C. Mycoplasma

Georgia will develop specific probes for differentiating live MG vaccines from field strains of MG. The probes will consist of a regions complementary to specific sequence within the intergenic region of the individual MG vaccines. The intergenic region between the 16 and 23S ribosomal RNA genes will be sequenced and differences reflecting intraspecies heterogeneity will be used to develop DNA probes that will allow rapid MG identification and differentiation. Intergenic regions of the DNA will be amplified using the PCR technique of Uemori et al. (1992) using the first set of primers (F1 and R1). The purified PCR product will be used in a second amplification using the nested primers. These products will be sequenced and the resulting data analyzed. Subsequent sequencing will be performed using internal primers designed from previously determined sequence. A search for restriction sites within the intergenic regions will be performed. Probes will be designed using results of the sequencing, labeled and used to visualize specific MG strains.

Alabama will develop a simple and specific test for MG diagnosis by cloning and expressing the agglutinating antigen gene. The gene will be identified and characterized. This will be accomplished by constructing an MG (strain PG31) DNA expression library in lambda gt11 and screening it with monoclonal antibodies (Snyder et al., 1987). **Delaware** will provide **Alabama** with an lambda gt11 MG S6 genomic library. The libraries will be screened with MAb M9 which recognizes the MG agglutinating antigen(s) by standard methods (Sambrook et al., 1989). The nucleotide sequence of the identified gene will be determined and the predicted amino acid sequence compared with amino acid sequences of other proteins available in the GenPept and Swiss-Prot databases. This information will be useful in suggesting the function of the agglutinating antigen. The nonstandard genetic code of mycoplasma species (Dybvig, 1990), poses a potential problem for screening expression libraries in *E. coli.* To minimize the effect of UGA tryptophan codons, screening of the expression library will be conducted in host *E. coli* strain Y1090-Su7UGA. A hybrid gene will be constucted to produce high levels of the entire or portions of the MG agglutinating antigen recognized by the MAb M9. The entire agglutinating antigen and the fragment encoding the M9 epitope will be separately cloned and expressed in an *E. coli* expression plasmid. Lysates of cells expressing the recombinant agglutinating antigens will be confirmed by Western blot using the MAb M9. Following purification, the MG agglutinating antigen will be utilized in the development of an ELISA and serum plate latex agglutination (SPLA) assay for routine laboratory MG diagnosis.

The SPLA procedure, previously designed for use with recombinant envelope polypeptide for detection of antibodies to HIV (Quinn et al., 1988), will be modified. The specificity, sensitivity, and stability of the assay reagents will be assessed. Efficacy of ELISA and SPLA assays will use sera specific for different mycoplasma (MG PG 31, MGR strain, MS *WVU* 1853, *M. gallinarum* Ala 22 isolate, *M. gallinaceum* Ala 6 , *M. iowae* strain 695) Serum from mycoplasma infected or suspect flocks will be obtained through the Alabama State Veterinary Diagnostic Laboratory. Tracheal swabs taken randomly from the corresponding flocks will be cultured and examined for the presence of mycoplasmas and select isolates will be speciated.

Alabama (Veterinary Diagnostic Laboratory) will obtain unidentified mycoplasma isolates from various diagnostic laboratories throughout the U.S., including **Alabama** (Dept. of Pathobiology), **Georgia, North Carolina** and **Connecticut.** The isolates will be cloned three times to assure purity. Each isolate will be initially tested with avian species-specific PCR assays (Boyle, et al., 1995; Kempf, et al., 1993; Lauerman et al., 1993, LHL and VLvS unpublished primers). Isolates that remain unidentified will be evaluated using PCR-restriction fragment length polymorphism (RFLP) analysis and grouped (Lauerman et al., 1995). The 16S rRNA gene will be sequenced from a representative in each group and the sequence information used to select species-specific PCR primers using sequence alignment. Other standard diagnostic techniques, such as biochemical tests (Goll, 1994), immunoassays (Goll, 1994; Imada et al., 1987), and immunoglobulin receptors (Lauerman and Reynolds-Vaughn, 1991, Lauerman et al., 1993) will be

utilized for further characterization. General mycoplasma PCR primers will be used to generate amplicon (DNA amplification product) from the down stream end of the 16S rRNA gene through the spacer region to the upstream end of the 23S rRNA gene of unidentified isolates. The PCR amplicons will be reacted with four restriction enzymes (*Mse*I, *Tsp*509I, *Dra*I, and *Rsa*I), and the electrophoretic patterns of RFLP evaluated (Lauerman, et al., 1995). Amplified DNA fragments will be isolated and the 16S rRNA sequence determined. For fragments longer than 500-600 bp, additional primers, derived from internal sequence will be used to complete the entire fragment. (Weisburg et al., 1989). Many mycoplasmal 16S rRNA sequences have been determined and provide the basis for a systematic phylogenetic analysis of these microorganisms (Weisburg et al., 1989). Species-specific PCR primers will be selected from 16S rRNA gene sequence information using computer alignment studies (Lauerman et al., 1993; LHL and VLvS, unpublished data). The 16S rRNA gene sequence information on various mycoplasmas will be obtained. The suitability of selected PCR primers will be determined by computerized sequence alignment studies. In addition, PCR assays with stock cultures of known avian mycoplasmas will be conducted.

The pathogenicity of selected mycoplasma isolates will be assessed in one-day-old SPF chickens inoculated by the footpad or ocular routes. Chickens will be observed for clinical signs, euthanatized and necropsied at 7, 14, and 28 days postinoculation. Specimens will be collected for histopathology, mycoplasma culture, PCR assays, and serological evaluation.

North Carolina will develop a database for avian Mycoplasma species and strain identification by RAPD analysis. The RAPD technique described by Fan et al. (Avian Dis., In press), will be modified as required to optimize all performance parameters, especially reproducibility. The ability of an optimized RAPD to distinguish among MG strains will be validated using known reference and vaccine strains. MG isolates from poultry and other birds e.g. house finches made by our laboratory and acquired from collaborators will be analyzed by RAPD. RAPD patterns will be photographed and the images digitized using a scanner, or digital images may be captured directly using a digital camera. A computer-assisted gel analysis system such as Dendron (Solltech Inc., Oakdale, IA) will be used to analyze RAPDs and build a database of MG strain identities. Briefly, the gel analysis system will correct, process and analyze RAPD gels in order to compare banding patterns. Similarity coefficients are computed between banding patterns and a dendrogram is generated. Results will be stored in a database which provides the capacity to retrospectively compare the fingerprint of every new strain with any or all strains previously analyzed.

An attempt will be made to finalize and standardize RAPD methodology and select a gel analysis system with input from collaborators at **Georgia**. Ideally, a uniform standard might emerge that could be widely utilized and whose data could be shared regionally, nationally and internationally via the Internet.

Initially, these efforts will focus on identification of MG vaccine strains and the molecular epidemiology of the MG outbreak in house finches. However, this capability will be readily expanded to impact on other aspects of MG disease, and then extended to other pathogenic avian Mycoplasma species (MS, *M. meleagridis, M. iowae*). **North Carolina** will exchange avian mycoplasma isolates with **Alabama** for the purposes of developing a database for avian Mycoplasma species and strain RAPD identification.

E. Infectious bursal disease virus (IBDV)

It is important to define the effects of host systems on antigenic configuration since this could have an enormous impact on any diagnostic reagents prepared from virus propagated in a fashion that would preferentially select for particular epitopes or other in vitro or in vivo characteristics. **Delaware** will assess the effects of host system(s) (bursa of Fabricius, embryo or cell culture) on the virulence and antigenic configuration of several standard- and variant-type infectious bursal disease viruses. Infectious bursal disease virus propagated in the bursa of Fabricius and adapted to grow in embryos and/or chicken embryo fibroblasts will be monitored for

changes in relative pathogenicity or shifts in antigenicity as evaluated with monoclonal and polyclonal antibodies. Currently-available vaccine strains and field isolates will be compared to one another and, in the case of the vaccine strains, to the "parent" virus when possible.

Ohio will concentrate on the molecular characterization and comparison of IBDV serotypes. IBDV strains will be cloned and the VP2 gene sequenced (Jackwood et al., 1989; Kibenge et al., 1990) to develop rapid diagnostic assays. These tests will employ specific cDNA probes in a dot blot hybridization test (Henderson and Jackwood, 1990; Jackwood, 1990; Jackwood et al., 1989; Jackwood et al. 1990), a reverse transcriptase polymerase chain reaction -restriction endonuclease (RT/PCR-RE) assay (Jackwood and Jackwood, 1994) (Henderson and Jackwood, 1990; Jackwood, 1990; Jackwood et al., 1989; Jackwood et al. 1990). The VP2 protein from the Del-A strain IBDV will be expressed in the baculovirus expression system (Henderson et al., 1992) for the purposes of developing a highly strain-specific enzyme-linked immunosorbent assay, the OSU-ELISA. Recombinant viruses expressing the VP2 protein from the Del-A strain of IBDV will be tested for specific neutralizing epitopes using defined monoclonal antibodies. The OSU-ELISA differs from commercial ELISA kits because it iws strain specific. **Alabama** (Veterinary Diagnostic Lab) will cooperate with **Ohio** in the evaluation of molecular diagnostic assays.

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

Ohio will import highly virulent strains of IBDV from The Netherlands, Turkey, and Taiwan. These strains have caused major epidemics in the old world in the last seven years but have not been detected in the U.S. The strains will be adapted to tissue culture and used as inactivated preparations to produce antibodies in chickens. Ohio's USDA importation permit limits use of live virus to *in vitro* study. Sera from immunized birds will be used to conduct *in vitro* cross neutralization studies. Chickens will immunized with the inactivated IBDV strains and will be challenged with American strains (classic and variant) to study the cross-protective characteristics of these viruses. Results from the above studies should provide a rational basis for prevention of these infections should they appear in the U.S. at some later time.

Delaware will evaluate the impact of virulent, intermediate and avirulent IBDVs on susceptibility to chicken anemia virus. Once defined, chickens that are potentially compromised immunologically by the dual infection(s) will be exposed to different pathotypes of infectious bronchitis virus (IBV) to assess the impact of relative immunosuppression on the emergence of increased susceptibility to IBV in vaccinated and unvaccinated birds. Results from these assessments will better define the role of common immunosuppressive agents, including IBDV vaccines, on the emergence of respiratory pathogens such as IBV.

Delaware will assess the impact of IBDV infections on the immune responses to two putative cytadhesins, MGC1 and MGC2, found surface exposed on MG (Dohms et al., 1992, Keeler et al., 1996). These proteins will be evaluated as vaccines in Objective 4. Inoculation of susceptible SPF chickens with IBDV given at hatching and three-weeks of age will use the variant A strain IBDV . At 4,6, and 8 weeks of age IBDV-infected and IBDV-noninfected controls will be inoculated with MGC1 and MGC2, recombinant peptides. At weekly intervals for 6-weeks post inoculation, the chickens will be bled and Harderian glands removed. The onset of local (GH extracts) and systemic antibody production to MGC1 and MGC2 will be evaluated using immune precipitation and immunoblotting procedures. At 6-weeks post immunization, remaining birds will be challenged with MG S6 strain via the air sac route. At 10-days post challenge, all birds will be bled and sampled for tracheal swabbings to detect MG. The incidence and severity of lesions will be evaluated.

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

A. Infectious bronchitis virus (IBV)

Georgia and **Delaware** will continue to monitor the various serotypes and variant viruses involved in outbreaks of the disease. The S-1 gene of "new" serotypes will be sequenced. **Georgia** and **Delaware** will examine the pathogenicity of new IBV isolates.

New York will study the pathogenesis of IBV infection in chickens to elucidate the mechanisms of intracellular virus persistence and cellular injury. IBV persistence and shedding following acute IBV infection has a definite impact on the epizootiology of the disease. The goals of this research are: 1) to identify cells which are infected by IBV during acute and persistent phases of infection, 2) to compare the cellular tropism of virulent and avirulent IBV strains, and 3) to identify the form(s) in which IBV exists intracellularly during acute and persistent phases of infection. Chickens, viruses and sampling schedule: Groups of 2-week-old chickens will be intranasally inoculated with IBV strains Delaware variant (DE-072) and Mass 41. Tissues (trachea, lung, cecal tonsil, bursae of Fabricius, and spleen) will be collected at post-inoculation days 3, 7, 10 and 14, and subsequently at weekly intervals until chickens are one-year old. Oviduct tissues will be collected only at certain specified intervals. Virus isolation, IBV antigen and genome detection. Virus isolation attempts will be performed in embryonated eggs using routine procedures (Gelb, 1989). IBV antigens will be detected using Immunochistochemical studies. These studies along with virus isolation data will provide a comprehensive picture of IBV tissue isolation data as well as tissue tropism, differences in the tissue tropism of different IBV strains and duration of persistence of IBV in the various tissues and cells. IBV genome will be detected by *in situ* hybridization (Poulet et al. 1995) using a DNA probe synthesized from IBV nucleocapsid (N) gene earlier cloned in plasmid pBT 327. Since IBV N gene sequences are highly conserved, we expect this probe to hybridize to 072 RNA as it is known to hybridize to M41 RNA. If this probe is found unsuitable for the detection of 072, we will use the 072 sequence data available from **Delaware** and synthesize a complementary oligo probe. Alternatively, *in situ* PCR will be performed if tissues from IBV infected chickens are found to be negative for IBV RNA by *in situ* hybridization. It is possible that IBV RNA may exist in quantities that are below the detection limits of the procedure.

B. Avian influenza virus (AIV)

USDA ARS, Ohio, and **Delaware** will collaborate to study the ecologic interrelationships of avian influenza viruses. **Ohio** and **Delaware** will provide fecal or cloacal swab samples of migratory waterfowl and shore birds (Delaware only) from the Midwest and Atlantic flyways to **USDA ARS**. Standard virologic procedures will be used for AIV isolation and characterization (Beard, 1989). All H5 and H7 isolates will have HA1 segment of the hemagglutinin gene sequenced and compared with standard poultry and wild bird isolates maintained at the Southeast Poultry Research Laboratory. Methodology for sequencing and analysis will use published procedures (Garcia et al., 1996; Perdue et al., 1995). Relationships between different isolates will be determined and reported.

The potential of avirulent AIVs to mutate and become highly virulent will be assessed. All H5 and H7 AIV isolates will be tested in a model system that favors emergence of highly pathogenic derivatives (Beck et al., 1995; Brugh and Beck, 1992). Sequence differences between mildly and highly pathogenic AIVs will be compared to determine the site(s) of change responsible for virulence shift and determine adjacent common sequences that favor virulence the molecular changes associated with high virulence.

C. Mycoplasmas

Alabama will conduct a retrospective study among poultry processing plants to determine rates of condemnation for airsacculitis. Results of the retrospective study will be used to develop a sampling frame for a prospective investigation of the involvement of mycoplasmas in airsacculitis condemnation. Monthly samples of respiratory tracts from birds with airsacculitis will be collected from a random sample of processing plants that are stratified by state. A systematic random sample of tissues will be collected from each plant. The samples will be based on the monthly condemnation for airsacculitis determined retrospectively, and 5% of the condemnation samples will be targeted for further evaluation. Veterinary diagnostic laboratories in the U.S., will be relied upon to supply unidentified avian mycoplasma isolates with flock histories to aid in epidemiology studies. Mycoplasma isolates will be obtained from **Alabama** and **Georgia**.

North Carolina will utilize the RAPD analysis to assess the epizootiology of emerging mycoplasmas. **Georgia** will use MG strain specific probes to assess the epizootiology of emerging mycoplasmas.

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

A. Infectious bronchitis virus (IBV)

Texas will examine the CTL responses to various U.S. strains of IBV in order to identify T-lymphocyte cross-reactive epitopes associated with the S1 and N proteins. Cross reactive epitopes from the S1 or the N proteins will be evaluated for their cross-protective potential against heterologous IBV strains. Heterogeneity of IBV strains will be determined by infection of chicks using a CTL assay with target cell expressing various strains of IBV or IBV proteins. Groups of 1-week-old chickens will be infected with one of various strains of IBV by intranasal intraocular routes (Collisson et al., 1990). They will be monitored for respiratory signs of infection. Spleens will be collected from four infected animals four to eight weeks later. Sera will also be collected and the presence of IBV specific antibody will be determined using ELISA and western blots (Parr and Collisson, 1993). Effector cells will be collected from spleens following Ficoll-paque gradient separation of single cell suspensions. Target cells generated from a syngeneic CK cell line, infected and labelled with ^{51}Cr. The spleens of age-matched uninfected chickens will serve as controls. The CTL assay will be done with target cells labelled by overnight incubation with ^{51}Cr (MacCubbin & Schierman, 1986). Background release will be determined. MHC restricted activity will be determined by using targets from heterologous (non-matched) birds. Specificity will be determined using heterologous viruses. In designing recombinant vaccines, it is necessary to choose viral proteins, if not epitopes, that will elicit maximum immunity. Texas will concentrate on the IBV S1 and the N proteins which have been cloned, sequenced and expressed in recombinant vectors. The SFV vector carrying S1 or N genes of Arkansas or other IBV strains will be used to transduce CK cells (Wang et al., 1994). Expression of the IBV proteins will be determined by IFA. In addition, the CTL response of chickens to the N and S1 genes will be determined. Chicks will be inoculated at 1-day of age using a gene gun supplied by Agracetus with plasmid DNA containing the N and S1 gene for which sequences are available. IBV-specific CTL and serum antibody responses will be examined prior to and after IBV challenge. IBV-specific CTL responses to homologous and heterologous serotypes will be evaluated in order to identify viral epitopes with cross-protective potential. **Delaware** will collaborate with **Texas** in performing challenge studies to evaluate the immunogenicity cross-reactive epitopes.

Georgia will clone the S1 and S2 glycoprotein genes of Ark IBV into the Baculovirus

expression vector using standard materials and procedures (Miller, 1988; O'Reilly et al., 1990; Yoden et al., 1989). Briefly, the IBV cDNA will be cloned into a transfer plasmid which has an insertion site flanked by sequences homologous to the polyhedrin gene promoter region of the Autographa californica nuclear polyhedrosis virus (AcMNPV), a Baculovirus used as an expression vector. The IBV gene will be cloned into the AcMNPV expression vector by co-transfecting an insect cell line (SF9 cells) with both the transfer plasmid carrying the IBV gene cDNA and the AcMNPV. Recombinant viruses will be detected in the insect cell culture due to the production of a visually selectable plaque phenotype which occurs when the polyhedrin gene of the AcMNPV is substituted with a foreign gene. Screening recombinant baculovirus vectors. Several recombinant expression vectors will be selected and evaluated for production of the IBV glycoproteins. Cells infected with a recombinant expression vector will be assayed with S1 and S2 specific monoclonal antibodies (Mab) using Western blotting. In addition, the infected SF9 cells expressing recombinant IBV S1 and S2 glycoproteins will be examined by electron microscopy to determine if spikes are being formed. If the nascent S glycoprotein is not cleaved into S1 and S2 subunits by the host cell enzymes, similar to reports in the literature for bovine coronavirus spike protein (Yoo et al., 1991), we will use site directed mutagenesis (Oste, 1988; Sambrook et al., 1989) to modify the proteolytic cleavage site so that SF9 cell enzymes will cleave the nascent S glycoprotein. Baculovirus expressed glycoprotein immunogenicity studies. Expression vectors which successfully produce the IBV S glycoproteins will be evaluated for antigenicity and immunogenicity in a pilot study involving SPF chickens. Chickens will be inoculated with the recombinant protein using several routes. Serum from these chickens will be tested using a commercially available enzyme-linked, immunosorbant assay (ELISA) to measure antibody specific for IBV. The recombinant glycoproteins will also be adsorbed to 96-well microtiter plates and used in an ELISA against chicken sera known to have positive titers to IBV. These tests will give a measure of antigenicity of the recombinant proteins. To evaluate the immunogenicity of the recombinant proteins, the vaccinated chickens will be given a challenge dose of virulent IBV by the intratracheal route. Five days later, some of the chickens will be sacrificed and their tracheas examined by histopathological examination for evidence of damage due to IBV. Tracheal tissue will be processed for isolation of IBV. The chickens will be examined for evidence of clinical signs of IBV infection. **Delaware** will cooperate with **Georgia** in the evaluation of promising IBV vaccines.

Georgia will evaluate nucleic acid (DNA) vaccines for IBV. The S1 gene from Ark IBV will be cloned into the pBC12/CMV/IL-2 expression plasmid (Dr. B.R. Cullen, Duke University)(Cullen, 1986). That plasmid containing the H7 gene of influenza virus under the control of the CMV immediate early promoter was used by other researchers to immunize chickens against lethal influenza challenge (Fynan et al.,1993). Standard cloning procedures will be used (Gubler and Hoffman, 1983). The human IL-2 cDNA sequence will be removed from the pBC1 2/CMV/IL-2 plasmid by digestion with the *Hind*III and *Bam*HI restriction enzymes. The IBV S1 gene will be obtained from the plasmid pMJTAS1 Ark (created in our laboratory) by digestion with *Eco*RV and *Bam*HI then ligated into the pBC12/CMV vector (with the IL-2 sequences removed) to create plasmid pMJCMV/S1Ark. The S1 gene will be under control of the CMV immediate early promoter. Expression of the S1 gene will be verified in vitro by transfecting several different cell lines with pMJCMV/S1 Ark using the Lipofectin reagent (Life Technologies, Gathersburg, MD) according to the manufacturers recommendations. The glycoprotein will be visualized by indirect immunofluorescence (IIF) using monoclonal antibodies or polyclonal antisera specific for the Arkansas serotype of IBV. Nucleic acid (DNA) vaccine efficacy studies. *In vivo* immunogenicity studies in SPF chickens will be conducted. At one-day of age chicks will be vaccinated with approximately 30 ug of plasmid DNA intramuscularly and at 14-days of age the birds will be bled then vaccinated intramuscularly with 100 ug of plasmid DNA. Half of the birds in each group will be necropsied at four weeks of age. The remaining birds will be challenged with pathogenic IBV (Ark DPI strain). The birds will be observed daily and necropsied at seven days post-challenge. At necropsy, clinical signs and lesions will be recorded, tracheal swabs will be collected for virus isolation/detection using a PCR-based DNA probe diagnostic test. Various tissues including

trachea, kidney, liver, and spleen, will be fixed in formalin for histopathology. Sera will be collected and tested for antibodies to IBV by ELISA. If protective immunity is not induced in the birds we are prepared to clone the S2 glycoprotein gene into the pBC12/CMV/IL-2 plasmid and use it in conjunction with the pMJCMV/S1 Ark plasmid in immunogenicity studies. **Delaware** will cooperate with **Georgia** in the in vivo evaluation of promising IBV vaccines.

Georgia will evaluate a eukaryotic subunit vaccine for IBV. A novel system for synthesizing the S1 and S2 glycoproteins of IBV will be utilized in an attempt to create an efficacious subunit vaccine. The eukaryotic transient-expression system described by Fuerst et al. (1986) utilizes recombinant vaccinia virus expressing the bacteriophage T7 RNA polymerase. When that virus is co-transfected into a continuous cell line along with plasmid DNA containing the S1 and S2 genes behind a T7 promoter, the T7 polymerase synthesizes the S1 and S2 mRNA and the cell machinery translates those genes into protein. Vaccinia virus VTF7-3 (ATCC 21 53-VR) was obtained from the American Type Culture Collection (Rockville, MD). The S1 and S2 genes from the Ark DPI strain of IBV will be obtained from plasmids pMJTAS1Ark and pMJTAS2Ark respectively, and cloned using standard procedures into the pET-17b plasmid (Novagen, Madison, WI) which contains the T7 promoter and terminator necessary for transcription of the genes into mRNA. The genes will be cloned into separate plasmids and they will be cloned into a single plasmid as the native spike gene containing the S1 and S2 subunits with the proteolytic cleavage sequence between the two subunits. Once the genes are cloned and verified by sequencing (Sanger et al., 1977) they will be co-transfected into cells using the Lipofectin reagent (GibcoBRL, Gaithersburg, MD) according to the manufacturers recommendations along with the VTF7-3 vaccinia virus. Several different cell lines will be used to express the S1 and S2 glycoproteins including vero cells and HeLa cells. The expressed proteins will be analyzed by IFA using Mab and polyclonal antibodies. Eukaryotic subunit vaccine immunogenicity studies. A pilot study will be conducted in two-week old SPF chickens to determine the immunogenicity of the expressed S1 and S2 proteins as described above. **Delaware** will cooperate with **Georgia** in the evaluation of promising IBV vaccines.

Delaware will determine the feasibility of using anti-sense oligonucleotides as a way of inhibiting IBV replication by targeting essential viral genes and non-coding regions that are common to all serotypes. Ultimately, the information generated could be used to develop genetically IBV-resistant commercial chickens when the technology to produce transgenic poultry is available. Antisense oligonucleotides will be used to inhibit two major transcriptional events in the replication of the Beaudette strain of IBV. The first transcriptional event that will be targeted is the RNA polymerase binding site at the 3' non-coding end of the IBV genome. Interference with the binding of the enzyme to the binding site by anti-sense oligos should inhibit the synthesis of the negative strand template and dramatically reduce replication at this critical early event. IBV conserved sequences at the non-coding region at the 5' end (+ strand) and 3' end (- complement) of the IBV Beaudette genome (NIH GenBank accession M95169) (Boursnell et al., 1987) have been identified using cluster analysis (Higgins and Sharp, 1988) (DNAStar, Inc., Version 1.03, 1993). An oligo complementary to the RNA polymerase binding site will be used to pre-treat Vero cells 2-4 hr prior to inoculation with the Beaudette strain of IBV. "Nonsense" oligos of the same length and composition will be included as controls. To achieve optimal specificity, we will initially use standard phosphodiester anti-oligos but these may be degraded by nucleases. liposomes. Various concentrations of anti-oligos will be incubated with Vero cells 2-4 hr prior to the addition of IBV at a moi of 0.1 and 1.0. Growth curves will be performed and infectivity titers will be determined (Cunningham et al., 1972). Total RNA (Favaloro et al., 1980) will be determined for infected aand control cultures. The second area of the replication cycle of IBV to be targeted is the homology regions at the initiation sites of the the A (nucleocapsid), B, C (membrane), D, E (spike), and F (RNA polymerase) genes in order to prevent the synthesis of m-RNAs. Conseved sequences at the initiation sites for leader primed trancription for m-RNAs A (nucleocapsid), B, C (membrane), D, E (spike), and F (RNA polymerase) have been identified. An anti-oligonucleotide to each of the six m-RNAs will be used alone and in various combinations

in Vero cells subsequently infected with the Beaudette strain of IBV. Oligos will be 18 mers designed to bind to the core homology region and to the four complementary nucleotides on each side of the homology region. Growth curves and mRNA synthesis will be analyzed as described above. The effect of oligos on the growth of heterologous IBV serotypes will be evaluated to assess the potential applicability of anti-oligo IBV strategies for poultry. Anti-oligos that inhibit the Beaudette strain, a Massachusetts serotype, will be tested against heterologous IBV serotypes, Arkansas and Connecticut. Unlike Beaudette, most IBV strains do not grow in Vero cells. Therefore, chicken kidney cells (CKC) or chicken embryo fibroblast (CEF) cell cultures with trypsin added to the culture media (Otsuki and Tsubokura, 1981) will be used. CEFs were suitable for demonstrating the antiviral effects of anti-oligos on the replication of Rous sarcoma virus (Zamecnik and Stephenson, 1978). CEF and CEK are routinely prepared in our lab using standard procedures (Gelb et al., 1987a;1987b). Growth curves using Arkansas and Connecticut will be performed to determine viral titers in cells treated with promising anti-oligos. Viral RNA levels will be assessed as described.

B. Infectious laryngotracheitis virus (ILTV)

Delaware and **Illinois** have developed considerable experience in constructing and testing recombinant strains of fowl pox virus expressing the ILTV glycoprotein B gene. Such a recombinant fowl pox virus has been shown to protect birds from a virulent ILTV challenge (Keeler and Poulsen, 1995). **Delaware** and **Illinois** will collaborate on constructing and testing strains of recombinant fowl pox expressing additional or multiple ILTV antigens. Likely gene candidates for insertion into fowl pox include the gD, gC and gH genes.

Successful completion of Objective 1 will be the first step in developing ILTV into a recombinant vaccine vector. Once a selectable foreign gene, like the lacZ gene, has been successfully inserted into a nonessential ILTV gene, such as gX, the next phase is to insert a gene encoding a protective antigen for another avian pathogen. Recombinant vaccines expressing the fusion (F) and/or hemagglutinin-neuraminidase (HN) genes of Newcastle disease virus (NDV) have been successfully constructed and tested using either fowl pox virus or herpesvirus of turkeys as the viral vector (Boursnell et al., 1990a; Boursnell et al., 1990b; Morgan et al., 1992). Neither of these vector systems induces adequate local immunity in the chicken respiratory tract. ILTV, being a respiratory pathogen, should be examined as an alternate vector system for use with avian respiratory pathogens. **Delaware** will use the techniques described above to clone the NDV F and HN genes into the gX gene of ILTV. Recombinant viruses will be evaluated for their ability to protect birds from challenge by ILTV and local or systemic challenges by NDV using established methods (Hastings, 1991; Morgan et al., 1992).

C. Mycoplasmas

Delaware will evaluate the immunogenicity and protective effects of MGC1 and MGC2 recombinant peptides following challenge with virulent MG S6 strain. The MGC1 peptide contains a 177 aa recombinant protein expressed in the pQE30 vector (Qiagen, Inc., Chattsworth, CA). The region expressed is exposed on the cell surface and contains no trptophan,TGA codons. MGC2, a 304 aa protein, was expressed as a fusion protein in pMALc following repair of a TGA codon by site-directed mutagenesis. Experiments will involve single entity (MGC1 or MGC2 peptides alone), and MGC1 and MGC2 combined as a single vaccine. Challenges will be after one to three immunizations using virulent MG S6 via the airsac route of inoculation. Controls will include non immunized chickens and chickens immunized with commercially available inactivated MG bacterin. The immune antibody response to both MGC1 and MGC2 will be monotored using immunoprecipitation and immunoblotting assays.

E. Infectious bursal disease virus (IBDV)

Delaware will cooperate with **Maryland** in the development of a subunit vaccine for CAV using recombinant techniques. The genomic ssDNA will be cloned and the complete nucleotide sequence determined. The VP1 and VP2 genes will be subcloned and expressed in a model baculovirus expression system. The expressed proteins will be characterized with available monoclonal antibodies and then evaluated as immunogens serologically and via challenge. If successful the proposed research would allow for the production of a safe CAV vaccine for use in breeding stock to protect progeny thereby minimizing immunosuppression associated with this agent and providing for increased resistance against respiratory pathogens.

Ohio will cooperate with **Illinois** to express IBDV genes in avian pox virus for the purposes of developing a recombinant vaccine.

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