks, M.S. Collins, R.J. Manville, K.M. Frost, E.C. Speidel, and E.W. Aldous. 1999. Antigenic and genetic characterisation of Newcastle disease viruses isolated from outbreaks in domestic fowl and turkeys in Great Britain during 1997. *Vet. Rec.* 145:417-21.

Alexander, D.J., R.J. Manville, P.A. Kemp, G. Parsons, M.S. Collins, S. Brockman, P.H. Russell, and S.A. Lister. 1987. Use of monoclonal antibodies in the characterisation of avian paramyxovirus 1 (Newcastle disease virus) isolates submitted to an international reference laboratory. *Avian Path.* 16:553-565.

Berinstein, A.B., H.S. Sellers, D.J. King and B.S. Seal. 2001. Use of the heteroduplex mobility assay to detect differences in the fusion protein cleavage site coding sequence among Newcastle disease virus isolates. Submitted.

Brown, C.C., D.J. King and B.S. Seal. 1999. Pathogenesis of Newcastle disease virus in chickens experimentally infected with viruses of different virulence. *Vet. Pathol.* 36:91-178.

Collins, M.S., J.B. Bashiruddin, and D.J. Alexander. 1993. Deduced amino acid sequences at the fusion protein cleavage site of Newcastle disease viruses showing variation in antigenicity and pathogenicity. *Arch. Virol.* 128:363-370.

Delwart, E.L., E.G. Shpaer, J. Louwagie, F.E. McCutchan, M. Grez, H. Rübsamen-Waigmann, and J.I. Mullins. 1993. Genetic relationships determined by a DNA heteroduplex mobility assay: analysis of HIV-1 env genes. *Science.* 262:1257-1261.

Domingo, E., C. Escarmis, N. Seilla, E. Baranowski. 1998. Population dynamics in the evolution of RNA viruses. *Adv. Exp. Med. Biol.* 440:721-727.

Espeseth, D.A., and M.L. Chapek. 1998. Report of the Committee on Biologics & Biotechnology. Proceedings of the 102nd Annual Mtg. of the U.S. Animal Health Assoc. Minneapolis, MN. pp. 112-119.

Glickman, R.L., R.J. Syddall, R.M. Iorio, J.P. Sheehan, and M.A. Bratt. 1988. Quantitative basic residue requirements in the cleavage-activation site of the fusion glycoprotein as a determinant of virulence for Newcastle disease virus. *J Virol.* 62:354-356.

Gotoh, B., Y. Ohnishi, N.M. Inocencio, E. Esaki, K. Nakayama, P.J. Barr, G. Thomas, and Y. Nagai. 1992. Mammalian subtilisin-related proteinases in cleavage activation of the paramyxovirus fusion glycoprotein: Superiority of furin/PACE to PC2 or PC1/PC3. *J. Virol.* 66:6391-6397.

Jestin, V., M. Cherbonnel, R. L'Hospitalier, and G. Bennejean. 1989. An ELISA blocking test using a peroxidase-labeled anti-HN monoclonal antibody for the specific titration of antibodies to avian paramyxovirus type 1. *Arch. Virol.* 105:199-208.

Jestin, V., and A. Jestin. 1991. Detection of Newcastle disease virus RNA in infected allantoic fluids by *in vitro* enzymatic amplification (PCR). *Arch. Virol.* 118:151-161.

King, D.J. 1993. Newcastle disease virus passage in MDBK cells as an aid in detection of a virulent subpopulation. *Avian Dis.* 37:961-969.

Locke, D. P., H.S. Sellers, J.M. Crawford, S. Schultz-Cherry, D.J. King, R.J. Meinersmann and B.S. Seal. 2000. Newcastle disease virus phosphoprotein gene analysis and transcriptional editing in avian cells. *Virus Res.* 69:55-68.

Lomniczi, B., E. Wehmann, J. Herczeg, A. Ballagi-Pordany, E.F. Kaleta, O. Werner, G. Meulemans, P.H. Jorgensen, A.P. Mante, A.L. Gielkens, I. Capua, and J. Damoser. 1998. Newcastle disease outbreaks in recent years in western Europe were caused by an old (VI) and a novel genotype (VII). *Arch. Virol.* 143:49-64.

Marin, M.C., Villegas, P., J. Bennett, and B. Seal. 1996. Virus characterization and sequence of the fusion protein gene cleavage site of recent Newcastle disease virus field isolates. *Avian Dis.* 40:382-390.

Nagai, Y., H.D. Klenk, and R. Rott. 1976. Proteolytic cleavage of the viral glycoproteins and its significance for the virulence of Newcastle disease virus. *Virology.* 72:494-508.

Office International des Epizootes. 2000.Newcastle disease. Ch. 2.1.15, 9th edition. International Health Code (http://www.oie.int/).

Ogasawara, T., B. Gotoh, H. Suzuki, J. Asaka, K. Shimokata, R. Rott, and Y. Nagai. 1992. Expression of factor X and its significance for the determination of paramyxovirus tropism in the chick embryo. *EMBO J.* 11:467-472.

Peeters, B.P.H., O.S. de Leeuw, G. Koch and A.L.J. Gielkens. 1999. Rescue of Newcastle disease virus from cloned cDNA: Evidence that cleavability of the fusion protein is a major determinant for virulence. *J. Virol.* 73:5001-5009.

Raumbaut, A., P.H. Harvey and P.H. Nee. 1997. End-Epi: an application for inferring phylogenetic and population dynamical processes from molecular sequences. *Comput. Appl. Biosci.* 13:303-306.

Russell, P.H., and D.J. Alexander. 1983. Antigenic variation of Newcastle disease virus strains detected by monoclonal antibodies. *Arch. Virol.* 75:243-253.

Sakaguchi, T., Toyoda, T., Gotoh, B., Inocencio, N., Kuma, K., Miyata, T., and Y. Nagai. 1989. Newcastle disease virus evolution; 1. Multiple lineages defined by sequence variability of the hemagglutinin-neuraminidase gene. *Virology.* 169:260-272.

Schloer, G. 1974. Antigenic relationships among Newcastle disease virus mutants obtained from laboratory strains and from recent California isolates. *Infect. and Immun.* 10:724-732.

Seal, B.S., J.M. Crawford, H.S. Sellers, D.P. Locke and D.J. King. 2001. Nucleotide sequence analysis of the Newcastle disease virus nucleocapsid gene and its phylogenetic relationship among the *Paramyxoviridae.* Submitted.

Seal, B.S., D.J. King and J.D. Bennett. 1995. Characterization of Newcastle disease virus isolates by reverse transcription PCR coupled to direct nucleotide sequencing and development of sequence database for pathotype prediction and molecular epidemiological analysis. *J. Clin. Microbiol.* 33:2624-2630.

Seal, B.S., D.J. King, D.P. Locke, D.A. Senne, and M.W. Jackwood. 1998. Phylogenetic relationships among highly virulent Newcastle disease virus isolates obtained from exotic birds and poultry from 1989 to 1996. *J. Clin. Microbiol.* 36:1141-1145.

Seal, B.S., D.J. King and R.J. Meinersmann. 2000. Molecular evolution of the Newcastle disease virus matrix protein gene and phylogenetic relationships among the paramyxoviridae. *Virus Res.* 66:1-11.

Toyoda, T., Sakaguchi, T., Hirota, H., Gotoh, B., Kuma, K., Miyata, T., Nagai, Y., 1989. Newcastle disease virus evolution. II. Lack of gene recombination in generating virulent and avirulent strains. *Virology.* 169:273-282.

Utterback, W.W., and J.H. Schwartz.1973. Epizootiology of velogenic of velogenic viscerotropic Newcastle disease in southern California, 1971-1973. J. *Am. Vet. Med. Assoc.* 163:1080-1088.

Westbury, H. 2001. Newcastle disease virus: an evolving pathogen? *Avian Path.* 30:5-11.

Werner, O., A. Römer-Oberdörfer, B. Köllner, R.J. Manvell and D.J. Alexander. 1999. Characterization of avian paramyxovirus type 1 strains in Germany during 1992 to 1996. *Avian Path.* 28:79-88.

Wilson, R.A., C. Perrotta, B. Frey, and R.J. Eckroade. 1984. An enzyme-linked immunosorbent assay that measures protective antibody levels to Newcastle disease virus in chickens. *Avian Dis.* 29:1079-1085.

Yang, C-Y., P-C. Chang, J-M. Hwang, and H.K. Shieh. 1997. Nucleotide sequence and phylogenetic analysis of Newcastle disease virus isolates from recent outbreaks in Taiwan. *Avian Dis.* 41:365-373.

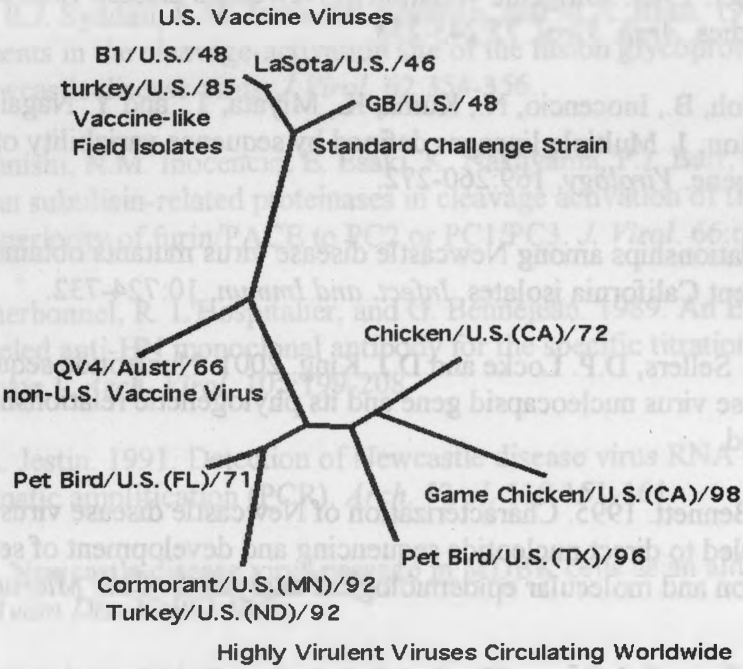

Fig. 1. Phylogenetic relationships among Newcastle disease virus isolates internationally.

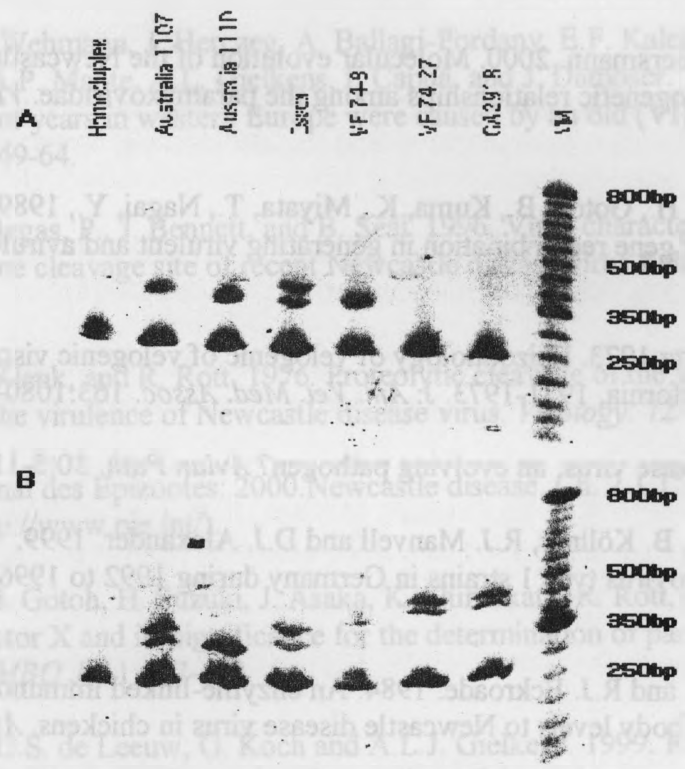

Fig. 2. Heteroduplex mobility assay to detect differences in the fusion protein cleavage site sequence. A. B1 as the reference virus. B. Ulster as the reference virus.

PNEUMOVIRAL INFECTION

Epidemiology of Avian Pneumovirus and Host range

K.V. Nagaraja, H.J. Shin, M.K. Njenga, B. McComb,
F.F. Jirjis, and D.A. Halvorson

Department of Veterinary PathoBiology
College of Veterinary Medicine
University of Minnesota
St. Paul, MN 55108

ABSTRACT

Avian pneumovirus (APV) has been a cause of a serious respiratory disease of turkeys in Minnesota. The first isolation of APV from turkeys was made in Minnesota in 1997 and since then it has posed a serious economic threat to the turkey producers of this state because of its impact on affected flocks (morbidity of up to 100 % and mortality of up to 20 %). Clinical signs of APV infection include: snicking, rales, sneezing, cough, discharge of nasal mucus, swollen sinuses, and mortality.

At the University of Minnesota, we have focussed on a number of studies on APV. With a highly sensitive and specific "M" gene based PCR test developed in our lab we have examined samples from turkey producers to confirm APV outbreaks and samples suspected to have any epidemiological link to the spread of APV. In addition, we found that waterfowl and wild birds are potential vectors of APV. We tested and found that APV can remain viable in a variety of harsh environmental conditions. Our experiments revealed that broiler chicks and ducklings are susceptible to infection by avian pneumovirus of turkey origin. Our studies indicated that avian pneumovirus RNA from wild and sentinel birds in the U.S. has genetic homology with APV isolates from domestic turkeys. A comprehensive survey was completed to look at the risk factors that may be associated with farms having a high prevalence of avian pneumovirus. We used the data from the APV survey study, which contained over 350 variables. The large number of variables did represent a challenge to identify factors which were truly significant. We tried to identify the variables that were not only statistically significant but also biologically plausible. We found three major themes that the statistically significant risk factors could be grouped into: 'handling of carcasses', 'movement between farms', and 'the commitment to biosecurity'.

Currently, there is a paucity of information on the epizootiology of avian pneumovirus. One interesting pattern, noted in APV outbreaks in Minnesota is its seasonal trend of occurrence. Most outbreaks occur in two periods of March through May, and October through November suggesting that environmental factors may contribute to the disease. One of the suspected sources of the outbreaks is wild birds.

INTRODUCTION

Avian pneumovirus infection is considered an emerging disease in the U.S. despite its presence elsewhere in the world in poultry for many years. In May 1996, a highly contagious respiratory disease of turkeys appeared in Colorado, and an APV was subsequently isolated at the National Veterinary Services Laboratory (NVSL) in Ames, Iowa (23). Prior to this time, the United States (20) and Canada

(11) were considered free of avian pneumovirus. Early in 1997, the presence of APV was detected serologically in turkeys in Minnesota and the virus spread rapidly among susceptible flocks. The disease was associated with catarrhal infections of the upper respiratory tract, foamy eyes, swelling of the sinuses in young poults and it was exacerbated by secondary infections. Morbidity in infected birds was often 100%. The mortality ranged from 1 to 90% and was highest in young poults. Avian pneumovirus is transmitted by contact. Nasal discharge, movement of affected birds, contaminated water, contaminated equipment, contaminated feed trucks and movement of affected poults can contribute to the transmission of the virus. Egg transmission and carrier birds also have been suggested. The virus is shown to infect the epithelium of the oviduct of laying turkeys.

History

Respiratory disease caused by an avian pneumovirus was first described in South Africa in the late 1970's (4) where it had a devastating effect on the turkey industry. The disease in turkeys was characterized by sinusitis and rhinitis and was called turkey rhinotracheitis (TRT). A similar disease was later reported as a major cause of respiratory disease in turkeys in Great Britain (2,12), France (10), Spain, Germany, Hungary, Italy, Netherlands (1), Taiwan (15), Japan (27), Mexico, Central and South America (13), Dominican Republic (19) and Brazil (3). The European isolates of APV have also been strongly implicated as factors in swollen head syndrome (SHS) in chickens (18). Swollen head syndrome was first described as a new disease of young chickens in South Africa in 1983 (16,4). Originally, the disease appeared in broiler chicken flocks infected with Newcastle disease virus (NDV) and was assumed to be a secondary problem associated with Newcastle disease (ND). Antibody against European APV was detected in affected chickens after the onset of SHS (8,28), thus implicating APV as the cause.

The avian pneumovirus often referred to as TRT virus, is a single stranded, non-segmented RNA virus that belongs to the sub-family *Pneumovirinae* of the family *Paramyxoviridae*, genus pneumovirus (5,14,29). The *Paramyxoviridae* family is divided into two sub-families: the *Paramyxovirinae* and *Pneumovirinae*. The subfamily *Paramyxovirinae* includes the genera: Paramyxovirus, Rubulavirus, and Morbillivirus. Recently, the sub-family *Pneumovirinae* was divided into two genera based on gene order, i.e. *pneumovirus* and *metapneumovirus* (17,21). The *pneumovirus* genus includes human respiratory syncytial virus (HRSV), bovine respiratory syncytial virus (BRSV), ovine respiratory syncytial virus, and mouse pneumovirus. The *metapneumovirus* genus includes European avian pneumovirus (subgroups A and B), which is distinguished from HRSV, the type species for the genus *pneumovirus* (17,21). The U.S. isolate of APV represent a third subgroup within *metapneumovirus* genus because it has been found to be antigenically (24) and genetically (22) different from European isolates.

Electron microscopic examination of negatively stained APV reveals pleomorphic, sometimes spherical, virions ranging from 80 to 200 nm in diameter with long filaments ranging from 1000 to 2000 nm in length (6). The envelope is made of a membrane studded with spikes 13 to 15 nm in length. The nucleocapsid is helical, 14 nm in diameter and has 7 nm pitch. The nucleocapsid diameter is smaller than that of the genera Paramyxovirus and Morbillivirus which usually have diameters of about 18 nm (6).

The subfamily *Pneumovirinae* generally encode ten proteins, including the nonstructural proteins (NS1 and NS2), nucleoprotein (N), phosphoprotein (P), matrix protein (M), small hydrophobic protein (SH), surface glycoprotein (G), fusion protein (F), second matrix protein (M2) and a viral RNA dependent RNA polymerase (L). The genome of European APV isolates lack the NS1 and NS2 genes, but have the M2 gene with structural characteristics similar to other mammalian pneumoviruses. Therefore, APV has tentatively been designated as a new representative of *Pneumovirinae*.

Serosurveillance in the U.S.

The U.S. Department of Agriculture (USDA) had been conducting serologic surveillance for APV in the U.S. Each year since 1988, between 500 and 800 serum samples from turkeys and chickens have been tested for the presence of antibody against both subgroups A and B. Prior to 1997, the U.S. surveillance samples had been free of APV antibody. Between 1997 and 1998, seropositive turkey flocks were found in Colorado, Minnesota, North Dakota, and South Dakota (Senne, 1999). All the chickens that were tested were negative for antibody to APV. Avian pneumovirus has been diagnosed in 92 Minnesota townships and in 22 counties, mainly in the areas of the state with the most dense turkey populations (9).

Host Range

Most isolations of avian pneumovirus have been made from turkeys and chickens implying that the host range is comprised of just these two species. When a similar but a different subtype of the virus from Europe (Turkey Rhinotracheitis virus- TRTV) was inoculated into turkeys, chickens, ducks, geese, pheasants, guinea fowl, and pigeons, the virus was recovered only from chickens and turkeys. The investigators in this study suggested that the lack of more severe effects may have been related to the use of an attenuated TRTV in this case along with the absence of important environmental factors.

However, other studies show indirect evidence of involvement of other hosts. Antibodies to TRTV have been demonstrated in guinea fowl that died of swollen head syndrome. Sera from ostriches sampled in the country - Zimbabwe were found to be positive for antibodies to TRTV. Seagulls trapped near the shorelines in Europe were found to have high titers of antibodies to avian pneumovirus in their blood.

Genetic homology of Avian Pneumovirus RNA from Wild and Sentinel birds in the U.S. with APV isolates from domestic turkeys

Samples collected from ducks, geese, owls, sparrows, swallows, and starlings, and from sentinel ducks placed next to turkey farms experiencing APV infections, were analyzed for APV genome and infectious particles. APV RNA was detected in samples examined from geese, sparrow, starlings, and sparrows. APV RNA and antibodies were also detected in two different groups of sentinel ducks. Infectious APV was recovered from the samples from sentinel ducks. The APV M gene isolated from the wild birds had over 96% predicted amino acid identity with APV/Minnesota 2A which was isolated earlier from domestic turkeys showing respiratory illness, suggesting that wild birds may be involved in spreading APV infection.

Susceptibility of broiler chicks to infection by avian pneumovirus of turkey origin

Studies were performed to evaluate the infectivity of a Minnesota turkey isolates of APV in chickens. Two-week-old broiler chicks free of antibodies to APV were exposed either by oculo-nasal or oral routes with a cell cultured APV of turkey origin. Chickens from both APV-inoculated groups exhibited clinical signs that included coughing, sneezing, nasal discharge and watery eyes during 2 to 8 days post-inoculation. Tissue samples from birds in the APV-inoculated group were positive for APV by

PCR up to 9 days post inoculation. Samples of blood from both oculo-nasally and orally infected chickens were positive for APV. Intestinal samples from chickens infected with APV orally were positive for the presence of APV on PCR up to 9 days post-inoculation. APV was re-isolated from samples taken from chickens in both groups inoculated orally and oculo-nasally. Sera from birds exposed by the oculo-nasal or by the oral route showed the presence of APV specific antibodies.

Airborne transmission of avian pneumovirus in turkeys

Although there is circumstantial evidence on possible airborne transmission of APV this has not been documented. We conducted a study to examine whether APV is transmitted through air. Four-week-old turkeys free of antibodies to APV were used in this study. Two isolators (1.2 X 1.2 m) completely enclosed in aluminum and Plexiglas-clad chambers connected by a rectangular duct (0.6 X 0.6m) that was 1 m long were used to conduct this experiment. The duct allowed ventilating air to flow from 1 isolator to the second isolator housing. Turkeys housed in one isolator were exposed to the Minnesota isolate of APV. Turkeys in the second isolator were kept as airborne-contacts. Choanal swabs from turkeys experimentally exposed to APV and those non-exposed to APV were collected at periodic intervals and examined by PCR for the presence of APV viral nucleic acid. Blood samples were collected at weekly intervals from birds in both groups to examine for the development of antibodies. It is important to note that by 3 days the choanal swabs collected from unexposed turkeys were positive for APV by PCR indicating a possible airborne transmission from the infected birds. Also, the birds in the unexposed group seroconverted to APV. Both PCR and serology results confirm airborne transmission of APV.

Homogeneity of U.S. viruses

To determine whether the outbreaks in the U.S. were due to the emergence of genetically different APV strains, 14 viruses isolated from the outbreaks in Minnesota over 3 years (1997 to 1999 were compared with the Colorado isolate (APV/CO). The current study suggests that there is high homogeneity within all U.S. isolates.

Epidemiological survey of APV infection to identify risk factors

This preliminary study looked into the risk factors that may be associated with farms having a high prevalence of avian pneumovirus. We used the data from the APV Survey Study, which contained over 350 variables. The large number of variables did represent a challenge to identify which were truly significant. We tried to identify the variables that were not only statistically significant but also biologically plausible. We found three major themes that the statistically significant risk factors could be grouped into. The themes identified were 'handling of carcasses', 'movement between farms', and 'the commitment to biosecurity'.

The analysis found that farms were more likely to be in the low prevalence group if they picked dead up multiple times in a day (chi-square=.021). We found that farms that buried their dead were more likely to be in the high prevalence group than farms that composted or incinerated them (chi-square=0.035). We found that farms with one company (not identified here) scheduling rendering trucks were more likely to be in the high prevalence group (chi-square=0.065). We found the farms that used their used litter as fertilizer within 0.5 miles of their farm were less likely to be in the high prevalence

group (chi-square=.071; odd ratio: 4.38 & Cl:1.125-17.021). Finally the use of vaccination crews were shown to be associated with high prevalence farms (chi-square=.065; odd ratio: 0.109 & Cl:.008-1.480).

There was evidence that farms with management committed to biosecurity had lower prevalence. Unfortunately this study has not yet identified specific risk factors. We have been able to get a glimpse into what areas we need to concentrate further analysis.

REFERENCES

1. Alexander, D. J. Newcastle disease and other avian paramyxoviridae infections. In: Disease of Poultry, 10th ed. B. W. Calnek, et al. Iowa State University Press, Ames, Iowa. pp. 669-673, 1997.

2. Anon. Turkey rhinotracheitis of unknown aetiology in England and Wales. Vet. Rec. 117:653-654, 1985.

3. Arns, C. W. and H. M. Hafez. Swollen head syndrome in poultry flocks in Brazil. In: Proc. 41st WPDC, Sacramento, California. pp. 81-83, 1992.

4. Buys, S. B. et al. A preliminary report on the isolation of a virus causing sinusitis in turkeys in South Africa and attempts to attenuate the virus. Turkey 28:36-46, 1980.

5. Cavanagh, D. and T. Barrett. Pneumovirus-like characteristics of the mRNA and proteins of turkey rhinotracheitis. Virus Res. 11:241-256, 1988.

6. Collins, M. S. and R. E. Gough. Characterization of a virus associated with turkey rhinotracheitis. J. Gen. Virol. 69:909-916, 1988.

7. Collins, P. L., et al. Respiratory syncytial virus. In: Field Virology; 3rd ed. Lippincott-Raven Publishers; Philadelphia; Field, et al.(Eds). Ch. 44: 1313-1351, 1996.

8. Cook, J. K. A., C. A. Dolby, et al. Demonstration of antibodies to turkey rhinotracheitis virus in serum from commercially reared flocks of chickens. Avian Pathol. 17: 403-410, 1988.

9. Friendshuh, K. Prevalence studies of avian pneumovirus. In: Proc. of the 50th Ann. Mtg. of the NCADC. October 3-5, Minneapolis, Minnesota. p. 31, 1999.

10. Giraud, P., et al. A possible viral candidate for the etiology of turkey rhinotracheitis. Vet. Rec.118: 81, 1986.

11. Heckert, R. A. and D. J. Myers. Absence of antibodies to avian pneumovirus in Canadian poultry. Vet. Rec. 132:172, 1993.

12. Jones, R. C., et al. Demonstration of a candidate virus for turkey rhinotracheitis in experimentally inoculated turkeys. Vet. Rec. 119:599-600, 1986.

13. Jones, R. C. Avian pneumovirus infection: Questions still unanswered. Avian Pathol. 25:639-648, 1996.

14. Ling, R., et al. Sequence analysis of the 22K, SH and G genes of turkey rhinotracheitis virus and their intergenic regions reveals a gene order different from that of other pneumoviruses. J. Gen. Virol. 73: 1709-1715, 1992.

15. Lu, Y. S., et al. Swollen head syndrome in Taiwan-isolation of an avian pneumovirus and serological study. Avian Pathol. 23:169-174, 1994.

16. Morley, A. J. and D. K. Thomson. Swollen-head syndrome in broiler chickens. Avian Dis. 28:238-343, 1984.

17. Naylor, C. J., et al. The ectodomains but not the transmembrane domains of the fusion proteins of subtypes A and B avian pneumovirus are conserved to a similar extent as those of human respiratory syncytial virus. J. Gen. Virol. 79:1393-1398, 1998.

18. O'Brien, J. D. P. Swollen head syndrome in broiler breeders. Vet. Rec. 117:619-620, 1985.

19. Pearson, J. E. Report of the committee on transmissible disease of poultry and other avian species. In: Proc. USAHA,Virginia. p. 334, 1990.

20. Pearson, J. E., et al. Viral turkey rhinotracheitis and swollen head syndrome of chickens: The current state of these diseases in the United States. In: Newly Emerging and Re-emerging Avian Diseases: Applied Research and Practical Applications for Diagnosis and Control. D.E. Swayne and M. Boulianne (eds). American Association of Avian Pathologists Symposium, Minneapolis, Minnesota, USA. pp. 78-83, 1993.

21. Pringle, C. R. Virus taxonomy. Arch. Virol. 143:1449-1159, 1998.

22. Seal, B. S. Matrix protein gene nucleotide and predicted amino acid sequence demonstrate that the first U. S. avian pneumovirus isolate is distinct from European subtypes. Virus Res. 58:45-52, 1998.

23. Senne, D. A., et al. Avian pneumovirus update. Proc. 134th Ann. Mtg., AVMA. July 21-24, Reno, Nevada. p. 190, 1997.

24. Senne, D. A., et al. Avian pneumovirus in turkeys: Have you seen it? In: Proc. 47th WPDC, California, pp. 67-68, 1998.

25. Senne, D. A., et al. Avian pneumovirus in turkeys: Preliminary findings. In: Proceedings and Technical Supplement of the Roche Avian Pneumovirus Workshop. June 30-July 1, St. Cloud, Minnesota. p. 51, 1998.

26. Shin, H. J. and K.V. Nagaraja. Specific Detection of Avian Pneumovirus (APV) US isolates by RT-PCR. Arch. Virol. 145:1239-1246, 2000.

27. Tanaka, M., et al.Turkey rhinotracheitis virus isolated from broiler chickens with swollen head syndrome in Japan. J. Vet. Med. Sci. 57:939-941, 1995.

28. Wyeth, P. J., et al. Antibodies to TRT in chickens with swollen head syndrome. Vet. Rec. 120:286-287, 1987.

29. Yu, Q., et al. Sequences and in *vitro* expression of the M2 gene of turkey rhinotracheitis pneumovirus. J. Gen. Virol. 73:1355-1363, 1992.

Pathogenesis and Diagnosis of Avian Pneumovirus

Daniel P. Shaw, DVM, PhD, Diplomate of ACVP and ACPV
Department of Veterinary Science
The Pennsylvania State University
Animal Diagnostic Laboratory
University Park, PA 16802-1110

Avian pneumovirus (APV) is a single stranded, non-segmented RNA virus that belongs to the subfamily pneumovirinae of the family Paramyxoviridae, genus pneumovirus.[8] Turkey rhinotracheitis virus (TRT) is in this group and is closely related to but distinct from US APV isolates.[9, 11] In the field, infection with this virus is associated with highly contagious respiratory disease and variable mortality rates (3 to 30 %).[10] Many of the clinical signs displayed by turkeys with APV infection are suggestive but not specific. There are no pathognomonic gross or microscopic changes. Laboratory testing is necessary for verification of the diagnosis. Clinical signs include snicking, coughing, gasping, nasal discharge, ocular discharge and swelling of the infraorbital sinuses. High rates of condemnation at processing due to airsacculitis and pneumonia have occurred.

Avian pneumovirus has been found to survive for 12 weeks at 4° C and 4 weeks at 22° C.[14] The virus survived less than 24 hours at 37° C. Most disinfectants effectively neutralize the virus. The complete host range has not been determined. Broiler chickens have been experimentally infected.[13] The virus has been detected in wild birds including sparrows and waterfowl.[7]

When three-week-old turkeys were experimentally inoculated via the oculonasal route with APV isolated from a Minnesota flock, clinical signs developed within 2 days.[5] The poults completely recovered by 10 days after inoculation. The clinical signs closely resembled those seen in the field and consisted of snicking, coughing, ocular and nasal discharge, and swelling of the sinuses. The postmortem changes included sinusitis and rhinitis. There was very mild inflammation in the tracheas. No significant changes were detected in the lungs although a few of the poults had mild airsacculitis. Antibody against APV was detected in many of the poults by 6 days postinoculation (PI) using the ELISA test. Virus was consistently detected in the nasal turbinates on days 4 through 6 PI using the PCR test. Virus was not as reliably detected by PCR in tracheal swabs during this period. The virus was isolated from the nasal turbinates during the same time period. The virus was not detected in lung or cloacal swabs using PCR or virus isolation.

When the disease first appeared in Minnesota, it was diagnosed by serology using the ELISA developed at the National Veterinary Services Laboratory (NVSL) in Ames, IA. The antigen used in the ELISA was from the virus that was isolated from the first outbreaks that occurred in Colorado in late 1996. As more cases appeared and demand for ELISA increased, the Minnesota Veterinary Diagnostic Laboratory began offering the test. Eventually, the ELISA was refined by using anti-turkey conjugate instead of anti-chicken conjugate.[1, 2] This modification greatly enhanced the sensitivity of the ELISA in turkey samples.

The reverse transcriptase-polymerase chain reaction (RT-PCR) assay was developed in1998 and adapted to the TaqMan (Perkin Elmer Corp./Applied Biosystems, Foster City, CA) system.[3] This test is based on the detection of a specific nucleotide sequence of the fusion (F) gene of the Colorado isolate of APV. This procedure is rapid, very sensitive (detects less than 0.01 TCID50) and the closed system of TaqMan greatly decreases the risk of cross-contamination in the laboratory. Another experimental RT-PCR assay for detection of APV has been developed and is presently being used on diagnostic specimens. This assay uses two sets of primers derived from the matrix (M) gene of APV.[12] It has been

shown to specifically detect the virus and the two primers are able to differentiate between isolates of APV from turkeys in Minnesota.

Choanal and tracheal swabs from live birds are excellent samples for PCR assay whereas nasal turbinates are the preferred tissue specimens for testing of dead birds. It has been found experimentally that there is a narrow window of time (2 to 6 days post-infection) for optimal opportunity to detect viral RNA by the RT-PCR procedures.[5] The optimal number of birds for sampling during an outbreak has not been determined. In the field, several birds are usually sampled at the earliest sign of the disease and up to 5 choanal swabs may be pooled for RT-PCR.

Virus isolation is a long process with this virus. Primary isolation is usually first attempted in chicken embryo fibroblasts[1, 4] or 9-day-old chicken embryos. This is followed by 6 or more blind passages in chicken embryo fibroblasts and vero cells. Once established, the virus produces distinctive cytopathic effects in vero cell cultures. Indirect fluorescent antibody staining of vero cells cultures provides specific detection of the virus within 3 passages. Avian pneumovirus can also be detected in formalin-fixed paraffin embedded tissues by the use of immunohistochemical staining.[6] This procedure has been used in research trials for studying the pathogenesis of the disease and shows promise for identification of the virus in field cases.

References

1. Chiang S: Isolation and seroprevalence of pneumovirus in Minnesota. M.S. thesis, University of Minnesota, 1998.

2. Chiang SJ, Dar AM, Goyal SM, Sheikh MA, Pederson JC, Panigrahy B, Senne D, Halvorson DA, Nagaraja KV, Kapur V: A modified enzyme-linked immunosorbent assay for the detection of avian pneumovirus antibodies. J.Vet. Diag. Inv. 12:381-384, 2000.

3. Dar AM, Tune K, Munir S, Panigrahy B, Goyal SM, Kapur V: PCR-based detection of an emerging avian pneumovirus in US turkey flocks. J. Vet. Diag. Inv. 13:201-205, 2001.

4. Goyal SM, Chiag SJ , Dar AM, Nagaraja KV, Shaw DP, Halvorson DA, Kapur V: Isolation of avian pneumovirus from an outbreak of respiratory illness in Minnesota turkeys. J.Vet. Diag. Inv. 12:166-168, 2000.

5. Jirjis FF, Noll S, Nagaraja KV, Halvorson DA, Shaw DP: Avian pneumovirus infection in turkeys: experimental reproduction of the disease. Avian Dis. 44:222-226, 2000.

6. Jirjis FF, Noll S, Nagaraja KV, Halvorson DA, Shaw DP: Immunohistochemical detection of avian pneumovirus infection in chickens. J. Vet. Diag. Inv. 13:13-16, 2000.

7. Nagaraja KV, Shin HJ, Njenga M, Shaw D, Halvorson DA: Host range and epidemiology of avian pneumovirus infection. Proceedings of the 51st Western Poultry Disease Conference, Davis, CA, pp.80-81, 2001.

8. Naylor CJ, Britton P, Cavanagh D: The ectodomains but not the transmembrane domains of the fusion proteins of subtypes A and B avian pneumovirus are conserved to a similar extent as those of human respiratory syncytial virus. J. Gen. Virol. 79:1393-1398, 1998.

9. Seal BS: Matrix protein gene nucleotide and predicted amino acid sequence demonstrate that the first U.S. avian pneumovirus isolate is distinct from European subtypes. Virus Res. 58:45-52, 1998.

10. Senne DA, Edson RK, Pederson JC, Panigrahy B: Avian pneumovirus update. Proceedings of the 134th Annual Convention of the Am.Vet. Med. Assoc., Reno, Nevada, p. 190, 1997.

11. Senne DA, Pedersen JC, Edson RK, Panigrahy B: Avian pneumovirus in turkeys: have you seen it? In: Proceedings of the 47th Western Poultry Disease Conference, Sacramento, California, pp. 67-68, 1998.

12. Shin HJ, Rajashekara G, Nagaraja KV, Jirjis FF, Shaw DP, Goyal S, Halvorson DA: Specific detection of avian pneumovirus US isolates by polymerase chain reaction (RT-PCR). Arch. in Virol. 145:1239-246. 2000.

13. Shin HJ, McComb B, Back A, Shaw DP, Halvorson DA, Nagaraja KV: Susceptibility of broiler chicks to infection by avian pneumovirus of turkey origin. Avian Dis. 44:797-802. 2000.

14. Townsend E, Halvorson DA, Nagaraja KV, Shaw DP: Susceptibility of an avian pneumovirus isolated from Minnesota turkeys to physical and chemical agents. Avian Dis. 44:336-342. 2000.