NORTHEAST REGIONAL RESEARCH PROJECT

PROJECT NUMBER: **NE-138**

TITLE: **EPIDEMIOLOGY AND CONTROL OF EMERGING STRAINS OF POULTRY DISEASE RESPIRATORY AGENTS**

DURATION: **OCTOBER 1, 1996 THROUGH SEPTEMBER 30, 2001**

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OF POULTRY RESPIRATORY DISEASE AGENTS

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EXECUTIVE SUMMARY

EPIDEMIOLOGY AND CONTROL OF EMERGING STRAINS OF POULTRY RESPIRATORY DISEASE AGENTS

Duration. October 1, 1996-September 30, 2001

This regional project addresses the need to counter a threat to the multi-billion dollar U.S. poultry industry caused by emerging and re-emerging viral and mycoplasmal respiratory pathogens. The project includes scientists from ten State Agricultural Experiment (SAES) and Veterinary Medical Experiment Stations (VMES), the Southeastern USDA-ARS laboratory, and the internationally-known Saint Jude Children's Research Hospital. A team of thirty-one senior scientists has been assembled with expertise in poultry respiratory infections that include infectious bronchitis, avian influenza, infectious laryngotracheitis and avian mycoplasmas. The team will address four objectives using discipline specializations in virology, bacteriology, immunology and pathology, and molecular biology.

Project Objectives.

1. Develop and evaluate rapid diagnostic capabilities for the identification of emerging agents causing mycoplasmosis, infectious bronchitis, avian influenza, and infectious laryngotracheitis virus.

2. Characterize immunosuppressive viruses (IBDV) and assess the interaction of underlying immunosuppressive agents (IBDV and CAV) on emerging respiratory agents.

3. Delineate epizootiology of emerging mycoplasma and the viruses that cause infectious bronchitis, avian influenza, and infectious laryngotracheitis.

4. Design and implement novel immune and genetic prophylactic strategies for effective control of respiratory diseases caused by emerging agents.

Project Interdependence. The procedures section outlines work that relies on extensive collaboration from the represented institutions. This involves a variety of exchanges to provide needed basic and applied information that directly impact our ability to diagnose and control these respiratory agents and the underlying immunosuppressive agents that contribute to field outbreaks. Diagnostic applications will require the sharing of newly developed reagents and technologies to develop the most rapid, sensitive and accurate tests. Control of the diseases under study will use a combination of novel molecular and conventional approaches. Importantly, this proposal also calls for avian influenza virus (AIV) surveillance studies to monitor AIV in wild bird populations in the Eastern and Midwestern regions of the U.S.

Application to Stakeholders. The cooperating institutions have ongoing, direct ties to the poultry industry in their respective states and regions. Well-established mechanisms will extend the results of the studies described herein to poultry veterinarians and other health specialists in the U.S. Consumers should also benefit from reduced disease losses through the continued cost-competitive nature of poultry and eggs.

Project Organization. The proposal outlines a organizational plan that will provide continuity from year to year. A Project Coordinator will be selected to help facilitate interactions between scientists working on the project and completion of the objectives. Annual meetings will continue to be held in conjunction with the North Central-116 (NC-116) Regional Project meeting in Chicago to exchange ideas and information on poultry disease control.

STATEMENT OF THE PROBLEM

Respiratory diseases in poultry are generally the outcome of complex interactions. Variability of causative agents, immunosuppression, host susceptibility, and the influence of environment affect the epizootiologic manifestation of respiratory disease. As a result, expression of respiratory disease can vary greatly. Understanding the pathogenesis and epizootic manifestations of respiratory disease is crucial for development of control strategies. Use of antibiotics and anti-microbials is being discouraged. This has resulted in pressure on the poultry industry to rely even more heavily on control methods such as vaccination as well as to explore other more long range alternatives such as antivirals. Because respiratory disease is seldom caused by a single entity, it is important to have rapid, highly sensitive, and specific methods to determine the presence of multiple agents. The application of molecular and conventional research approaches is needed to identify/diagnose and control emerging respiratory pathogens. Because of the complexity of the problem, namely many contributing agents, mixed infections, environmental impact, and variation in host susceptibility, cooperative and coordinated planning and research by the scientists and staffs of the participating institutions greatly increases the probability of success in reducing losses from avian respiratory diseases.

JUSTIFICATION

Poultry meat and eggs are major protein sources in the American diet. In 1992, total egg production in the U.S. was 70.5 billion eggs with a total value of $3.4 billion (Georgia Agricultural Statistics Service, 1993). That same year, 6.6 billion chickens and 290 million turkeys were produced with a combined value of $15 billion (Poultry Slaughter, USDA, 1992-1993). Poultry meat consumption increased 37 percent from 60.2 lb. to 96.6 lb. In contrast, per capita beef and veal consumption declined 105 lb. to 96.1 lb. during the same time span (USDA Livestock and Poultry, Situation and Outlook, 1993). Consumption of poultry meat is projected to continue to increase for the foreseeable future. Improved efficiency has paralleled the rise in poultry production. The American consumer has benefited from the intense competition within this non-subsidized industry. Poultry retail prices have remained considerably lower and more stable than those for pork and beef. Broiler retail prices rose from $1.417/kg in 1975 to $1.881/kg in 1988, a 25 % increase over a 13 year period. During the same time period, beef rose from $3.141/kg to $5.613/kg, a 44% increase. Turkey retail meat prices have been remarkably stable fluctuating around $ 1.00/lb. (range $.96 - $1.07) over the last decade (Duewer et al., 1993; Putham and Allshouse, 1993).

The increase in efficiency and productivity achieved by the poultry industry is not without associated problems. The necessity for mass rearing of single and multiple aged flocks has created conditions that favor the rapid transmission of disease agents. Diseases are the major cause of lost productivity in U.S. flocks. Losses occur from mortality, condemnation, decreased egg production, poor feed conversion and increased medication (antibiotics) costs. Poultry producers operate on thin margins, with market forces sometimes causing losses over extended periods. The economic impact of diseases frequently has devastating effects on the well-being of the industry.

Respiratory disease continues to be the most common and economically important contributor to overall disease losses in poultry in the U.S. and many other countries. It is difficult to assess respiratory disease impact on total mortality, nevertheless it is widely thought to contribute to the 9.5% mortality in young turkeys and the 4% growing mortality in broiler chickens. One measure of impact of respiratory disease is the condemnation losses reported as airsacculitis and septicemia by the Federal Meat and Poultry Inspection Service (USDA-FSIS). Airsacculitis and septicemia condemnations from October 1,1992-September 30,1993 represented 58.2% of the total condemnations of broiler chickens in the U.S. The 31.6 million broilers condemned for respiratory disease during this 12 months period resulted in retail loss of approximately $124 million assuming a cost of $.90/lb. and 4.37 lb. average market weight (Duewer et al., 1993). Airsacculitis and septicemia condemnations are higher in turkeys than chickens. In the month of September 1993, respiratory disease condemnations in all federally inspected turkeys was 77.8% of all condemnations (Georgia Agricultural Statistics Service, 1993). The impact of respiratory disease on health and egg productivity of adult hens is more difficult to estimate since statistics for egg losses by cause are not maintained.

The respiratory disease losses described above do not reflect total losses. In a study of the Delmarva broiler industry, the nations most concentrated and 10th largest poultry region, researchers showed that disease losses could have multiplier effects ranging from 1.33-2.56 in broiler industry sectors that included grower payment, feed grains, chicks started, vaccines, sanitation, poultry processing (e.g. line slowdown due to parts reclamation), utilities, professional services and insurance premiums (Buckner and Hastings, 1984). Thus, when significant disease losses occur in the poultry industry, there is multiplied impact on regional and national economics. Given the complexity of measuring total losses, it can be argued that USDA statistics, showing millions of dollars lost annually to respiratory disease, represent only a fraction of the real economic loss.

Infectious bronchitis virus (IBV) continues to be the most common cause of respiratory disease in the U.S. Variant serotypes of IBV have been associated with major outbreaks in broilers and layers. The emergence of IBV variant serotypes through virus mutation and selective pressures associated with poultry management and vaccination favor the establishment of novel serotypes capable of causing losses. IBV itself generally does not cause high mortality, but the

infection readily predisposes the host to secondary infections that negatively impact growing mortality and airsacculitis condemnations.

The potential for considerably higher mortality exists with virulent infectious laryngotracheitis virus (ILTV) and highly virulent (high path) avian influenza viruses. The incidence of ILTV outbreaks involving pathogenic strains has increased dramatically since 1994 in the Delmarva peninsula region, Georgia and Arkansas. AIV continues to threaten the U.S. poultry industry via contact with live poultry markets in cities of the northeastern states, feral waterfowl and shore birds migrating from South and Central America, and most recently, the outbreak of highly pathogenic AIV in Mexico (see Attachment C). The close proximity of Mexico is a concern for several reasons. Waterfowl and shore birds seasonally migrate from Canada and the U.S. to (through) Mexico and back. These birds may carry AIV subtypes capable of causing infection in poultry. Prevention of avian influenza in domestic poultry needs a more detailed understanding of influenza virus ecology in relation to different wild bird populations as well as domestic poultry. A more complete understanding of the AIV sources and their potential for exchange will assist in the development of husbandry and farming strategies to decrease potential interspecies and intraspecies AIV transmission. Secondly, the removal of trade barriers with Mexico resulting from the North American Free Trade Agreement (NAFTA) has increased the potential for poultry, eggs or products derived from them to enter the U.S. In addition, the improved relations between the two countries, along with the continued economic problems in Mexico has contributed to increased immigration of Mexicans to the U.S. to seek employment. Potentially, these individuals could provide a vehicle for bringing AIV to the U.S. if they come in contact with poultry.

Mycoplasma infections, resulting in lowered production and management-related problems, accounted for an estimated $132 million loss to the U.S. layer industry in 1988, while disease alone cost the broiler industry $588 million (Anon., American Association of Avian Pathologists, AAAP News Letter, 1989). Chronic respiratory disease, synovitis and sinusitis, caused by *M. gallisepticum* (MG) and *M. synoviae* (MS) infections present serious economic problems to the poultry industry. Although these diseases caused by MG do not necessarily account for high mortality, morbidity is high. The damaging effects of disease are consequent to lowered egg production, retarded growth, poor carcass quality and predisposition to secondary bacterial and/or viral infections during the prodromal stages (Hopkins and Yoder, 1982; Springer et al., 1974; Timms, 1972). Over the years, the American Association of Avian Pathologists (AAAP) has recommended the eradication of MG and MS from chicken and turkey growing and laying flocks as the ultimate goal. If this goal is to be realized it will require continual serological monitoring and prophylactic immunization of poultry flocks. Efforts targeted to control mycoplasmal disease have been hampered due to a lack of effective vaccines, the use of live vaccines in multiplier flocks, and the inadequacy of available diagnostic tests for specific detection of MS infections. The latter problem is compounded by the presence of commensal antigenically-related mycoplasmal species in most intensive poultry farming operations. The consensus of most researchers is that the success of any serodiagnostic test for MG detection will rest on the availability of a reliable and consistent antigen (Avakian et al., 1988; Bradbury, 1988; Kleven et al., 1988; Talkington et al., 1985)

RELATED CURRENT AND PREVIOUS WORK

A CSREES search was conducted and showed several projects with objectives involving the agents studied in this project. The majority of the projects currently being conducted are with researchers listed in this project. It is the intent of our group to expand our participation with other researchers over the project period by continuing to interact with colleagues working with these agents. For instance, members of the North Central 116 (NC-116) regional project working with turkey influenza virus at Minnesota AES could easily conduct work under this project's objectives. The joint meeting with the NC-116 group in Chicago will serve to assist us in project recruitment and collaboration (see **ORGANIZATION** section).

A. Infectious Bronchitis Virus (IBV)

Infectious bronchitis (IB) is a highly contagious disease of chickens that causes significant economic losses in commercial broilers, layers and broiler breeders. The many different recognized serotypes and emerging variant serotypes (Gelb et al., 1991) make IB among the most difficult poultry diseases to control. Variant IBV's pose a major threat to commercially raised poultry throughout the world. In the U.S. in 1992, serious yet unrelated outbreaks occurred in broilers in the Delmarva peninsula area, caused by the Delaware variant (92-072), and the Southeastern region caused by the Arkansas serotype and the closely-related Georgia variant. Although recognized since the late 1980's in the Central Valley region, the California variant still causes considerable losses in broilers. IBV causes acute respiratory disease that is commonly followed by secondary bacterial infection most often due to *Escherichia coli*, particularly in broiler chickens. The combination often results in elevated growing mortality and airsacculitis condemnation rates in affected flocks. Layers and broiler breeders also may be highly susceptible to variant IB. Egg production and losses are common particularly on farms with multiple-aged flocks. Layer and broiler flocks infected with pathogenic mycoplasma experience more severe IBV-related egg production and respiratory disease associated losses. The high incidence of IBV infections in commercial chickens underscores the importance of developing vaccination programs that afford maximal protection under field conditions.

IB is perhaps the most "explosive" of all avian diseases. The virus spreads extremely rapidly in flocks due to its short incubation period (18-48 hours) and highly contagious nature (King and Cavanagh, 1991). The virus is present in respiratory excretions and feces and is disseminated by aerosol transmission. Mechanical transmission of the virus by humans on contaminated clothing and poultry equipment can be very important in IB spread.

IBV enters the chicken by inhalation or ingestion of contaminated feed or drinking water and replicates initially in the upper respiratory tract. Viral damage to respiratory tissues includes deciliation of tracheal epithelial cells and impairment of the production and secretion of mucus from glands lining the trachea ultimately resulting in predisposition to secondary bacterial infections. Pneumonia involving the bronchial and pleural surfaces of the lung may occur with more virulent strains and result in higher mortality. Virus is also disseminated to enteric tissues, such as proventriculus, intestine and cecal tonsil (Ambali and Jones, 1990; Gelb, 1989), as well as kidney (Cumming, 1969), and oviduct (Crinion and Hofstad, 1972). Damage to the oviduct produces egg production losses in the form of a cessation or decrease of egg production and an increase in egg quality problems (wrinkled shells and lost pigmentation, watery albumin). Virus is shed from the cecal tonsil and is present in feces for several weeks or months after the acute disease episode (Cook, 1968; Alexander and Gough, 1977). Cellular tropism of mammalian coronaviruses can be correlated to their virulence and *in vivo* persistence (Fleming et al.,1994; Stuhler et al.,1994).

Immunity following recovery from natural infection or live IBV vaccination is of variable duration and specificity depending on the virulence of the field or vaccine strain, age of infection or vaccination, immunocompetency of the flock, the presence of potentially interfering maternal antibodies, and the serotype and virulence of a subsequent challenge strain. Vaccinal protection results following the application of IBV live attenuated strain(s) onto the respiratory mucosal

surface by the aerosol (spray), eyedrop, or drinking water (exposure via the palatine cleft) routes. Vaccines given by these "natural" routes of application mimic the natural portal of infection and induce local tissue immune responses in the upper respiratory tract. On the other hand, inactivated oil emulsion vaccines given by injection do not stimulate significant respiratory immunity, but are effective in stimulating systemic blood-borne resistance in live IBV-primed birds by providing protection to the internal target tissues, kidney and oviduct.

Immunosuppression associated with IBDV infection, particularly during the first two weeks of life, is known to increase the severity of viral respiratory diseases, contribute to prolonged shedding of live Newcastle disease virus (NDV) and IBV, as well as impair the response to vaccination with NDV, laryngotracheitis virus, and IBV (Allan et al., 1972; Faragher et al., 1974; Pattison and Allan, 1974; Rosenberger and Gelb, 1978). Research has also shown that IBDV destroys local antibody-producing plasma cells in the gland of Harder, and thus may directly compromise upper respiratory tract immunity (Dohms et al., 1981). Field observations over the years have also clearly demonstrated the relationship between early (< 2 week) infection in broilers and poor flock performance in terms of elevated airsacculitis condemnations and a greater incidence and severity of respiratory disease challenges and mortality. As a result, much effort is directed at protecting baby chicks from the immunosuppressive effects of IBDV infection. The impact of early infection has been reduced by providing passive protection (IBDV maternal antibody) to chicks through the use of IBDV immunization programs in breeder flocks. This strategy has been successful in preventing early infection and long-lasting immunosuppression, however it does not prevent infection after maternal immunity wanes at 2-4 weeks of age. In fact, infection at 2-4 weeks produces a more transient immunosuppression than one-day infection but still has the potential to impact respiratory disease immunity. IBDV infection of 3-week broiler chickens produced plasma cell necrosis in the gland of Harder, from 5-14 days after inoculation and reduced antibody production to sheep red blood cells and *Brucella abortus* antigens (Dohms et al., 1988; Dohms and Jaeger, 1988).

Diagnosis of IBV is accomplished by isolating and serotyping the causative field isolate. The virus-neutralization (VN) and hemagglutination-inhibition (HI) tests have been until very recently the only procedures used for serotyping (Gelb, 1989). These procedures are expensive, tedious, and time-consuming and are not widely available to the poultry industry. Serodiagnosis of IBV using serum from recovered flocks can not be used to identify the causative serotype of IBV because chickens produce cross-reacting antibodies following multiple infections (Gelb and Killian, 1987). Serotype-specific monoclonal antibodies (Mabs) developed for the vaccinal serotypes, Mass, Ark, and Conn have been used to identify vaccinal serotype(s) involved in IB outbreaks (Karaca et al., 1992). The polymerase chain reaction (PCR) has been used successfully to diagnose viral infections in humans and animals (Andreasen et al., 1991; Homberger et al., 1991). The technique is particularly attractive in that it is extremely sensitive and is capable of detecting minute amounts of virus in clinical specimens. The PCR is also highly specific in that it utilizes oligonucleotide primers that potentially may recognize specific gene sequences that encode important epitopes of viral proteins responsible for initiating infection through attachment and inducing protective immunity. In addition, the PCR is a rapid test giving results in a matter of days. PCR is currently used for the diagnosis of IBV serotypes. Two reverse transcritption (RT) PCR approaches have been used to identify IBV serotypes. Restriction fragment length polymorphism (RFLP) was developed by Kwon et al. (1993). Alternatively, RT PCR using serotype-specific primers has been used to identify IBV (Reed et al., 1992).

The structure and replication of IBV and other coronaviruses have been reviewed (Boursnell et al., 1989; Spaan et al., 1988). The virion has a helical symmetry and is 80-160 nm in diameter. The ss RNA 27.6 kb IBV co-linear genome is arranged into six regions, each containing one or more open reading frames which are separated by intergenic sequences containing signals for transcription of multiple subgenomic m-RNAs. Genomic RNA is of a positive polarity and as such serves as a template for the production of full-length negative strand RNA. The 5' and 3' ends of the genome contain noncoding homology sequences that on the 3' end have been proposed to serve as a RNA polymerase binding site for production of the negative strand (Boursnell et al., 1989). The negative strand serves as template for the production of

genomic RNA and subgenomic m-RNAs. Subgenomic m-RNAs A (nucleocapsid), B, C (membrane), D, E (spike), and F (RNA polymerase) form a 3' coterminal nested set that are structurally multicistronic but are functionally monocistronic. Only the gene located at the 5' end of each m-RNA is translated. These m-RNAs are synthesized in constant but nonequimolar concentrations by a unique leader-primed transcription process. Analysis of the 5' end of genomic and m-RNAs has revealed a common leader sequence, CUUAACAA (Brown and Boursnell, 1984) or the very similar CUGAACAA (Boursnell et al., 1987). Leader sequences of about 60-65 nucleotides are transcribed discontinuously from the 3' end of the negative strand template by viral RNA polymerase. The conserved (i.e., homology) sequences are thought to be the initiation sites for transcription by the leader-RNA polymerase complex. The degree of complementarity (base-pairing) between the free leader and the different initiation sites may regulate the expression of the m-RNAs. The core homology is conserved in all the intergenic regions, however the region around the core is not conserved for different genes even in a single IBV serotype. When the intergenic region for the matrix gene was compared for several Mass strains and four other European serotypes, including some recently isolated variants, high homology was found around the identical core homology (Cavanagh and Davis, 1988).

The virus possesses three major structural proteins; a 45kD nucleocapsid protein (N), a (23kD) membrane or matrix (M) glycoprotein and an envelope spike (S) glycoprotein peplomer. The N protein is involved in encapsidation of genomic RNA and has been implicated in the process of RNA replication (Spaan et al., 1988). The M protein is required for viral maturation and assembly interacting with N and perhaps genomic RNA. The S gene encoding for subunits S-1 (gp 92) and S-2 (gp 84) has received considerable attention since S-1 is required for virus attachment to host cells and erythrocytes (Cavanagh and Davis, 1986) and the induction of immunity (Cavanagh et al., 1986;1988). Sequence analysis of the S-1 gene of IBV has identified hypervariable regions characterized by nucleotide substitutions, sequence insertions and deletions that are not conserved among serotypes (Binns et al., 1986; Kusters et al., 1989). The hypervariable sequences in S-1 have been mapped to regions encoding serotypic determinants (Cavanagh et al., 1988; Kusters et al., 1989).

Non-structural proteins encoded by the coronavirus genome include an RNA polymerase (mRNA F) used in the synthesis of genomic RNA and m-RNAs. The RNA polymerase, however has yet to be identified. In addition, coronavirus infected cells have two m-RNAs (B and D) for which proteins have yet to be identified. m-RNA B potentially encodes two proteins (MW 7.5 and 9.5 Kd) and mRNA D may encode three proteins (MW 6.7, 7.4, and 12.4 Kd). The 12.4 Kd protein has been detected in IBV infected chicken embryo kidney cells by immunoprecipitation and is thought to be virion associated (Liu et al., 1991). The other two open reading frames associated with mRNA D have been transcribed *in vitro* (Liu et al., 1991). The role(s) of these mRNAs or proteins is not known (Spaan et al., 1988).

Antisense oligonucleotides (anti-oligos) have been used to study gene function by blocking gene expression in eucaryotic and procaryotic systems (Stein and Cheng, 1993). Anti-oligos have been used to selectively inhibit DNA and RNA viral gene expression and replication (Agrawal, 1992). The technology provides an useful alternative to standard (homologous recombination) mutational analysis which is difficult with large RNA viruses like coronaviruses. Anti-oligos inhibit viral replication by blocking translation through binding to viral m-RNA complementary bases (Stephenson and Zamecnik, 1978; Vickers et al., 1991). Effective anti-oligos have been directed at the 5' non-coding translation initiation sites of several viruses (Gupta, 1987; K. C., 1987; LeMaitre et al., 1987; Offensperger et al., 1993; Storey et al., 1991). Alternatively, transcription may also be affected through base pairing with the genomic RNA serving as a template for the replication of virion RNA (LeMaitre et al., 1987). Specific antiviral activity has been reported for human and avian retroviruses (HIV, Rous sarcoma virus, HTLV III), herpesviruses (HSV), rhabdoviruses (VSV), influenza virus, and papilloma virus (Agrawal, 1992). The antiviral chemotherapeutic potential of anti-oligos *in vivo* was demonstrated in poultry using duck hepatitis virus (Offensperger et al., 1993).

The stability and efficiency of uptake of exogenously-applied anti-oligos by cultured cells has been studied. Phosphodiester oligodeoxyribonucleotides maybe degraded by nucleases

(Thierry and Dritshilo, 1992). Nuclease-resistant phosphorothioate and methylphosphonate analogs have been extensively used (Hoke et al., 1991;Storey et al. 1991). Concentrations of anti-oligos required for antiviral activity range from uM for phosphorothioate analogs (Vickers et al., 1991) to nM for poly-L-lysine-conjugated anti-oligos (LeMaitre et al., 1987). Uptake of oligomers fom 10-25 nucleotides in length may occur spontaneously or be facilitated by liposomes (Thierry and Dritshilo, 1992).

B. Infectious laryngotracheitis virus (ILTV)

Infectious laryngotracheitis virus (ILTV), an herpesvirus causes an acute respiratory disease (ILT) of world-wide importance to the poultry industry. Twice in the last ten years severe outbreaks of the disease have occurred on the Delmarva peninsula, causing severe financial losses to the poultry industry. In 1994/1995, the Delmarva peninsula and other areas of the southeastern United States experienced a serious epornitic of ILT. Approximately 370 cases were submitted to the University of Delaware's Poultry Diagnostic Laboratory from September 1994 through June of 1995. There were an additional 185 cases reported in Georgia in 1995. The disease was also reported in Alabama and Arkansas. Fortunately, ILT is a slowly spreading, controllable disease, which can cause high mortality and condemnation. Unfortunately, the presence of ILT prevent the export of U.S. poultry to some foreign markets.

There is considerable interest in developing safe and easily monitored ILTV vaccines. Vaccination 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. 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. It is thought that present live virus vaccines may mutate and become responsible for disease outbreaks. A series of reports by Guy et al. (1989, 1990, 1991) suggested that vaccine viruses played a role in outbreaks of ILT and that vaccine viruses increased in virulence after bird-to-bird passage. In contrast, another group (Hastings, 1991; Keeler et al., 1993) has proposed that ILTV vaccine strains are genetically stable and that the inability to differentiate vaccine strains from field isolates is due to the limitations of restriction fragment length polymorphisms (RFLPs) analysis.

Infectious laryngotracheitis was first described in 1925 (May and Tittsler, 1925). In its acute form, ILT is characterized by signs of respiratory distress in birds, accompanied by gasping and expectoration of bloody exudate (Hanson, 1991). In addition, the mucous membranes of the trachea become swollen and hemorrhagic. The epizootic form of the disease spreads rapidly and can affect up to 90-100% of an infected flock. Mortality generally averages between 10-20%. Milder forms of the disease are characterized by watery eyes, conjunctivitis, persistent nasal discharge and a reduction in egg production (Cover and Benton, 1958).

Several groups have tried to differentiate strains of ILTV on the basis of RFLPs. In the U. S., Andreasen et al. (1990), Guy et al. (1989), Keeler et al. (1993) and Keller et al. (1992) have reported that most field isolates of ILTV have restriction patterns which are identical or very similar to those observed for embryo-propagated ILTV vaccine strains. However, with the exception of Guy et al. (1989), the other three groups reported the presence of field isolates of ILTV that exhibited unique restriction patterns. The general similarity in ILTV DNA restriction patterns tends to confuse the issue regarding the source of virus resulting in field outbreaks of ILT.

The avian immune response to ILTV has been extensively studied. There is no evidence that neutralizing antibodies correlate with protection. A prominent role of cell-mediated immunity has been suggested. Chickens surgically bursectomized at one-day of age and subsequently treated with cyclophosphamide resisted virulent ILTV challenge without the presence of mucosal antibody (Fahey et al., 1983; Fahey and York, 1990). In addition, adoptive transfer of spleen cells from immune donors conferred protection (Fahey et al., 1984). Furthermore, recent experiments using inbred lines of chickens have also suggested an association of the chicken MHC with ILTV resistance (Loudovaris et al., 1991a; Loudovaris et al., 1991b). However, it is not known

whether protection is mediated directly by cytotoxic T-lymphocytes (CTL), by soluble factors released by activated T-cells, or a combination of the above. It is also not known what components of the mature virion are responsible for eliciting this protective immune reaction.

ILTV contains a linear, double-stranded DNA genome approximately 155 kilobase pairs (kb) in length (Bagust and Johnson, 1995; Kotiw et al., 1982; Johnson et al., 1991). ILTV exhibits the physical characteristics of class 2 herpesviruses such as pseudorabies virus (PRV) and equine herpesvirus 1 (EHV-1). In the last five years, a number of ILTV genes have been identified and sequenced. For a review see Bagust and Johnson, 1995. The University of Delaware laboratory has identified the thymidine kinase, glycoprotein B, glycoprotein C, and glycoprotein H gene homologues in the unique long region of the genome, a SORF3 gene homologue in the inverted repeat region, and nine unique short region genes (Keeler et al., 1991; Kingsley et al., 1994; Poulsen et al., 1991).

The host range of ILTV is limited to the chicken. Non-avian cell lines cannot be used to propagate the virus. ILTV grows in chicken embryos and chicken cell cultures. Fortunately, within the past few years, two groups have reported the ability to adapt ILTV to grow on continuous avian liver cell lines (Schnitzlein et al., 1994; Scholz et al., 1993).

C. Avian Influenza Virus (AIV)

Avian influenza (AI) is a disease associated with several clinical syndromes and infected chickens exhibit various clinical signs. AI virus (AIV) isolates can be categorized into 14 hemagglutinin (H1-14) and 9 neuraminidase (N1-9) subtypes (Easterday and Hinshaw, 1991; Kawaoka et al., 1990). In most birds species, especially free-living or captive wild bird species of the orders Anseriformes (migratory waterfowl) and Charadriformes (shorebirds), AIV infections are asymptomatic (Slemons et al., 1974; Stallknecht and Shane, 1988). Waterfowl and shore birds are considered to be primary reservoirs for AIVs. Recently, ratites have been shown to harbor AIV and could serve as a source of infection of commercial poultry. In poultry, AI is frequently subclinical, but can be associated with mild to moderately severe clinical disease syndromes such as respiratory disease, decreases in egg production and renal disease, especially in commercial poultry (Alexander and Gough, 1986; Alexander et al., 1981; Halvorsen et al., 1980; Johnson and Maxfield, 1976; Selleck et al., 1994; Skeeles et al., 1974). Typically, these diseases have been accompanied by low mortality and were self-limiting, but in some outbreaks, the mortality has been high, especially when accompanied by exacerbating factors such as concurrent infections, physiologic stresses or certain environmental factors (Newman et al., 1981).

Since 1950, 13 outbreaks of H5 or H7 AIVs have been associated with severe systemic disease in chickens and turkeys with accompanying high morbidity and mortality (Easterday and Hinshaw, 1991). These diseases were historically termed fowl plague, but the more accurate and current designation is highly pathogenic (HP) AI (Bankowski, 1981). Such viruses express high lethality in pathogenicity tests for chickens.

No avian or mammalian reservoir for HPAIVs has been identified. By contrast, epidemiologic and molecular genetic information suggests that HPAIVs arise via mutation of non-pathogenic (NP) or mildly pathogenic (MP) AIVs. For example, in the 1983-84 outbreak of H5N2 AIV in the northeastern U.S., the disease began as an acute respiratory syndrome associated with low mortality and reduced egg production (Eckroade and Silverman-Bachin, 1986). Initial virus isolates were categorized as NP AIV based on failure to kill 4-week-old chickens in laboratory pathogenicity tests. Six months later, the disease had changed character to become a severe systemic syndrome producing total cessation of egg production within a few days, 50-90% mortality and nervous signs in affected chickens. These new isolates were experimentally pathotyped as HP. Analysis of the hemagglutinin of AIVs from Pennsylvania identified the loss of a glycosylation site at amino acid residue 13 as being associated with a change from NP-MP to HP (Kawaoka et al.,1984). Subsequently, emergence of HP variants from AIVs of lower pathogenicity were derived following in vitro, in ovo, and/or in vivo laboratory passage (Brugh and Beck, 1992; Brugh and Perdue, 1991; Brugh and Perdue, 1993; Beck et al., 1995; Horimoto

and Kawaoka, 1995; Ohuchi et al., 1989; Perdue et al., 1995; Swayne et al., 1995). However, no systematic examination has been accomplished to determine if all NP/MP H5 and H7 AIVs have equal potential for emergence of HP AIVs.

The acquisition of high virulence for NP and MP H5 and H7 AIVs is most consistently related to molecular changes at the cleavage site of the hemagglutinin surface glycoprotein (Kawaoka and Webster, 1988). First, AIVs of low virulence have a maximum of two basic amino acids within the cleavage region. By contrast, AIVs of high virulence have a minimum of four basic amino acids out of five residues in the HA1 subunit immediately upstream from the cleavage site. The increase in basic amino acids at the cleavage site, either as substitutions or insertions, correlated with cleavability of hemagglutinin in trypsin-free CEF cultures and with expression of high lethality in chickens (Bosch et al., 1979). Second, the loss of a glycosylation site at amino acid 13, adjacent to the proteolytic cleavage site of the HA molecule allowed enhanced cleavability and increased virulence. This occurred with the shift in virulence during the 1983/84 AI outbreak in Pennsylvania poultry (Kawaoka et al., 1984).

In November 1993, respiratory problems of undetermined etiology were reported in broiler flocks in Mexico (Charon and Ramirez, 1995). In central Mexico in May 1994, H5 AIV was isolated from 6-week-old broilers exhibiting respiratory signs and 12-18% mortality (Charon and Ramirez, 1995). These viruses were categorized as NP AIVs based on chicken pathogenicity tests (Charon and Ramirez, 1995; Senne et al., 1995). In November 1994 in Puebla, Mexico, laying hens exhibited depression and torticollis (Salem, 1995) (M.V.Z.E. Rivera-Cruz, personal communication). Mortality rates (1.2-15.6%) in the field and following laboratory pathogenicity tests (J. Pearson and D. Senne, personal communication) were not consistent with HPAI. However, the amino acid sequence of the AIVs hemagglutinin cleavage site were compatible with HPAI (Garcia et al., 1995; Garcia et al., 1996) (J. Pearson and D. Senne, personal communication). In January 1995 in Queretaro, Mexico, H5N2 AIV was diagnosed in a broiler breeder operation with total cessation of egg production in 6 days and presence of gross lesions compatible with HPAI. Pathogenicity testing in chickens identified this virus as highly lethal and the amino acid sequence of AIV hemagglutinin cleavage site was compatible with HPAI. Furthermore, sequence homology of the HAI segment of various H5 AIVs indicated that the Mexican AIVs were most closely related to an H5 AIV isolated from a ruddy turnstone in Delaware in 1991 (Garcia et al., 1996; Horimoto et al., 1995). However, the origin of the AIV for the Mexican AI outbreak of commercial poultry is unknown. Possibilities include: 1) direct transmission from migratory bird populations, 2) maintenance of AIVs in backyard poultry, and 3) and transmission from a non-migratory wild bird population.

D. Mycoplasmas

Mycoplasmosis in commercially reared chickens is primarily caused by *M. gallisepticum* (MG) and *M. synoviae* (MS). The chronic respiratory disease, synovitis and sinusitis caused by these infections present serious economic problems to the poultry industry. Although these diseases do not necessarily account for high mortality, morbidity is high, and the damaging effects of disease are consequent to lowered egg production, retarded growth, poor carcass quality and predisposition to secondary viral and bacterial infections (Hopkins and Yoder, 1982; Springer, et al., 1974; Timms, 1972). Complicated infections can be severe with associated high mortality (Yoder, 1991).

MG and MS share similar epizootiology. Both are respiratory pathogens of chickens and turkeys (Yoder, 1991), although their host range is not limited to these avian species. MG has been isolated from natural outbreaks in pheasants, partridge, peafowl, quail, parrots, ducks, geese, and house finches. Likewise, MS has been isolated from outbreaks in guinea fowl, ducks, geese, pigeons, quail, and partridge (Kleven, 1991). When spread horizontally, they are transmitted by direct contact with infected poultry, via fomites, or by aerosols. Once respiratory epithelium is colonized by virulent strains, clinical signs include tracheal rales, nasal discharge and coughing. Additionally, MS causes infectious synovitis. Vertical transmission occurs when both MG and

MS infects chicken and turkey reproductive tracts with subsequent spread in hatchlings. Reproductive tract infections in laying chicken flocks can cause lowered egg production and economic loss.

For years the National Poultry Improvement Plan (NPIP) and the American Association of Avian Pathologists (AAAP) have recommended erradication of both MG and MS. Substantial progress has been made in elimination of these agents from primary breeding flocks. Strategies to reduce the adverse impact of MG and MS infection in commercial poultry include, 1) surveillance and eradication programs, 2) use of antimicrobials, and 3) vaccination (Yoder, 1991).

Persistent MG and MS reserviors threaten transmission to commercial mycoplasma-free flocks. They include the large multiple-age egg layer operations where MG is controlled by vaccination with live-attenuated vaccines. Although these immunizations may prevent disease manifestations, they do not prevent infection (Yoder, 1991). Live F strain MG vaccine has been used extensively in pullets to reduce egg production losses that otherwise occur when they are moved to multiple-age layer complexes which have endemic MG (Yoder, 1991). However, following experiental infection, the vaccinal MG F strain was pathogenic for turkeys (Yoder, 1991), and has been associated with MG outbreaks in meat and breeder turkeys under field conditions (Ley et al., 1993). Recently, MG strains 6/85 and ts-11 were produced commercially as live vaccines, and it was reported that both vaccines are poorly transmissible from bird to bird and possess little or no virulence for chickens and turkeys (Abd-el-Motelib and Kleven, 1993; Evans and Hafez, 1992; Whithear et al., 1990). It has not been determined if these live vaccines are virulent under field conditions, particularly when birds are exposed to immunosuppressive viral agents. Determining this necessitates a means to identify each vaccine strain.

Small "backyard" multiple species flocks have long been recognized sources of MG and MS. Today, in addition to these flocks, there is an increasing number of ratite species reared in a variety of conditions. Numerous ratite mycoplasma isolates have been made but are incompletely characterized. This source may present an additional threat for the U.S. poulty industry.

House finches with conjunctivitis leading to blindness and mortality have been reported since 1994 in many eastern states. Between June and September 1994, seven accessions from Virginia, North Carolina and Delaware representing 23 birds (21 house finches, 2 blue jays) were studied. Eleven MG isolations were made (Ley et al., in press). Isolates from the first two positive accessions were confirmed as MG by a commercially available PCR-based test kit (FlockChek™, IDEXX Laboratories, Westbrook, Maine). In addition, immunofluorescence assays identified the isolates as MG and not closely related species such as *M. imitans* (Bradbury et al., 1993). An additional PCR-based test indicated that these isolates were not the MG vaccinal F strain. The discovery of MG in house finches presents a new facet to the epizootiology of the disease in poultry and possibly susceptible wild bird populations.

The ability to identify and distinguish current and emerging strains of avian Mycoplasma species is critical to our understanding of the epizootiology of the diseases they cause and effectiveness of interventions such as vaccination. This understanding is required to reduce losses resulting from these diseases.

Diagnosis of mycoplasma infections, particularly MG and MS infections, has had a troubling history. Currently there are serological techniques (hemagglutination inhibition, serum plate agglutination and ELISA) available to detect MG. These tests cannot differentiate between vaccines or field strains. Isolation of mycoplasma from infected flocks and its species identification is very time consuming, taking several weeks. The isolation and speciation of the mycoplasma does not give information on its potential source. For instance are the isolates from a live vaccine or wild bird? Rapid and accurate adjuncts to traditional cultural identication are needed.

With the application of molecular genetic technology, new methods have been developed such as DNA fingerprinting (Kleven, et al., 1988; Morrow et al., 1990), hybridization with species and/or strain-specific mycoplasma DNA (Geary et al., 1988; Hyman et al., 1989; Krause et al., 1990; Santaha et al., 1987; Zhao and Yamamoto, 1990), and use of ribosomal RNA gene probes (Yogev, 1988). DNA probes have been shown to be highly specific for detection of MG and MS (Khan et al., 1987, 1989; Zhoa and Yamamoto, 1990). Yet the stringent criteria for

hybridization (Huynh et al., 1985; Nauschurtz et al., 1990; Santha et al., 1987), the requirement for an optimal concentration of organisms (Hyman et al., 1989), and the intricacies of the technology, have not simplified or improved routine laboratory diagnosis of mycoplasmal infections.

Newly developed polymerase chain reaction (PCR) technology has been applied to infectious disease diagnosis of many agents. The assays are rapid, sensitive and specific. These assays build on the information provided by the nucleic acid sequence of defined MG and MS genes. Southern blots and RFLP can differentiate between vaccine strains (Lauerman et al., 1995). These assays are not practical for a diagnostic lab. The arbitrary primed-PCR or random amplified polymorphic DNA (RAPD) analysis (Fan et al., 1994), can also differentiate between the three MG live vaccines. The use of DNA probes in conjunction with PCR is more sensitive than other molecular tests. The IDEXX F-Strain PCR probe assay (IDEXX Laboratories, Inc, Westbrook, ME) can specifically identify F strain. The development of probes specific to ts-ll and 6/85 will allow rapid identification of these two vaccines. Uemori et al. (1992) were the first to develop nested PCR assays to detect pathogenic mycoplasmas in humans and animals. In their study, a nested set of primers was used to amplify an intergenic region between the 16 and 23S ribosomal structural genes. The first set of primers were designed to amplify a conserved sequence within the structural genes of 12 mycoplasma species. A second set of primers amplified the intergenic region itself. Later research showed that these same two primer sets could be used to specifically amplify contaminant mycoplasmas in animal cell cultures (Harasawa et al., 1993). Using this technique, the authors observed that along with variations in length, nucleotide sequences of the spacer regions varied among mycoplasma species. They concluded that this might be useful in species identification (Uemori et al., 1992). This PCR technique has been applied to pathogenic avian mycoplasmas.

The RAPD technique amplifies random DNA sequences using short primers in the PCR. The products of amplification are visualized directly in a gel after electrophoretic separation of fragments and staining with ethidium bromide. RAPD analysis is regarded as a time-saving, cost-effective method for studies of genetic diversity, genetic relationships, genetic mapping, DNA fingerprinting and population genetics (Van Buren et al., 1994; Vierling and Nguyen, 1992). Fan et al. (in press) have developed RAPD primers capable of distinguishing among 25 MG strains. Using a slightly modified procedure, others confirmed the utility of RAPD analysis for MG strain identification (Ley et al., in press). Specifically, the RAPD technique can distinguish among the reference strain R and the vaccine strains F, 6/85 and ts-11.

In summary, there is a need to apply these PCR assays in routine diagnostics in veterinary diagnostic laboratories to confirm and establish their efficacy and efficiency using clinical specimens.

Results of conventional serological tests such as the serum plate agglutination (SPA) test, the hemagglutination inhibition test, and the enzyme-linked immunosorbent assay (ELISA) have frequently been confounded because of cross reactions between shared MG and MS epitopes (Avakian et al, 1988; Bradbury and Kleven, 1987; Kleven, 1975; Olson et al., 1965; Opitz and Cyr, 1985, Vardaman and Yoder, 1971). Recent studies have demonstrated a genetic and antigenic relationship between MG and MS (Nauschuetz et al., 1990; Yogev et al., 1989), thus confirming the presence of epitopes common to both species and thereby the difficulty in distinguishing MG from MS. Spurious serological reactions resulting from vaccination of poultry with mycoplasma-unrelated bacterins and the presence of antibodies to such agents as *Staphylococcus aureus* and *Streptococcus faecalis* have been cited as factors that frequently complicate the interpretation of test results (Avakian et al., 1988). A number of other factors have been implicated as likely sources of erroneous test results e.g. presence of undesirable contaminating media components in mycoplasma test antigens (Kleven, 1975; Opitz and Cry, 1985; Vardaman and Yoder, 1971; Yogev et al., 1988) and the presence of antibodies to serum components in poultry vaccines (Ahmad et al., 1988; Avakian, 1988; Hopkins and Yoder, 1982; Ross et al., 1990; Springer et al., 1974; Timms, 1972).

The consensus of most researchers is that the success of any serodiagnostic test for MG detection will rest on the availability of a reliable antigen (Avakian et al., 1988; Bradbury, 1988;

Kleven et al., 1988; Opitz and Cyr, 1985; Talkington et al., 1985). This is a high priority, since serological survellience is the cornerstone of eradication programs.

A monoclonal antibody (MAb M9) identified a MG agglutinating antigen. This antigenis a potential test antigen. There is observed antigenic variability, both between different strains and among variants of a single species (Kleven et al., 1988; Panangala et al., in press, Rosengarten and Wise, 1990; Thomas and Sharp, 1988). Amid this surface variability, there are some antigenic determinants that are conserved among all mycoplasmas of a given species (Bradly et al., 1988; Hwang et al., 1989; Kenny, 1978; Nichols and Kenny, 1984), thus enabling serological characterization of mycoplasmas into serovars (ICSB, 1979; Khanna et al., 1989; USDA, 1985). One antigen detected in MG is highly conserved in all strains (Morsy et al., 1991; Panangala et al., in press). To date, isolates from different geographic locations in the U.S. react with Mab M9 in Western blot procedures suggesting the agglutinating antigen's conservation among all MG strains.

A wide array of antigenic mycoplasmal proteins have been successfully cloned and expressed as recombinant proteins (Riethman et al., 1987; Stevens and Krause, 1990; Strasser et al., 1991; Su et al., 1988; Taylor et al., 1984). One troubling aspect of recombinant mycoplasma protein expression is that mycoplasma species have a nonstandard genetic code; tryptophan is specified by the codon UGA , the stop codon in all other organisms (Dybvig, 1990).

Despite the tryptophan codon problem, a number of researchers have successfully cloned and expressed mycoplasmal genes (Geary et al., 1988; Khan et al., 1987; 1989; Zhao and Yamamoto, 1989). In a recent study, a genomic fragment coding for the amphiphilic surface lipoprotein p65 of *M. hyopneumoniae* was successfully cloned and expressed in E. *coli* (Kim et al., 1990). A 19 kDa recombinant protein corresponding to the carboxy-terminal region of p65 was recognized by both monoclonal and monospecific polyclonal antibody to the gel-purified p65. Serum from swine, obtained after experimental M *hyopneumoniae* infection preferentially recognized the native amphiphilic p65 lipoprotein and also bound to the recombinant product (Kim et al., 1990). Similar studies with MG have revealed the presence of specific antigens cloned and expressed in E. *coli* that were recognized by serum from MG -infected chickens (Krause et al., 1990). Precise analysis of many antigen-coding genes that signal events in mycoplasmal pathogenesis have been made possible with innovative molecular biologic procedures (Feldmann et al., 1992; Jacobs et al., 1988; 1990; Lai et al., 1990).

Recently a two candidates for test antigens have been studied. The putative MG cytadhesin (attachment) gene, *mgc1* , has been cloned and sequenced (Dohms et al., 1992; Keeler et al., 1995). This gene shows 29% amino acid (aa) homology with the major P1 cytadhesin of the human respiratory pathogen *M. pneumoniae*. In addition, the two genes flanking *mgc1* have been sequenced. The upstream gene, *mgc2* shows 41.6% amino acid homology to *M. pneumoniae* cytadhesin p30 (Hnatow et al., 1995). Sequence of the gene downstream from *mgc1* is near completion, and it shows 27% aa homology with *M. pneumoniae* ORF5, an cytadhesin-accessory protein encoded in the P1 cytadhesin operon. Portions of MGC1 have been expressed as peptides. Specific polyclonal antibodies have been prepared against MGC1 and MGC2 using peptide immunogens. Using these antibodies, MGC1 was found to be trypsin sensitive and surface exposed on the MG cell.

E. Infectious bursal disease virus (IBDV)

Infectious bursal disease (IBD) has been observed in chickens since 1957 (Cosgrove, 1962). This immunosuppressive disease of young chickens is enzootic in many areas of the U.S. (Faragher, 1972; Winterfield, 1969). Chicks infected during the first week of life are permanently immunosuppressed, are more susceptible to pathogenic microbial agents (Fadly et al., 1976; Giambrone et al., 1977; Lucio and Hitchner, 1980; Rosenberger and Gelb, 1978) and do not respond adequately to vaccinations (Allan et al, 1972; Faragher et al., 1974; Giambrone et al., 1977; Winterfield et al., 1978).

The bursa of Fabricius (BF) is the primary target organ of IBDV. IBDV replicates in the immature bursa-derived lymphocytes (B-lymphocytes) of the chicken (Hirai and Calnek, 1979;

Ivanyi, 1975; Yamaguchi et al., 1981). Birds infected with IBDV at one-day of age are completely devoid of serum immunoglobulin G (IgG) and produce only a monomeric IgM (Ivanyi and Morris, 1976). A permanent decrease in the number of peripheral blood B cells was observed following IBDV infection (Hirai et al., 1979). IBDV has been observed in association with T-lymphocytes (Kauier and Weiss, 1980, Sivanandan and Maheswaran, 1980, Winterfield et al., 1972) but its effect on these cells has not been elucidated. Studies on turkey poults indicated that IBDV infects cells in the BF epithelium at the apex of the plica (Nusbaum et al., 1988). The virus was not observed in cells within the BF follicle. This study suggests that in the turkey, IBDV infects epithelial cells and not lymphocytes; which is consistent with the lack of immunosuppression observed in turkeys (Jackwood et al., 1982; Jackwood et al., 1984). The nonenveloped IBDV virion displays icosahedral symmetry and is approximately 58-60 nm in diameter (Becht, 1981). The genome of IBDV consists of two segments of double-stranded RNA (MacDonald, 1980, Muller et al., 1979; Steger et al., 1980). Molecular cloning and *in vitro* translation studies (Hudson et al., 1986) have shown that each RNA segment encodes a single mRNA. The larger mRNA codes for a polyprotein which is cleaved following translation into the maior structural proteins of the virus. The smaller mRNA encodes an RNA polymerase.

Birnaviruses have at least four structural proteins. The designation and molecular weights of the structural proteins observed in IBDV are: VP1 (90,000), VP2 (41,000), VP3 (35,000), and VP4 (28,000) (Becht, 1981; Dobos, 1979; Dobos et al., 1979). Additional proteins have been identified (Becht, 1981; Dobos, 1979; Nick, et al. 1976; Todd and McNulty, 1979). It was suggested that they have a precursor-product relationship with the four structural proteins observed in infectious virions (Muller and Becht, 1982). Molecular studies on the largest RNA segment support this hypothesis (Hudson et al., 1986).

Initially IBDV was thought to be a disease only of chickens. However a second serotype of IBDV, isolated from turkeys, (Jackwood et al., 1982; Jackwood et al., 1984; Lukert et al., 1981, McFerran et al., 1980; McNulty et al., 1979) was determined to be a different serotype. Chicken-derived IBDV were designated serotype 1 and the turkey-derived IBDV were termed serotype 2. The serotypes were designated based on antigenic differences observed in virus-neutalization and cross-challenge tests (Jackwood et al., 1982). At least one shared antigen exists among serotype 1 and 2 IBDVs viruses and can be demonstrated by immunofluorescence. Both serotypes are present in commercially-reared chickens but only serotype 1 viruses cause disease (Ismail et al., 1988; Jackwood et al., 1982; Jackwood and Saif, 1983; Jackwood et al., 1984; Jackwood et al., 1985). Studies have shown antigenic subtypes (classical and variant) exist within serotype 1 IBDV (Ismail et al., 1990; Jackwood and Saif, 1987; Rosenberger and Cloud, 1985; 1986; Rosenberger et al., 1987; Saif, 1984;1987). Vaccination of chicks with one of these subtypes does not insure birds will be protected from infection and disease from another serotype 1 subtype (Rosenberger and Cloud, 1985; Saif, 1984). Ismail and Saif (1991) demonstrated that vaccination with one serotype 1 subtype did not always protect chickens from challenge with another serotype 1 subtype particularly if a vaccine dose, containing low virus titers, was used. The some vaccination programs to protect against IBD may be due to differences between antigenic subtypes of serotype 1 IBDV (Ismail and Saif, 1991; Jackwood and Saif, 1987; McFerran et al., 1980; Saif et al., 1987).

The distribution of serotype 1 IBDV strains IM and 2512 in chickens has been described (Kaufer and Weiss, 1980; Sivanandan and Maheswaran, 1980; Winterfield et al., 1972). The BF and spleen contained the highest quantities of virus as determined by titration on the chorioallantoic membrane of embryonated eggs (Winterfield et al., 1972). Other tissues which contained virus were the blood, liver, kidney, lung, and thymus. The CU-1 IBDV strain (serotype 1) was at its highest titer in the chicken bursa and spleen at 2 days post-inoculation (Kauser and Weiss, 1980). This virus also spread to the thymus, and liver. Six days following inoculation, IBDV could not be detected in these tissues using a virus plaque assay.

Passage of IBDV in chick-embryo-fibroblast (CEF) cells changed both the biologic properties and structural proteins of the virus (Muller et al., 1986). When virus was passaged at a high multiplicity of infection (MOI), a small plaque variant was observed and at a low MOI, a large plaque variant was observed. The small and large plaque variant populations were stable after the

ninth passage in CEF cells. The small plaque morphology remained stable even when the virus was further passaged at a low MOI. Muskett et al. (1985) also reported on the emergence of small plaque variants. Recently, Saijo et al. (1990) plaque purified the small and large plaque variants and observed stable populations through 10 passages in CEF cells. Lange et al. (1987) also observed stable large and small plaque morphologies. They reported differences in structural proteins between a population of viruses grown in bursa tissue compared to the small and large plaque viruses grown in CEF cells. It was speculated that cellular factors such as proteases were important in the maturation of IBDV particles and differences in the micro-environment among different host cells could account for the development of defective virus particles. The antigenicity of the small and large plaque viruses was reported to be similar (Saijo et al., 1990). These studies were conducted using a VN assay. The pathogenicity and immunogenicity of two antigenic variant IBDV strains was studied following 30-40 passages in BGM-70 cells (Tsai and Saif, 1992). Although both viruses lost pathogenicity, their immunogenicity was not altered. Since no attempt was made to separate large and small plaque viruses both plaque sizes were present in the population of virus used for these vaccination /challenge studies. Saijo et al. (1990) reported that both plaque sizes retained immunogenicity in chickens but the large plaque virus had a higher potency compared to the small plaque virus. The large and small plaque viruses were less pathogenic for chicks compared to the wild type virus grown in bursa tissue (Saijo et al., 1990) and the small plaque virus was more attenuated than the large plaque virus (Lang et al., 1987; Saijo et al., 1990).

Detection of IBDV antibodies has been accomplished using a number of different assays. ELISA is often used because it is economical and can accomodate large numbers of samples. Many improvements have been made to ELISA first described by Marquardt et al. (1980). Case et al. (1983) optomized the test using purified antigen. Another modification of the IBDV ELISA used a linear regression equation to predict the ELISA titer of a serum sample from a single dilution (Briggs et al., 1986; Snyder et al., 1986; Snyder et al., 1984). Thayer et al. (1987) reported a correlation between serum ELISA antibody titers and the virus-neutralization (VN) assay for IBDV. Since the development of commercially available ELISA kits for IBDV antibodies, little improvement of this technology has been reported.

Commercially available IBDV ELISA kits detect antibodies to both serotypes 1 and 2 (Ismail and Saif, 1990). Identification of antibodies to different antigenic subtypes of IBDV is currently only possible using the VN assay (Jackwood and Saif, 1987; McFerran et al., 1980). ELISA systems employ whole virus particles as antigen. One approach to the development of a more specific assay includes expression the IBDV structural protein VP2 for use as an ELISA antigen. Oppling et al. (1991) reported that VP2 contains at least three independent epitopes. Using monoclonal antibodies, Whetzel and Jackwood (1995) showed that differences in neutralizing epitopes on VP2 exist between variant and classic viruses. These results suggest that it may be possible to express IBDV epitopes which will only bind antibodies to specific subtypes of IBDV. Several expression systems have been utilized to produce IBDV proteins. Azad et al. (1987) expressed VP2 in *Escherichia coli.* A recombinant fowlpox virus expressing VP2 protected birds challenged with IBDV against mortality, but not against damage to the bursa (Bayliss et al., 1991). Vaccination of specific-pathogen-free (SPF) hens with a VP2 fusion protein produced in yeast provided passive (maternal antibodies) protection to IBDV challenged progeny (Fahey et al., 1991). IBDV proteins have also been expressed using the baculovirus system (Oppling et al., 1991; Vakharia et al., 1993). Proteins expressed in baculovirus have been produced in amounts of 1 to 500 mg/ml (Luckow and Summers, 1988a; Luckow and Summers, 1988b; Niikura et al., 1991; Smith et al., 1983). There are no reports of using these expression products as antigen in an ELISA.

OBJECTIVES

1. Develop and evaluate rapid diagnostic capabilities for the identification of emerging IBV, ILTV, mycoplasmas, and IBDV.

2. Characterize immunosuppressive viruses (IBDV) and assess the interaction of underlying immunosuppressive agents (IBDV and CAV) on emerging respiratory agents.

3. Delineate epizootiology of emerging mycoplasma and the viruses that cause infectious bronchitis, avian influenza, and infectious laryngotracheitis.

4. Design and implement novel immune and genetic prophylactic strategies for effective control of respiratory diseases caused by emerging IBV, ILTV, mycoplasmas, and IBDV and CAV.

PROCEDURES

Objective 1. Develop and evaluate rapid diagnostic capabilities for the identification of emerging IBV, ILTV, mycoplasmas, and IBDV.

A. Infectious bronchitis virus (IBV)

Delaware, Georgia and **Connecticut** will cooperate in the development and evaluation of direct automated sequencing for identifying field isolates. **Delaware, Georgia,** and **Connecticut** will design primers that anneal to conserved sequences flanking hypervariable regions in the S1 gene. Primer sequences will be evaluated for their ability to anneal all IBV serotypes. For the purposes of designing improved primers, **Delaware** and **Georgia** will share unpublished IBV S1 sequence data for the Delaware and California variant serotypes. The primers ability to amplify an unknown (blind) panel of diverse IBV serotypes will be determined. The PCR products will be directly sequenced using a sequencer to verify the identity of the IBV serotype. This method for identifying IBV serotypes will be an improvement over exisiting approaches because a new or previously unrecognized serotype can be immediately identified and compared to recognized serotypes.

Connecticut will construct an IBV genomic cDNA library as a basis for development of group and strain-specific probes and RT-PCR techniques. IBV strains Massachusetts (Mass 41), Connecticut (Conn 46), Arkansas (Ark 99), and Delaware variant (072) will be initially grown in chicken embryo kidney cells (CEK), plaque-purified and characterized by oligonucleotide fingerprinting. The viruses will then be grown in 10-to-11-day-old chicken embryos, genomic viral RNA isolated by standard procedures, copied into cDNA by reverse transcription and cloned into the plasmid pUC 18 (Sambrook et al., 1989). The screening and identification of IBV specific segments of the cDNA will be done by cross-hybridization (DNA:RNA) against a panel of IBV field, and variant strains, and other avian pathogenic viruses of Newcastle disease, avian influenza, infectious laryngotracheitis, reovirus, fowlpox and infectious bursal disease. Clones containing unique sequences will be selected as strain-specific probes. Sequence analysis of the cDNA fragments will be performed (Sambrook et al., 1989) in order to identify DNA primers specific for strains of IBV. Efficacy of the cDNA probes as well as RT-PCR's will be tested for their sensitivities and specificities in experimental infections. Eight-week-old SPF chickens will be inoculated intratracheally with IBV strains, Mass, Conn, Ark, and Delaware variant. Fecal swabs will be taken for 8 weeks to monitor IBV infection using nucleic acid hybridization (labeled cDNA probes) and by standard virus isolation procedures (Lukert, 1980). Blood samples will be taken at the same intervals for serologic monitoring using HI, VN, and ELISA (King, 1988; Lukert, 1980). Isolates of IBV from various geographic locations of the U. S. will also be provided by **Delaware**

and **Georgia**. After a successful identification of cDNA probes and RT-PCR primers, additional IBV isolates from field outbreaks and their suspected source of infection will be studied on a comparative basis.

Georgia will improve the RT PCR RFLP test by designing better primers. The procedures for the rapid serotype identification of IBV using RT PCR RFLP have been published (Andresen et al., 1991; Jackwood et al., 1992; Kwon et al. 1993). **Georgia** and **Delaware** will attempt to streamline RT PCR procedures by modifying the IBV RNA extraction and PCR methods so that samples taken from the field can be examined directly without the necessity for embryo passage.

Delaware will characterize IBV antigenic determinants. This project will identify serotype-specific peptides for diagnostic purposes. Peptides common to several IBV serotypes will be evaluated as novel vaccines (see Objective 4). A random hexapeptide library constructed in the phagemid, *fd-tet* (Parmley and Smith, 1988; Scott and Smith, 1990), will be used as a source for the IBV epitopes. This peptide library is unique, such that the hexapeptides are expressed as fusion proteins with the minor coat protein, pIII on the surface of the filamentous phage, fd. The recombinant phage library was amplified in the host *Escherichia coli* strain, *K91Kan* . The titer of the genomic library was 3.7×10^{11} transductants/ml. To elucidate the epitopes of IBV, various strain-specific chicken polyclonal antisera will be used (at least 2 antisera/IBV strain). Through the process termed "panning", phage which bind to the antibodies and therefore, express specific IBV-specific peptides will be separated from unbound phage. The bound phage will then be eluted, amplified in *K91Kan* host cells and subjected to two more rounds of panning. After the third round of panning, phage will be mixed with *K91Kan* host cells and then plated at low density on medium containing tetracycline to allow growth of transductants. Phagemid DNA from these recombinant bacterial colonies will then be prepared for sequencing. It is expected that at least 50 recombinant clones per antiserum will be sequenced for determination of the consensus peptide sequences (epitopes), as described previously (Cwirla *et al.*, 1990). Phage which bind to IBV-specific antibodies and contain unique peptide sequences will then be tested for their ability to inhibit virus neutralization in homologous assays. The specificity of peptides will also be tested in heterologous neutralization assays.

B. Infectious laryngotracheitis virus

Delaware has identified and sequenced a number of ILTV genes. Several of these genes are structural glycoproteins (gB, gC, gD, gE, gI, gH, gX, gp60, gp67). Efforts will continue to characterize the proteins encoded by these genes. The development of specific immunologic reagents will be used to characterize ILTV glycoprotein maturation and processing. These reagents will also be used to develop specific ELISA assays to monitor ILTV infections in the field.

In addition, a genetically-tagged, live attenuated vaccine strain of ILTV will be constructed by deleting a non-essential glycoprotein gene. Without the gene, birds infected with the mutant virus will not develop antibodies to the corresponding protein. This property can be used to subsequently differentiate the vaccine virus from a field isolate. Methods for constructing mutant strains of ILTV have been developed and published (Guo et al., 1994; Okamura et al., 1994; Schnitzlein et al., 1995). The ILTV gX gene will be targeted for disruption. The gX gene is nonessential and is not involved in the pathogenicity of other herpesviruses. Consequently, its removal should not effect the efficacy of a vaccine strain. The gene will be disrupted by deleting a portion of the coding sequence and replacing it with a gpt-lacZ gene cassette. The resulting plasmid will be co-transfected with ILTV viral DNA into a chicken liver cell line and recombinants will be identified by their ability to make beta-galactosidase and their lack of gX antigen.