PATHOBIOLOGY OF aMPV/CO/97 AND THE ROLE OF WILD BIRDS IN THE ECOLOGY OF AVIAN METAPNEUMOVIRUS

by

ELIZABETH TURPIN

(Under the Direction of DAVID E. SWAYNE)

ABSTRACT

Avian metapneumoviruses (aMPV) cause upper respiratory disease, primarily in turkeys. aMPV belongs to the family Paramyxoviridae, subfamily Pneumovirinae, and genus Metapneumovirus. aMPVs have been identified among poultry in Europe, Asia, Africa, and South America for some time. Since first identified in Colorado in 1997, aMPV subtype C has been a recurring problem in Minnesota turkey flocks. The emergence of this new aMPV, has lead to a need for greater understanding of basic aMPV pathology and ecology. These studies assessed aMPV/CO/97’s ability to cause disease in vivo and in vitro, as well as determined the role wild birds in virus dissemination. The inoculation of turkeys or ducks with aMPV/CO/97 resulted in little to no clinical disease. However, the inoculation of turkeys with aMPV/CO/97 three days prior to Newcastle disease virus (NDV) challenge resulted in the manifestation of clinical signs similar to what has been reported in the field. These findings suggest routine use of live NDV vaccine in aMPV endemic areas may exacerbate disease. In vitro, cell surface proteins involved in viral binding were examined by treating cells or virus with various compounds and measuring their effect using flow cytometry. Heparin, heparan sulfate, heparinase I and III were able to reduce aMPV binding to Vero cells, indicating a role for heparan sulfate in aMPV binding. In addition, treatment of cells or virus with anti-CX3CR1, anti-fractalkine, or recombinant human fractalkine, resulted in reduced binding, indicating CX3CR1 is also important for virus attachment. The final aspect of aMPV pathobiology examined was the ability of wild birds to serve as a reservoir. Wild bird serum samples were screened for the presence of aMPV antibodies and oral swabs were assayed for the presence of aMPV. A blocking enzyme linked immunosorbent assay (bELISA) was developed for testing the wild bird serum samples. Fifteen species of wild birds were examined, and five species had antibodies to aMPV as detected by the bELISA. These species included, American coot, American crow, Canada goose, cattle egret, and pigeon. aMPV was detected in oral swabs collected from coots and geese using RT-PCR with primers specific to the matrix gene.
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CHAPTER 1

INTRODUCTION

Avian metapneumovirus, (aMPV), is responsible for upper respiratory tract infections of poultry, characterized by nasal discharge, tracheal rales, foamy conjunctivitis, and sinusitis, primarily in turkeys. Decreased egg production has also been reported in laying hens (27, 75). The disease syndrome has been termed turkey rhinotracheitis (TRT) in turkeys and swollen head syndrome in chickens. Mortality and increased levels of morbidity are often associated with secondary bacterial or other viral infections.

aMPV belongs to the Paramyxoviridae family, subfamily Pneumovirinae, and genus Metapneumovirus. These viruses have a nonsegmented, single-stranded, negative-sense RNA genome. The aMPV genome consists of a nucleoprotein (N), phosphoprotein (P), matrix gene (M), small hydrophobic (SH), glycoprotein (G), fusion (F), second matrix (M2), and an RNA-dependent RNA polymerase (L), found 3’ to 5’ respectively. aMPV differs from other paramyxoviruses by the lack of neuraminidase and hemagglutinin activity, and from other pneumoviruses by the lack of nonstructural genes, NS1 and NS2 (26).

The disease caused by aMPV was first reported in South Africa during 1978 (12, 18). The virus was isolated and characterized as an aMPV in Europe during 1986 (19, 51, 94, 95, 141, 144). After the initial identification, the virus was isolated throughout Europe, South America, Israel, and Asia (2, 40, 74, 98, 107, 133). The viruses isolated
from these countries were placed in two subtypes, A and B (27). During 1997, an aMPV was isolated from turkeys in the United States, and has since been classified as a subtype C aMPV (44). Subtype D virus has recently been identified from historical tissues collected during 1985 in France (9). Viruses are placed into these different subtypes based on sequence analysis and cross-reactivity using serological assays.

The present study focuses on understanding the pathobiology and ecology of subtype C aMPV was based on two hypotheses. That aMPV can cause disease in multiple species of domestic poultry that can be exacerbated by secondary infections and that migratory and other wild birds serve as a reservoir for aMPV. The first objective was to determine the pathobiology of the aMPV/CO/97 isolate. The initial step was to determine the ability of the virus to replicate and cause disease in turkeys followed by evaluation of the role of secondary pathogens for increasing the severity of clinical signs. The second step was to determine the ability of aMPV to replicate and cause disease in ducks. The third step was to evaluate cell surface proteins involved in aMPV binding. The second objective of this study was to determine the role of wild birds in the ecology of aMPV in the United States. To achieve this objective, serum samples were collected from various wild bird species and tested for the presence of aMPV antibodies to determine which free living species were potential hosts. Following identification of wild bird species with antibodies to aMPV, individual birds from these species were swabbed and samples analyzed for the presence of aMPV or viral nucleic acids. Detected viruses were analyzed genetically and compared to turkey isolates to determine their phylogenetic relationships to the aMPV found in the US and abroad.
Emergence

During 1978, the first report of aMPV infection occurred among turkeys in South Africa with respiratory signs characterized by sneezing, rales, and watery nasal discharge (12, 18). After the initial description of aMPV infection in South Africa, similar clinical signs were observed among commercial turkeys in Europe. A paramyxovirus-like agent was isolated and proven to be the etiological agent. The virus was classified as a pneumovirus based on ultrastructural morphology, biochemical properties, antigenic reactivity, and sequence analysis (19, 51, 95, 141, 144). The identification of aMPV allowed for the development of diagnostic tests and the subsequent detection of aMPV in poultry within other countries. Antibodies to aMPV or the virus have been detected in poultry in most European countries, South Africa, Israel, Japan, Morocco, Chile, Taiwan, and Brazil (2, 40, 74, 98, 107, 133). Viruses isolated from these countries fall mainly into A and B subtypes (63). Currently, of the countries tested, only Australia and Canada have not reported aMPV infection in commercial poultry (11, 65).

Respiratory infections consistent with aMPV were first reported in the United States among commercial meat turkeys in Colorado during 1996. Clinical signs reported included coughing, rhinitis, and sinusitis with mortality rates from 0.5 to 20%, due to secondary bacterial infections. Initially, the Colorado aMPV infection was not detected as available reagents for detecting aMPV antibodies were limited to the subtype A and B
viruses. An aMPV strain was isolated from the turkeys in Colorado by the National Veterinary Services Laboratories in 1997 and an ELISA, virus isolation, as well as RT-PCR assays were developed (44, 125). The prototype virus isolated from Colorado differed at the antigenic and genomic level enough to be classified as new subtype, C (34, 119, 120). During 1997, using newly developed diagnostic assays, aMPV and anti-aMPV antibodies were detected in Minnesota turkeys, where aMPV continues to circulate today (56). Seropositive turkeys have been detected primarily in Minnesota, but positive flocks have been identified in North Dakota and South Dakota. Currently, 35-40% of the turkey flocks in Minnesota are infected with aMPV, resulting in losses of $15 million annually (44). Turkey flocks in Arkansas, California, Colorado, Indiana, Iowa, Michigan, Missouri, Nebraska, North Carolina, Ohio, Pennsylvania, Texas, Virginia, West Virginia, and Wisconsin have all tested negative for aMPV subtype C antibodies (109). To date, natural aMPV infection has not been reported in chickens in the United States (44).

Avian metapneumoviruses are divided into four subgroups (A, B, C, and D), based on the molecular sequence of the glycoprotein (G) gene and neutralization tests using monoclonal antibodies or virus specific serum (25, 36, 74, 79, 80). Isolates from the United Kingdom were initially limited to subtype A viruses, while isolates from France, Hungary, Italy, the Netherlands, and Spain were classified as subtype B (36, 80). Currently, both subtypes can be found throughout Europe, indicating spread or introductions of both viruses into new areas, however, subtype B isolates are most frequent today (63, 101, 138). Subtype C viruses were first isolated in the United States in 1997 and differed genetically from the A and B subtypes sufficiently to be placed in
their own subgroup. Currently, subtype C viruses have been found only in the United States (44). Finally, a unique aMPV has been isolated from archived turkey tissues from France and found to differ from previously identified viruses. The phylogenetic analysis of the F, G, and L genes suggests that the French isolates from turkeys should be placed in a separate group, subtype D (9).

Classification

Avian metapneumoviruses belongs to the order Mononegavirales, family Paramyxoviridae, subfamily Pneumovirinae, genus Metapneumovirus (113). The Paramyxovirdnae family consists of the Paramyxovirinae and Pneumovirinae subfamilies. The Paramyxovirinae subfamily consists of the genus Morbillivirus, Paramyxovirus, and Rubulavirus. The Pneumovirinae subfamily consists of respiratory syncytial virus (RSV), mouse pneumovirus, human metapneumovirus (hMPV), and avian metapneumovirus (2).

Avian metapneumovirus, like other members of the paramyxovirus family, contain a non-segmented, single-stranded, negative-sense RNA genome. Morphologically, pneumoviruses are pleomorphic, enveloped viruses. Most of the pneumovirus genomes encode eight to ten genes including non-structural (NS1 and NS2), nucleoprotein (N), phosphoprotein (P), matrix (M), small hydrophobic (SH), surface glycoprotein (G), fusion (F), a second matrix (M2), and a RNA-dependent RNA polymerase (L), found 3’ to 5’ respectively. Members of the pneumovirus subfamily differ from the paramyxovirus subfamily in their lack of hemagglutinin and neuraminidase activity (26). Avian and human metapneumoviruses differ from other members of the pneumoviruses family due to the lack of NS1 and NS2 genes and a
smaller L gene (24, 114, 145). The putative gene order for aMPV and hMPV is 3’-N-P-M-F-M2-SH-G-L-5’ (85, 88) which differs from that of other mammalian pneumoviruses 3’-NS1-NS2-N-P-M-SH-G-F-M2-L-5’ (88).

**Surface Proteins**

aMPVs have three surface proteins, including the surface glycoprotein, fusion protein, and short hydrophobic protein, which function in virus binding and fusion with the cell surface. The surface glycoprotein (G) is believed to be the major attachment protein of aMPV and also a major antigenic determinant. The G protein is a type II integral membrane protein with a mucin-like composition (9). This protein has potential for N and O linked glycosylation, as well as a high concentration of cysteine residues, all of which likely contribute to secondary structure. The protein can be found in virus-infected cells in both membrane-bound and cleaved soluble forms for mammalian viruses (9, 84).

aMPVs are divided into subtypes based on properties of the G protein. The first aMPV G gene sequence was reported to be 1193 nt in length and encodes a predicted protein of 391 aa (88). The aMPV subtype C viruses have a G gene of 1321 nt with one ORF encoding a protein of 435 aa (7). Within each subgroup the predicted G proteins are highly conserved (98.5 to 99.7% for A and B viruses, respectively, and 72-97% for C viruses), while the different subtypes of the aMPV have less than 40% identity (28). The amino acid sequence identity between the A and B subtypes is only 38% (80). The G protein of subtype D aMPV shares 56.6% identity with subtype A and 31.2% with subtype B (9). The aMPV subtype C virus has even less identity to the other subtypes (4
to 16.5%). This is less than the aa sequence identity of hMPV (21%) with that of the aMPV subgroup C (7).

The fusion (F) protein is another major viral antigen, which mediates cell fusion (102). The F protein is a single spanning type I integral membrane protein (84). The F protein has characteristics that are found in other pneumoviruses including: N-terminal hydrophobic sequence predicted to function as a signal sequence for membrane translocation, a highly basic sequence where cleavage may occur, a hydrophobic N-terminus of the F1 characteristic of the fusion-associated moiety, a hydrophobic sequence of 22 residues near the C terminus characteristic of membrane-spanning sequences, and a 25 residue hydrophilic sequence at the C terminus. The fusion protein is cleaved by proteases to form the F1 (436aa) and F2 (102aa) molecules (145).

The F genes of subtype A and B aMPV are both 538 aa in length with 74-83% amino acid identity between the two subtypes (28, 42, 102, 120). The subtype C aMPV F protein is 537 aa in length, and shares 98% nucleotide identity within the subgroup, but only 72% sequence identity to subtype A and 71% identity to subtype B viruses (28, 120). The F gene of subtype D viruses has 70-80.5% nucleotide identity with subtype A and B and 77.6-97.2% identity with subtype C (9).

The SH protein is a minor surface protein with an unknown function. It has a hydrophobic region with N-linked glycosylation sites on the carboxy-terminal side of the hydrophobic region. The SH gene is 589 nt in length and encodes a predicted protein of 173 aa (88).
Internal Proteins

The matrix protein (M) is the most abundant protein and is believed to be the central organizer of viral morphogenesis. The M protein is an internal protein that is associated with the nucleocapsid and the envelope. The self-association of the M protein and its contact with the nucleocapsid may be the driving force in forming a budding virus (84). The M gene encodes a protein of 254 aa, which has a highly hydrophobic sequence of 14 aa (147). The M gene of the A and B subtypes of aMPV share 90% aa identity with one another and only 60% identity to subtype C viruses (28, 115, 119). The M gene has 98% nucleotide identity with only one nonsynomous change among the type C viruses (120). The gene abundance and high level of nucleotide identity make this gene an ideal target for detection by RT-PCR.

The second matrix protein (M2) is a nonglycosylated membrane associated protein. The function of M2 is unknown, although evidence indicates that the M2 protein is an inner component of the viral envelope (84). The M2 gene has 2 open reading frames (ORF) one consists of 558 nt encoding a polypeptide of 186 aa, while the second ORF is comprised of 219 nt (146).

The RNA-dependent RNA polymerase protein (L) is the least abundant of the structural proteins. Together with the P and N proteins, it forms a complex required for genome synthesis and transcription. This complex can make mRNA in vitro that is capped at the 5’ end and contains a poly(A) tail (84, 87). The aMPV polymerase (L) gene is 6099 nt long and encodes a single ORF of 2004 aa. This makes the aMPV L protein the smallest to be described of all the nonsegmented, negative-sense RNA viruses (116). The N protein is comprised of 1197 nt and has a single ORF encoding a protein of
391 aa. The subtype A phosphoprotein gene has 855 nt and encodes a polypeptide of 278 aa (84).

**Virus Binding**

The identification of viral receptors and epitopes important for binding would help determine the initial steps in the pathogenesis of the virus infection. This could explain tissue distribution of virus replication. The aMPV G protein has been identified as the attachment protein based on similarities to the RSV G protein and the ability of antibodies specific for the protein to neutralize the virus (9). Although the cellular receptor for aMPV has not been identified, studies with RSV, which has similar surface proteins, have implicated a role for cell surface glycosaminoglycans (GCG) as an initial step in attachment (48, 64, 83). The major cell surface GCGs includes heparin, heparan sulfate, chondroitin and dermatan sulfate. Heparin, which is synthesized by mast cells and basophils, is a linear repeat copolymer of 1→4-linked uronic acid and glucosamine residues. Heparin has a high negative charge density due to its high level of sulphate and carboxylate residues. Heparan sulfate (HS) is similar to heparin in that it also has a repeating linear copolymer of variably sulphated uronic acid and glucosamine. Heparan sulfate differs from heparin by containing less that 1 sulphate per disaccharide and is predominantly glucuronic acid 1→4 linked glucosamine. HS is localized on the external surface of cell membranes and in the extracellular matrix. It plays a role in cell-cell and cell-protein interactions. Binding to heparin by various substances appears to be an ionic-based interaction, without specific hydrophobic or hydrogen bonding interactions (67). Studies with RSV indicate that heparan sulfate and chondroitin sulfate B are
important for efficient infection *in vitro* (48, 64, 83). Heparin binding domains have been identified in the RSV G protein (48, 129).

Most viruses utilize multiple binding sites for attachment to host cells. The aMPV G protein has both heparin binding and a CX3C motifs (7). This CX3C motif is present on the G protein of RSV and is capable of binding to CX3CR1 on the surface of Vero cells (134). Fractalkine (Fkn) is the only CX3C chemokine that has been described to date. Interaction of the CX3C motif of the G glycoprotein of RSV with CX3CR1 appears to be capable of modulating immune response and facilitating infection (134). Although the G protein is the major attachment protein, infectious mutants lacking both the G and SH proteins remain infectious. This indicates the ability of the F protein to bind to cells (64). The F protein of RSV can independently interact with immobilized heparin and can attach to cells via interaction with cellular heparan sulfate (47). Both the heparin binding domain and the CX3C motifs have been identified in the G protein of aMPV, indicating a potential role for virus binding to these substrates (7). The similarities among the surface proteins of RSV and aMPV indicate a possible role for G, F and possibly SH in the attachment of aMPV to the cell surface.

**Clinical Signs**

*Subtype A and B in Turkeys*

aMPV, initially called turkey rhinotracheitis virus, affects turkey flocks of all ages. Although disease has been reported in many species it has been most often reported in turkeys (53). Signs of infection include rapid onset, high morbidity, depression, gasping, snicking, coughing, catarrhal inflammation of the upper respiratory tract, nasal and ocular discharge, foamy conjunctivitis, sneezing, tracheal rales, swollen infraorbital
The disease appears to be more severe in winter than summer. Experimental inoculation of turkeys with aMPV generally resulted in gasping, difficulty breathing, watery eyes, and nasal discharge. At necropsy, clear to grayish exudates were noted in the turbinates, and excess mucous were present in the trachea. Transient lesions occurred in the trachea, however other tissues including the lung and conjunctiva were rarely affected. Histologically, rhinitis with destruction of the epithelium and loss of cilia, hyperemia, and mild mononuclear infiltration was often observed in the submucosa of infected turkeys. Viral antigens were associated with the cilia of the epithelial cells of the turbinates, trachea, and lung, leading the investigators to speculate that the virus replicates in the epithelial cells of the nasal cavity and trachea. No antigen or histopathologic changes were detected in the conjunctiva, air sac, liver, spleen, kidney, hypothalamus, blood, or nervous system of the birds with infections by subtype A or B viruses.

Breeder turkeys with aMPV infections have mild respiratory distress with accompanying decreased egg production and lower eggshell quality. Drops in egg production have been reported following intravenous inoculation, while oculonasal inoculation failed to induce drops in egg production. aMPV antigen was detected in the
oviduct epithelium of the IV inoculated birds (28, 37, 38). Other investigators have also detected viral antigens by weak antibody staining in the uterus epithelium and on the surface epithelium of the oviduct. Most of the infected surface epithelial cells were in the lower magnum and vagina (78).

**Subtype A and B in Chickens**

In contrast to the disease in turkeys, naturally occurring aMPV infection in chickens is generally mild. Antibodies to aMPV have been detected in flocks with a history of respiratory problems as well as healthy flocks, indicating that aMPV infection of chickens does not always result in clinical disease (29). However, virus has been found consistently in the epithelium of the upper respiratory tract 2-3 days after experimental inoculation. Damage caused by the virus was minimal and recovery was rapid (16). Viral antigen were detected in the cytoplasm and associated with the cilia of the nasal turbinate epithelial cells resulting in severe alterations to cell surface and ciliary apparatus of the turbinate epithelium. Other abnormalities reported include cytoplasmic blebs, clumping and intracytoplasmic inclusions (93). In other studies, *in vitro* work in organ culture of oviduct epithelium showed that they were susceptible to aMPV infection while the *in vivo* studies failed to show aMPV replication in the oviduct of chickens even after intravenous inoculation (81).

In chickens, a disease generally termed swollen head syndrome (SHS) has been associated with subtype A and B aMPV infection (103, 110, 112, 143). SHS was first reported among broiler chickens in South Africa during 1971. SHS has also been reported in Europe, Japan, Israel, Yemen, and Taiwan (43, 62, 77, 89, 98, 103, 112, 126). Antibodies to aMPV have been found in many of the cases of SHS mentioned above and
aMPV has also been isolated from chickens with SHS indicating that aMPV may be involved in some of the cases of SHS (14, 55, 77, 89, 90, 98, 110, 112, 126, 128). Clinical signs of SHS include sneezing with nasal discharge, coughing, swelling of the periorbital and infraorbital sinuses, torticollis, and incoordination (143). The gross lesions included extensive yellowish gelatinous to purulent edema in subcutaneous tissues of the head and congestion of the subcutaneous vasculature of the head, neck, and wattles (89). Morbidity generally ranges from 0.5 to 20 % (128). Although aMPV has been isolated from chickens with SHS, inoculation with the virus alone does not consistently reproduce the syndrome (3, 32, 56, 75, 97, 99, 112). Along with aMPV, *E. coli*, Newcastle disease virus, infectious bronchitis virus, avian reovirus, *Morganella morganii*, and *Proteus mirabilis* have been isolated from chickens with SHS (89, 97, 126, 128). Also, *E. coli* in conjunction with aMPV can result in typical lesions of SHS including facial swelling due to cellulitis, blepharitis, periophthalmitis, and infraorbital sinusitis. This suggests that the *E. coli* used in this study had the ability to invade into facial subcutaneous tissues from the blepharitis and rhinitis (99).

Although commercial broiler chickens with clinical signs similar to infection with aMPV including SHS were reported in California during 1994, aMPV or antibodies were not detected. Clinical signs observed included snicks, swollen heads, and severe depression. *E. coli* and IBV (Mass serotype) were isolated from trachea/nasal cavity pools. Birds were negative for aMPV using ELISA tests specific for European isolates (43). aMPV has never been detected or isolated from chickens in the United States (44). This indicates SHS is not a specific disease syndrome of aMPV infection but probably is a generic clinical syndrome caused by a variety of etiological agents.
**Subtype C in Turkeys**

Infection with subtype C aMPV are generally less severe than with subtype A or B viruses. Experimental infections generally result in limited clinical signs in turkeys and no clinical signs in chickens. Clinical signs associated with some of the Minnesota aMPV isolates included nasal discharge, swelling of the infraorbital sinuses and frothy ocular discharge. Mild inflammation was seen in the mucosa of the nasal turbinates and infraorbital sinuses (70, 71, 121). Turkeys inoculated with the Colorado isolate had no gross lesions in the turbinates, infraorbital sinuses or trachea, but microscopic examination revealed acute rhinitis, sinusitis, and tracheitis visualized as congestion, edema, lymphocytic and heterophilic infiltration and loss of ciliated epithelium (109). In tracheal organ cultures, ciliastasis has not been observed with the subtype C virus, as occurs commonly with infections by subtype A and B viruses (34). The lack of clinical signs with the aMPV isolate from Colorado and the reduced signs with the other subtype C viruses as compared to subtype A and B viruses indicate a role of secondary agents in the presentation of disease seen in the field.

**Dual Infections**

Inoculation with aMPV alone does not consistently produce severe clinical signs as observed in natural infections. Numerous dual infection studies have been preformed to determine the role of secondary avian pathogens in contributing to the field clinical disease associated with aMPV infections. When chickens were inoculated with live IBV vaccine prior to aMPV, the inclusion of the aMPV vaccine did not have any deleterious effect on the response to the IBV vaccine or level of protection against IBV (39). Similar results were reported when turkey poulets were inoculated with aMPV field strains.
followed by *Mycoplasma synoviae* (Ms) or turkey herpesvirus. Dual infection did not result in increased severity of clinical disease, virus replication, gross or microscopic lesions. The patterns of aMPV and Ms reisolation were similar in the single and dual infected group and no differences were detected in quantity of antibody produced against aMPV (82, 137).

In other studies, co-infecting with other avian pathogens increased severity of clinical signs. Turkeys inoculated with aMPV followed by hemorrhagic enteritis virus (HEV) vaccination, and challenged with HEV had high mortality. This suggests the aMPV infection reduced the efficacy of the HEV vaccines (20). Dual infection of turkeys with aMPV and *E. coli* resulted in higher incidence of gross lesions compared to either of the single infection groups. aMPV was detected in the respiratory tract of all aMPV-inoculated birds and *E. coli* was recovered from the turbinates, trachea, lungs, heart, and liver of all birds receiving dual infections. These results indicate that aMPV may act as a primary agent and predispose birds to *E. coli* colonization and invasion. This would result in enhanced susceptibility of epithelium cells to secondary bacterial infections (1, 92, 93, 139). *Bordetella avium* and a Pasteurella-like organism were also more invasive in the presence of aMPV (32). Infections with *Mycoplasma imitans* or *Mycoplasma gallisepticum* and aMPV dual infection resulted in a significant increase in clinical signs and lesions in the dual infected groups. The *Mycoplasma sp.* were also found in the lung and air sacs in the presence of virus infection (49, 100). In addition, these studies mimicked what has been observed in the field with morbidity rates as high as 100% and mortality rates up to 30% reported among infected turkey flocks with accompanying secondary infections (101). From these infected turkeys, *E. coli*,
Mycoplasma gallisepticum, M. meleagridis, and Newcastle disease virus were also isolated (12, 18).

Other Species

Although aMPV is most often reported in chickens and turkeys, other avian species are susceptible to infection. aMPV has been isolated from Muscovy ducks exhibiting egg-drop production and coughing. Increased mortality to 2% was noted and a dead bird had general congestion, splenomegaly, and tracheitis (130). In Pekin ducks, experimentally inoculated with subtype C aMPV, virus was recovered from nasal turbinates, blood, lungs, and trachea indicating that domestic ducks are capable of supporting virus replication (123). Experimentally, pheasants and guinea fowl were susceptible to infection with subtype A and B viruses with the absence of disease, while pigeons, geese, and ducks were resistant to infection (2, 53).

Wild Birds

Antibodies to aMPV have been detected in other avian species including gulls from the Baltic Sea, ostriches from Zimbabwe, and pheasants in Italy (15, 17, 27, 54). aMPV has also been isolated from farm reared and free-living pheasants in Italy (17). In the United States aMPV was detected in nasal turbinates collected from geese, sparrows, starlings, and sentinel ducks located on aMPV infected turkey farms. The detected viruses were found to be 96% similar to aMPV isolates from turkeys in Minnesota (122, 124). The initial spread of aMPV from South Africa to Europe and to other poultry producing areas, lead investigators to hypothesize a role for migratory birds in the transmission of the virus (28, 124). This is further supported by the sudden appearance of a unique aMPV in the United States and the detection of aMPV in wild birds in
Since the origin of aMPV is unknown, it is tempting to suggest that wild birds may be a natural reservoir for the virus (74). The isolation of poultry pathogens from wild birds has been reported with both avian influenza and Newcastle disease virus (117, 127). More studies are needed to determine if infections in wild birds resulted from transmission from turkeys to wild birds or if wild birds can be a true reservoir of aMPV.

**Diagnosis**

Accurate and rapid detection of aMPV is critical to make a diagnosis and to prevent the spread of infection to other areas. The variation in clinical signs as well as the increased evidence for antigenic and genetic variation within the aMPVs make the diagnosis difficult (28). Detection of aMPV focuses on virus isolation or the identification of antigen, nucleic acid or antibody (28).

*Virus Isolation*

Many diagnostic tests have been developed for the detection of virus in clinical and experimental samples. Tracheal organ culture (TOC) and inoculation of chicken or turkey embryonating eggs are the preferred methods for initial virus isolation (12, 34, 95, 141, 144). The aMPV A and B subtypes cause ciliastasis in TOC by 10 days post inoculation, while the C subtype does not produce ciliastasis (32, 34). Primary isolation should consist of several blind passes in embryonating eggs, possibly supported by passage in TOC or Vero cells (27).

Virus can also be recovered from the allantoic fluid and yolk sac membrane of chicken or turkey embryos inoculated via the yolk sac (12, 34). Mortality may occur along with hemorrhages in the embryos (28). Isolated viruses can easily be adapted to chicken embryo fibroblasts (CEF), chicken embryo liver (CEL), Japanese quail
fibrosarcoma cell line (QT-35), or Vero (an African green monkey kidney cell line) cell cultures (12, 23, 56, 57, 118, 142). Using routine virus isolation methods in cell culture, virus is normally detected 3-5 days post inoculation by the presence of cytopathic effect (CPE) characterized by syncytia formation (32, 36, 100).

Antigen Recognition

aMPV antigen can be detected in embedded tissue sections or infected cell monolayers. Micro-indirect immunofluorescent antibody tests are reportedly as effective as virus neutralization assays or presence of CPE for the detection of aMPV infection, while being easier to use for multiple samples with a more rapid diagnosis (72, 135). Immunohistochemistry (IHC) methods are utilized to detect antigen in formalin-fixed, paraffin-embedded tissues using streptavidin-biotin immunoperoxidase staining techniques and antibodies specific for each subtype. Using IHC, viral antigen has been detected in the ciliated epithelial cells of nasal turbinates and infraorbital sinuses (69, 105).

RT-PCR

Using routine virus isolation methods, the earliest virus detection is between 3-5 days post inoculation (32, 36, 100). RT-PCR allows for detection at earlier time points and as long as 17 days post inoculation (40, 41, 68, 86, 94, 101, 111). Diagnostic RT-PCR primers to the M and F gene of aMPV have been used in Minnesota (4, 68, 86, 94, 108, 125). A multiplex RT-PCR assay has also been designed to detect and distinguish between aMPV and NDV infection, which both infect the respiratory tract and produce similar clinical signs (5). Nested RT-PCR was reported to be more sensitive than a single RT-PCR reaction (10, 111). Similarly, an immunochemiluminescent Southern blot RT-PCR was used to detect the F gene in two European and two Brazilian isolates. This
technique was comparable to nested RT-PCR and was more sensitive than single PCR (41). RT-PCR protocols have been developed that can detect and subtype A and B aMPV based on primers to the F, G, and N genes (10, 131). RT-PCR detection including RNA extraction, amplification, and electrophoresis has taken less than 2 days. It was specific for aMPV, while virus isolation is not specific and can take from 7-10 days (68).

**Serology**

Antibodies to aMPV have been detected using virus neutralization, indirect immunofluorescence (IIF), and ELISA assays (8, 21, 28, 46, 57, 66, 104). Antibodies to aMPV were detected using IIF antibody staining of aMPV infected tracheal or nasal turbinate sections (76, 106). Virus neutralization tests have been used to determine which subtype of aMPV turkeys have been exposed (27). Commercially available ELISA kits vary in sensitivity for turkeys and chickens due to the use of an anti-chicken antibody for detection (50, 96). The use of an anti-turkey antibody with turkey serum samples results in increased sensitivity (22). In addition, commercial serological diagnostics do not detect antibodies to all four subtypes of aMPV. Consequently results are often difficult to interpret with weakly positive sera (34, 125). To overcome these problems a variety of antigen preparations and ELISAs have been developed. Many ELISA assays use a double well format to overcome the non-specific binding of avian serum to the ELISA plates or contaminating proteins. This format uses both infected and noninfected cell culture lysates as antigens in the ELISA (104). Additionally, antigen preparation with NP-40 extracted antigens resulted in less variation than that seen with untreated cell monolayers (25). Discrepancies have commonly been detected using ELISA tests from Europe, where both A and B aMPV subtypes are endemic, depending
on the viral antigen used to coat plates. Antigens should be prepared depending on the subtype of viruses that predominate in a given geographical area (45, 46, 132). Such variability has not been noted in the United States due to the high level of sequence identity among the subtype C isolates (22).

ELISAs using recombinant aMPV proteins have been evaluated for the detection of aMPV/C antibodies. The M and NP genes of aMPV type C was cloned and expressed in *E. coli* then used as antigens in ELISA plates (58, 59). When compared to standard indirect ELISAs, both the M and NP ELISAs were more sensitive and specific for aMPV antibodies than routine diagnostic ELISA assay (58, 59).

**Sample Collection**

Samples for virus isolation should be taken early in the infection process because isolation is generally unsuccessful at later times when birds exhibit severe clinical signs (52). Successful isolation of virus is rare in field cases due to the short virus replication period with titer peaks occurring between 3 to 5 days post infection. Isolation should be attempted at the first signs of disease in the flock, selecting birds not yet showing clinical signs (27, 28). For virus isolation, 20% tissue suspensions of nasal exudate or homogenized tissues in phosphate buffered saline (PBS) can be inoculated into TOC or embryonating eggs, and subsequently cultivating in cell culture (52). Swabs can be placed in PBS or dried and held for several days before testing and will still give more reproducible results than tissue extracts (68). aMPV replication occurs primarily in the nasal tissue or trachea, with limited replication in the lung (28). aMPV needs to be differentiated from other agents including NDV, IBV, AIV, bacteria, or mycoplasma that can cause a similar upper respiratory disease (52).
Control

The most important preventative measures for aMPV include good health management and enhanced biosecurity. Transmission of aMPV generally occurs through direct bird-to-bird contact; however aMPV on fomites such as personnel, equipment, feed or in contaminated water, along with the movement of infected poults have been implicated in the spread of this disease (2). aMPV infection is exacerbated by poor management practices such as inadequate ventilation, over stocking, poor litter conditions, poor hygiene, and mixed age groups. Improper debeaking and vaccination with NDV has been associated with increased severity of aMPV (2). There are no treatments for aMPV infection, but the administration of antibiotics does reduce the affects of secondary bacterial agents (61).

Live attenuated vaccines against the A and B subtypes are commercially available in Europe (136). Single vaccination may protect chickens and turkeys, but a second vaccination is recommended for turkeys reared beyond 10-12 weeks of age, since reinfections can occur as immunity declines (27, 138). aMPV passaged through TOC generally results in less attenuation of the virus such that clinical signs can occur after vaccination. By contrast, viruses that have been passaged in Vero or CEF cells have greater attenuation and produce no clinical signs (30, 31, 33, 79, 140, 142). Tissue culture adaptation of aMPV subtype C has resulted in an attenuated virus that has been approved for limited vaccination in the United States. Two Minnesota aMPV isolates have been passaged in cell culture and tested for vaccine efficiency and both isolates appear to be attenuated. One virus (passage 41) was reported to protect from subsequent
challenge, while the other virus (passage 63) requires a higher vaccine dose to provide protection (60, 73).

Duration of cross-protection between A and B subtypes is important for countries where multiple subtypes are predominant (136). Experimentally, investigations demonstrated that homologous and heterogenous vaccination can protect from challenge with virulent strains of A and B viruses (33, 35). Although the virulent and attenuated virus vaccines were able to prevent clinical signs, virus was recovered during heterologous challenge (136). The recent reports of antigentically different aMPVs from the USA and France show the virus is capable of emerging in new forms. Although the existing vaccines do protect against all known types, this may come to an end (27, 34, 136). Although maternal antibodies do not provide complete protection against infection or appear to interfere with primary immune response following vaccination, less severe clinical signs occur in birds with maternal antibodies following exposure to aMPV (6, 33, 140).

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CHAPTER 3

EXPERIMENTAL INFECTION OF TURKEYS WITH AVIAN PNEUMOVIRUS AND EITHER NEWCASTLE DISEASE VIRUS OR *ESCHERICHIA COLI*¹


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SUMMARY. Avian pneumoviruses (APV) are RNA viruses responsible for upper respiratory disease in poultry. Experimental infections are typically less severe than those observed in field cases. Previous studies with APV and *Escherichia coli* suggest this discrepancy is due to secondary agents. Field observations indicate APV infections are more severe with concurrent infection by Newcastle disease virus (NDV). In the current study, we examined the role of lentogenic NDV in the APV disease process. Two-week-old commercial turkey poults were infected with the Colorado strain of APV. Three days later these poults received an additional inoculation of either NDV or *E. coli*. Dual infection of APV with either NDV or *E. coli* resulted in increased morbidity rates with poults receiving APV/NDV having the highest morbidity rates and displaying lesions of swollen infraorbital sinuses. These lesions were not present in the single APV, NDV, or *E. coli* groups. These results demonstrate that coinfection with APV and NDV can result in clinical signs and lesions similar to those in field outbreaks of APV.

Key words: avian pneumovirus, turkey rhinotracheitis, turkey, RT-PCR, Newcastle disease virus, *Escherichia coli*

Abbreviations: APV = avian pneumovirus; CFU = colony forming units; CPE = cytopathic effect; DPI = days postinoculation; EID$_{50}$ = 50% egg infectious dose; ELISA = enzyme linked immunosorbent assay; HA = hemagglutination; HI = hemagglutination inhibition; IBV = infectious bronchitis virus; Ig = immunoglobulin; i.n. = intranasal; NDV = Newcastle disease virus; O.D. = optical density; PBS = phosphate buffered saline; PCR = polymerase chain reaction; RT = reverse transcriptase; TCID$_{50}$ = 50% tissue culture infective dose; TRT = turkey rhinotracheitis;
INTRODUCTION

Avian pneumoviruses (APVs) are negative-sense single-stranded RNA viruses that belong to the family *Paramyxoviridae* and the genus *Metapneumovirinae* (34). APVs cause turkey rhinotracheitis (TRT), a respiratory disease in turkeys, and have been associated with swollen head syndrome in chickens (12). The disease TRT was first described in South Africa during the 1970s (9). In 1986, APVs were isolated from clinically affected turkeys (29,44). Today APV is found in South Africa, Japan, Europe, South and Central America and the United States (3,12,24,40,41).

APVs have been tentatively designated as subtype A, B, C, or D on the basis of virus neutralization and sequence analysis (7). Subtype A and B viruses currently are found in Europe, Japan, and South and Central America and generally share approximately 83% amino acid sequence similarities in their fusion and matrix proteins (38, 39). APVs isolated in the US to date are subtype C viruses. Subtype C viruses are 78% similar to subtype A and 71% similar to B viruses with the fusion protein for comparison (39). Subtype D viruses, isolated in 1985, were recently characterized and found to be 70%-80.5% similar to subtype A and B viruses and 77.6%-97.2% similar to subtype C viruses (6).

Experimentally, APV is able to induce respiratory disease in turkeys, with clinical signs and lesions that include sneezing, depression, and rhinotracheitis with nasal exudates (12). More severe clinical signs of infection, coughing, head shaking, swollen sinuses, and increased morbidity and mortality, have been reported with natural infections. As with many infections, secondary agents and/or poor husbandry will prolong the disease and result in increased morbidity and mortality (12).
Dual infections with various bacteria and viruses have been shown to induce more severe clinical signs of infection. *E. coli* can worsen the effects of APV infection in both chickens and turkeys (33,42). Clinical signs, including nasal exudates and frothy discharge from the eyes, are most severe when bacteria were inoculated within 3 or 7 days post-APV infection, although no signs of swollen heads or sinuses have been observed (1,28,30,42). Other organisms examined during coinfection studies include *Bordetella avium*, *Pasteurella*-like organisms, *Mycoplasma synoviae*, *Mycoplasma gallisepticum*, *Mycoplasma imitans*, infectious bronchitis virus, and turkey herpesvirus (13,15,20,26,31,43). Results from these studies indicated that many agents may be involved in the disease process, although severe disease characterized by swollen sinuses and a swollen head appearance was not typically reproduced experimentally.

In naturally occurring APV infections in turkeys, concurrent infection with Newcastle disease virus (NDV) from either vaccination or field outbreaks contributes to the severity of clinical disease (Dr. Hugo Medina, pers. comm.). The effect of NDV on APV infection has not been determined. Mesogenic and lentogenic strains of NDV are endemic in wild birds, circulate in many poultry populations, and are thought to impair clearance of other respiratory pathogens (9,18,19). Lentogenic NDV vaccine strains may result in tracheal lesions that are postulated to decrease mucociliary clearance, leading to decreased resistance to environmental pathogens and depressed phagocytosis by respiratory macrophages (18,19). The presence of NDV from naturally circulating or vaccine strains in a turkey population makes NDV a potential secondary pathogen in APV outbreaks.
Experimental APV infections are typically less severe than those observed in field cases. Severe APV infections in the field are often concurrent with live NDV vaccination of turkeys, suggesting a role for lentogenic NDV in the APV disease process. To test the role of secondary agents in exacerbating APV infection, we compared infection with APV alone to coinfection with either a lentogenic strain of NDV or an intermediately pathogenic *E. coli* isolate. Poult s receiving dual infections were compared with negative control poult s and poult s receiving single NDV, APV, and *E. coli* infections for clinical signs and virus isolation from oral swabs and tissues.

**MATERIALS AND METHODS**

**Viruses.** The APV isolate used in this study, APV Colorado, was obtained from the National Veterinary Services Laboratories, Ames, IA (courtesy of Dennis Senne). The virus was isolated from commercial turkeys in Colorado having clinical respiratory disease (32). Upon receipt, the isolate was passaged through Vero cells, and the titer was determined by the 50% tissue culture infective dose (TCID$_{50}$) (35).

The lentogenic NDV isolate, TK/VA/695/85 (obtained from D. J. King, Southeast Poultry Research Laboratory, Athens, GA), was isolated from turkey flocks in Virginia during 1985. The virus has an intracerebral pathogenicity index of 0.49, an intravenous pathogenicity index of 0.00, and deduced amino acid sequence similar to the La Sota strain of NDV (27). The virus titer was determined as 50% egg infectious dose in specific-pathogen-free chicken embryos.

**Bacteria.** *E. coli* O35 (obtained from J. K. Rosenberger, University of Delaware, Newark, DE) was isolated from a broiler flock (37). The bacteria were cultured overnight on MacConkey agar at 37 C. One colony was transferred to brain heart infusion medium
and cultured overnight at 37 °C with shaking (37). The bacterial culture was titered through dilutions and plating on MacConkey agar plates 1 day prior to inoculation. This isolate has been characterized as intermediately pathogenic on the basis of its ability to cause 75%-100% mortality with lesions in 1-day-old chickens; however, 14-day-old chickens are relatively resistant, as previously described (36).

**Turkeys.** Conventional 1-day old turkey poults (British United Turkeys of America, Lewisburg, WV) free from maternal antibodies to APV and NDV were housed at Southeast Poultry Research Laboratory in isolation cabinets under negative pressure within a biosafety level 3 agriculture facility. Poults were provided free access to food and water (5).

**Experimental design.** *Experiment 1.* Two-week-old turkeys were distributed into six groups of 12 birds each. Turkey poults were numbered prior to inoculation and sacrificed numerically to induce randomization in the sampling procedure. Group 1 (negative control) received 0.2 ml of phosphate buffer saline (PBS) via an intranasal (i.n.) inoculation on days 0 and 3. Group 2 (*E. coli*) received 10^7 colony-forming units (CFU) of *E. coli* i.n. in a 0.1 ml dose on day 3. Group 3 (NDV) received 10^5 EID<sub>50</sub> of NDV i.n. in a 0.1-ml dose on day 3. Group 4 (APV) received 10^{4.5} TCID<sub>50</sub> of APV i.n. in a 0.2-ml dose on day 0. Group 5 (APV/*E. coli*) received 10^{4.5} TCID<sub>50</sub> of APV i.n. in a 0.2-ml dose on day 0 and 10^7 CFU of *E. coli* i.n. in a 0.1-ml dose on day 3. Group 6 (APV/NDV) received 10^{4.5} TCID<sub>50</sub> i.n. of APV in a 0.2-ml dose at day 0 and 10^5 EID<sub>50</sub> of NDV i.n. in a 0.1-ml dose on day 3. The turkey poults were monitored daily for clinical signs. The turkey poults were orally swabbed 2, 4, 6, 8, 10 and 14 days post inoculation (DPI). Oral swabs were placed in 1.0 ml of PBS (Invitrogen, Carlsbad, CA) containing antibiotics
(1000 units/ml of penicillin, 10 µg/ml of gentamicin, and 5 µg/ml of amphotericin B) (Sigma, St. Louis, MO). On 2, 4, 6 and 8 DPI two poults from each group were sacrificed by intravenous injection of sodium pentobarbital (100 mg/kg), and tissues were collected for histologic examination and virus isolation. On day 14, the four remaining turkeys were sacrificed and tissues were collected. Lung and trachea were collected under aseptic conditions, ground using mortar and pestle, and prepared with a final suspension of 20% (w/v) in PBS with antibiotics. Serum samples were collected at 14 DPI. All serum, swab, and tissues samples were tested individually.

Experiment 2. Two-week-old turkeys were distributed into four groups with five birds per group in an attempt to reproduce clinical signs observed in Experiment 1. Group 1 (negative control) received 0.2-ml of PBS i.n. on days 0 and 3. Group 2 (NDV) received 0.1-ml of NDV i.n. for a final dose of $10^5$ EID$_{50}$ on day 3. Group 3 (APV) received 0.2-ml i.n. of APV for a final dose of $10^{4.5}$ TCID$_{50}$ on day 0. Group 4 (APV/NDV) received $10^{4.5}$ TCID$_{50}$ i.n. of APV in a 0.2 ml dose at day 0 and $10^5$ EID$_{50}$ of NDV i.n. in a 0.1 ml dose on day 3. The turkey poults were monitored daily for clinical signs. No oral swab or tissues samples were collected for virus isolation. Serum samples were collected at 14 DPI.

Virus isolation. Vero cells (ATCC CCL-81) were inoculated with 200 µl of oral swab, trachea, or lung samples and incubated for 45 min, and the medium was replaced (21). Cells were observed daily for cytopathic effect (CPE), characterized by scattered focal areas of cell rounding and syncytia formation (3,11,14). At 7 DPI cells were subjected to three rounds of freeze thawing and 200 µl of fluid passed onto fresh Vero cells. All samples were passed through Vero cells three times. Virus isolation results
were verified by APV and NDV reverse transcriptase (RT) polymerase chain reaction (PCR).

**RNA extraction.** RNA was extracted from experimental samples with TRIZOL® reagent (Invitrogen). Vero cells inoculated with experimental samples were subjected to freeze thawing and 200 µl of material added to 1 ml of TRIZOL®. RNA was extracted by following the manufacturer’s protocol, resuspended in 30 µl of Rnase-free water, and stored at −70 C.

**RT-PCR.** RT-PCR was used to confirm virus isolation results as well as to distinguish between the presence of NDV or APV in experimental samples. APV primers were developed to detect viruses isolated in the United States (38). The avian pneumovirus primers, APVMLT95 5′-CTGCCTGCAAGGTTAACAGT-3′ and APVMLT745 5′-GTGGCTCCAGTTCCTGCAGA-3′, amplified a 650 base pair (bp) product of the matrix gene of APV. NDV primers to the nucleoprotein gene were included to perform a multiplex RT-PCR as previously described (4). The NDV primers amplify a 309 bp product. RT-PCR was carried out with the One-Step RT-PCR kit (Qiagen, Valencia, CA). Five microliters of sample RNA was added to the RT-PCR mixture with 10 units of ribonuclease inhibitor (Invitrogen) and 0.6 µM of each primer in a final volume of 25 µl. The RT reaction was carried out at 50 C for 30 min with a 15-min initial PCR activation step at 95 C. PCR parameters consist of 94 C for 3 min, 30 cycles of 94 C for 45 sec, 55 C for 30 sec, 72 C for 1 min, followed by an additional extension step of 72 C for 7 min. An additional PCR reaction was included to amplify low levels of viral nucleic acid not detected with the initial RT-PCR reaction (7). For the second PCR reaction, 5 µl of the first reaction was added to a Taq PCR Master Mix
(Qiagen) along with 0.5 µM of each primer in a total volume of 25 µl. RNA extracted from viral stocks was used as a positive control, and no template and no RT reactions served as negative controls. RT-PCR products were visualized by ethidium bromide staining of 1% tris borate (0.045M) EDTA (0.001M) electrophoresis buffer (TBE) agarose gels.

**Histology.** Tissues taken for histopathology included upper and lower trachea, lung, heart, air sac, bursa of Fabricius, nasal cavity, lower eyelid, spleen, thymus, thyroid, Harderian gland, and lacrimal gland. The tissues were fixed in 10% buffered formalin solution, sectioned, and stained with hematoxylin and eosin.

**Serology.** APV antibodies were detected by an indirect enzyme-linked immunosorbent assay (ELISA) specific for APV Colorado. APV Colorado was sucrose purified and coated onto ELISA plates. The antigen was purified as previously described (22). Plates were blocked by the addition of 1% polyvinylpyrrolidone (Sigma). The turkey serum was heat inactivated (37°C for 30 min) and tested at five-fold dilutions from 1/25 to 1/15625. A horseradish peroxidase conjugated goat anti-turkey IgG antibody (Southern Biotech, Birmingham, AL) was added to the ELISA for detection and o-phenylenediamine dihydrochloride (Sigma) served as substrate. Substrate development was stopped with sulfuric acid by following the manufacturer's protocol, and the optical density (OD) was determined at 490 nm. Turkey serum samples were considered positive if their OD reading was 3 standard deviations above the mean of the negative control sera as previously described (8).

Serum titers to NDV were determined by hemagglutination inhibition (HI) tests at 14-DPI. Test sera were diluted twofold in PBS, 4 hemagglutination (HA) U of antigen
(TK/VA/695/85) was added and the mixture was incubated at room temperature for 30 min. An equal volume of 0.5% chicken red blood cells in PBS was added. The HI endpoint was determined as the last dilution with complete inhibition of HA activity (27).

**Statistics.** Antibody titers were analyzed by nonparametric analysis of variance test (Kruskal-Wallis), and, for significantly different groups (P<0.05), Student-Neuman-Keuls multiple comparison test was performed with PC-based software (SigmaStat, Jandel Scientific, San Rafael, CA).

**RESULTS**

**Clinical signs.** No clinical signs were observed in the negative control, NDV- or *E. coli*-inoculated groups throughout Experiment 1. The APV inoculated group had increased mucous in the nasal cavity as compared with negative control poult on 6 and 8 DPI (days post APV inoculation). At day 4, one poult in the APV*/E. coli* group was gasping for breath and was noticeably depressed. This poult died at day 5 with lesions compatible with septicemia and ventricular dilation. The remainder of the APV*/E. coli* infected poult were mildly depressed at days 8-10 postinoculation, but recovered by day 14. Feeders for the APV/NDV group required less feed than those of other groups indicating decreased food consumption for the group from day 8 to the end of the experiment. At day 10, three of the remaining four poult in the APV/NDV group had noticeable nasal exudates with infraorbital, periocular and submandibular swelling that were evident to the end of the experiment (Figure 3.1).

In Experiment 2, no clinical signs were observed in the negative control or NDV-inoculated poult. The APV group was mildly depressed at days 4-6 postinoculation. The poult in the APV/NDV group had reduced food consumption and were lethargic by
day 10 through the remainder of the experiment. Four of the five birds had infraorbital, periocular, and submandibular swelling noted at day 11 through the end of the experiment.

**Virus isolation and RT-PCR.** CPE in cell culture was from lung, trachea and swab samples on all days sampled detected in all groups receiving NDV or APV. Multiplex RT-PCR resulted in the expected 650 bp product for APV and the expected 309 bp product for NDV (Figure 3.2). No APV nucleic acid was detected from the negative control or *E. coli*-inoculated poult at any sample times (Table 3.1). APV was identified in oral swabs of the APV-inoculated group at days 3, 6, 8, and 10 post challenge, with positive trachea and lung samples at days 2, 4, 6 and 14. The APV/*E. coli*-inoculated group was positive for APV early in infection, on days 2 and 4 postinoculation. APV was detected throughout the experiment in the group that received APV/NDV (Table 3.1).

NDV nucleic acid was not detected by RT-PCR in the negative control, *E. coli*, APV or APV/*E. coli* group (Table 3.2). NDV was detected only from oral swabs at day 6 and in lungs on days 4, 6, and 14 from poult s in the NDV group. In the APV/NDV dual infection group, NDV was detected on days 4, 6, and 8 from oral swabs and days 6, 8, and 14 from lung and trachea.

**Histopathology.** Inoculation of poult s with *E. coli*, NDV, or APV produced only mild lesions in the respiratory tract. Poult s inoculated with *E. coli* alone had mild lymphoplasmacytic hyperplasia of the nasal lymphoid tissue and a mild increase in luminal mucus. These lesions were observed primarily at 8 and 14 DPI. Conversely, poult s inoculated with NDV initially developed a mild necrotizing to necropurulent
rhinitis, which eventually matured to lymphoplasmacytic in nature and was accompanied by mild epithelial hyperplasia by 8 DPI. In addition, the NDV poults also developed a mild lymphoplasmacytic airsacculitis that was observed at 8 and 14 DPI. Lesions in the nasal cavity of poults inoculated with APV alone consisted of only mild lymphoplasmacytic inflammation at 8 and 14 DPI. However, APV also induced mild heterophilic conjunctivitis with edema in poults sampled at 4 and 6 DPI. Mild airsacculitis also was observed in both APV-inoculated poults sampled at 6 DPI.

More pronounced lesions were observed in poults inoculated with either APV/NDV or APV/E. coli. Inoculation of poults with APV/E. coli resulted in a heterophilic to lymphoplasmacytic rhinitis and sinusitis with epithelial hyperplasia, with the severity of these lesions varied from mild to marked. In addition, mild conjunctivitis was observed in one poult collected at 4 and 6 DPI. The inoculation of poults with APV/NDV produced the most consistent and severe lesions in the nasal cavity and sinuses, air sacs, and conjunctiva. Lesions in the nasal cavity and sinus consisted of moderate to severe mixed heterophilic to mononuclear inflammation with epithelial hyperplasia and an accumulation of mucus within the cavity. Lesions in the air sacs and conjunctiva in APV/NDV inoculated poults were typically mild to moderate and morphologically resembled those previously described.

**Serology.** Negative control, E. coli- and NDV inoculated groups had no detectable antibodies to APV at 14 DPI. All three groups that received APV had detectable antibody titers at day 14-post infection (Figure 3.3). No significant differences were detected in the titers to APV among the three APV inoculated groups in either Experiment 1 or 2.
No NDV titers were detected in the negative control, APV/E. coli, E. coli, or APV groups. Both the NDV and APV/NDV groups had HI titers to NDV at 14 days post infection (Figure 3.4). No significant differences were detected in the titers to NDV between the two NDV inoculated groups in either Experiment 1 or 2.

DISCUSSION

Natural infections with APV are more severe than experimental infections, suggesting that secondary agents or other factors play a prominent role in the clinical disease process. Many studies have focused on the effects of bacteria and viruses on APV infection with various degrees of success at reproducing the clinical signs seen in the field (13,11,20,26,28,31,43). Experimentally, APV infections are similar in both chickens and turkeys, although some factors appear to affect host susceptibility (10). Experimental infections with the subtype C APV isolates have resulted in various degrees of clinical signs, with the Colorado isolate resulting in milder clinical signs and microscopic lesions than the APV Minnesota 2A isolate (14,23,32). This study demonstrated that coinfection with APV/NDV resulted in reproducible clinical signs comparable with descriptions of APV subtype C field infections, including infraorbital, periocular and submandibular swelling (16). These clinical signs were not observed in the poults receiving APV alone or in the negative control, E. coli, or NDV groups. Poults receiving APV/E. coli exhibited mild clinical signs early during infection, but no swelling of sinuses was observed, as previously described (13, 27). This finding supports the hypothesis that APV infection is exacerbated by dual infections and suggests lentogenic NDV may play a role in the more severe field cases of APV.
The gross and histologic lesions in the APV/NDV-inoculated poult's most closely paralleled those previously reported in turkey flocks naturally infected with APV (12). Poult's inoculated with NDV developed histologic lesions in the nasal cavity and air sacs analogous to those previously described in field cases of lentogenic NDV, including deciliation to epithelial necrosis and edema followed by a lymphoplasmacytic inflammation and epithelial hyperplasia (2,17). The histologic lesions in the upper respiratory tract in the APV group were only minor in comparison with those previously reported for field cases of APV infection (23). Gross lesions of turkeys observed during an APV infection reported from field cases have included the accumulation of mucus in the nares and infraorbital sinuses accompanied by cloudiness and thickening of the air sacs, pericardial sac and peritoneum (23). Histologic changes in the nares and trachea occurring with natural APV infection have consisted of an early loss of cilia followed by submucosal edema and hyperemia, heterophilic infiltrates with exocytosis, and an accumulation of inflammatory exudates within the lumen (7,16,25). After the acute infection, the cellular inflammation matures from heterophilic to mononuclear (25). Only the poult's inoculated with APV/NDV in this investigation had gross and histologic lesions of comparable distribution and severity with those previously reported with spontaneous APV infection. This finding again suggests a synergistic relationship between APV and other respiratory pathogens such as NDV.

No difference in serologic responses to APV and NDV were noted in the single or dual infection groups. This finding differs from those in previous experiments where Ganapathy et al. (20) showed that higher serum ELISA titers for humoral response were noted in dual infection with APV and *Mycoplasma imitans* as compared with single
infection of APV. Because APV generally results in a localized infection of the upper respiratory tract, systemic antibodies may not be the optimal parameter to measure protection or exposure. The ELISA used in these experiments tests only for levels of IgG, whereas measuring local IgA levels may help monitor response to local respiratory infections more efficiently.

APV can generally be detected by virus isolation from 3 to 5 DPI in nasal and sinus tissues (14). In this study, we used RT-PCR to confirm the virus isolation results, differentiate between APV and NDV infections, and extend the detection time as described (6). In the current study, the majority of virus isolation and RT-PCR positive results in the APV group occurred early during infection. The poults in the APV/NDV group had longer periods of virus recovery and a higher percentage of birds were positive at each sampling. The increased severity of clinical signs as well as the increase in duration and number of poults shedding virus indicates that dual infection of APV and NDV resulted in disease more closely resembling field outbreaks. This finding supports the hypothesis that APV disease pattern in the field may be linked to concurrent NDV infections and potentially even live NDV vaccines.

Cook et al. (11) have demonstrated that vaccination with live infectious bronchitis virus (IBV) vaccine and concurrent infection with a pathogenic subtype B APV isolate in chickens resulted in reduced APV recovery and antibody production. Investigators that used IBV and NDV demonstrated that competition or interference occurs when two viruses are known to replicate in the same tissue (11). The competition for the replication site and the knowledge that APV replicates slower in tissue culture than does IBV may explain the apparent decrease in APV virus recovery and subsequent antibody production.
This situation does not appear to have occurred in the present experiment with APV and NDV in turkeys. The differences in the IBV and NDV experiments may be due to host response, differences in inoculation schemes, or the interaction between APV/NDV or that of APV/IBV. Although NDV also replicates more quickly than APV in cell culture systems, the inoculation of APV 3 days prior to NDV appears to have allowed the APV time to infect epithelial cells but did not block the ability of NDV to establish an infection. This is demonstrated by the similar rates of virus recovery and antibody detection in the groups that received single or dual APV and NDV inoculations. Instead, a synergistic effect appears to occur with increased virus recovery detected at each time point as well as increased length of viral shed. Dual infection with APV and *E. coli* caused mild morbidity in the turkey poult on days 8-10 post-APV inoculation. This was also evident by the increased lesions noted upon necropsy and histologic examination. *E. coli* inoculation appears to have decreased APV replication as noted by decreased APV detection in the APV/*E. coli* group compared with the APV alone group. Further studies are needed to completely understand the kinetics and synergistic effects of APV and NDV described in this study.

The results of these experiments demonstrate that dual infection with APV and NDV produces what has been diagnosed as clinical APV. The correlation between increased clinical signs and coinfection with a lentogenic NDV isolate suggests a need for better monitoring of NDV vaccination programs. Reports of some field outbreaks of APV have been associated with increased respiratory disease associated with live NDV vaccination (H. Medina and M. Kumar, pers. comm.). The vaccination of a turkey flock with an underlying APV infection may result in an increase of both NDV and APV
disease. Care should be taken to control or prevent APV infections prior to NDV vaccinations. Increased biosecurity and restricted movement of people and material between APV-infected and noninfected farms is needed to prevent and control APV infections (45).

ACKNOWLEDGMENTS

We would like to acknowledge Joan Beck, Roger Brock, Jack King, Bruce Seal and Darrell Kapczynski for assistance and scientific advisement. This research was supported by USDA/ARS CRIS project 6612-32000-022.

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Table 3.1. RT-PCR results for APV. Time points are listed as days post APV inoculation (DPI). NDV and *E. coli* were inoculated at 3 DPI.

<table>
<thead>
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<th>Group</th>
<th>Inoculum</th>
<th>Sample</th>
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<sup>a</sup>S = oral swab (n=10 for 2 and 4 dpi, n=8 at 6 dpi, n=4 for 10 and 14 dpi), T = trachea (n=2), L = lung (n=2)

<sup>b</sup>- = negative, + = 1 positive, ++ = 2 positive, +++ = 3 positive samples

<sup>c</sup>1 mortality at 5 dpi

<sup>d</sup>NT=not tested
Table 3.2. RT-PCR results for NDV. Time points are listed as days post APV inoculation. NDV and *E. coli* were inoculated at 3 DPI.

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\(^a\)S = oral swab (n=10 for 2 and 4 dpi, n=8 at 6 dpi, n=4 for 10 and 14 dpi), 
\(^b\)T = trachea (n=2), L = lung (n=2) 
\(^c\)1 mortality at 5 dpi  
\(^d\)NT=not tested
Figure 3.1. Turkey poults at 10 days post APV inoculation. A. Poults inoculated with APV/NDV with infraorbital swelling. B. Negative control poult.

Figure 3.2. Multiplex RT-PCR demonstrating the ability to detect both APV and NDV in one sample, representative of experimental data. Lanes 1=molecular weight marker, 2=APV, 3=NDV, 4=APV+NDV, 5=negative control, and 6=molecular weight marker.
Figure 3.3. Serologic response against APV in turkeys at 14 days post inoculation determined by an indirect ELISA.

Figure 3.4. Serologic response against NDV infection in turkeys at 14 days post inoculation determined by HI titers.
CHAPTER 4

EXPERIMENTAL INFECTION OF DUCKS AND TURKEYS WITH SUBTYPE C AVIAN METAPNEUMOVIRUS

SUMMARY. Avian metapneumoviruses (aMPV) cause upper respiratory disease in turkeys. It was originally reported in South Africa and Europe, and subsequently spread to Africa, Asia, and South America. aMPV was first reported in the United States during 1996 and is distinct from aMPV isolates reported from other parts of the world. Migratory waterfowl have been implicated in virus spread. To help determine the role these birds may play in the spread of aMPV, Pekin ducks were intranasally inoculated with aMPV Colorado 1997 and monitored for infection. Virus was isolated from oral swabs, lung, and trachea samples from inoculated ducks. Clinical signs, virus isolation, and seroconversion detected among inoculated ducks were compared to experimentally infected turkeys. This study demonstrated that Pekin ducks were susceptible to aMPV infection.

INTRODUCTION

Avian metapneumoviruses (aMPV) are single-stranded negative-sense RNA viruses that belong to the family Paramyxoviridae, and have recently been placed in the genus Metapneumovirinae (15). Avian metapneumoviruses cause a respiratory disease in turkeys, often called turkey rhinotracheitis (TRT), and have been associated with swollen head syndrome in chickens (7). The disease TRT was first described in South Africa during the 1970’s (6). Avian metapneumoviruses were isolated from clinically infected
turkeys and shown to cause disease in Europe in 1986 (6, 13, 27). Today aMPVs are found in South Africa, Japan, most of Europe, South and Central America, and the United States (7, 12, 22, 26).

Experimentally, aMPV is able to induce respiratory disease in turkeys with clinical signs of infection including sneezing, nasal exudates, depression, and rhinotracheitis (7). The sporadic appearance of aMPV world wide and the recent emergence of the virus in the United States has lead to the speculation that wild bird populations may be involved in the transmission of the virus (7, 12). This study describes the clinical signs, virus isolation, and seroconversion seen in Pekin ducks inoculated with the aMPV Colorado 1997 isolate, as compared with inoculated specific pathogen free (SPF) turkeys.

MATERIALS AND METHODS

Virus. The aMPV isolate from Colorado isolated in 1997 (aMPV/CO) used in this study was obtained from the National Veterinary Services Laboratories (NVSL), Ames, Iowa. The virus was isolated from commercial turkeys in Colorado exhibiting respiratory disease (14). Upon receipt, the isolate was passaged through Vero cells and titer determined by 50% tissue culture infective dose (TCID₅₀) (16).

Animals. Specific pathogen free (SPF) Small Beltsville White turkey embryos were obtained from an in house flock and fertile SPF Pekin duck embryos were obtained from Cornell University, Ithaca, NY. Duck and turkey embryos were incubated until hatch and moved to isolator units housed at Southeast Poultry Research Laboratory and housed in stainless steel isolation cabinets located within negative pressure isolation.
rooms of a Biosafety Level 3 Agriculture facility (1). Feed and water were provided *ad libitum*.

**Experimental design.**

**Experiment 1.** Fifteen four-week-old turkeys were divided into control and experimental groups. Group 1 consisted of 6 control birds, which were sham inoculated via the intranasal route with 200ul of phosphate buffered saline (PBS) (Life Technologies). Group 2 consisted of 9 experimental birds, which received $10^{3.5}$ TCID$_{50}$ of aMPV/CO via intranasal route. Animals were monitored daily for clinical signs of disease. Oral swabs were collected from all groups on days 1, 2, 3, and 5 post inoculation. Sterile tissues were collected from the control birds at 2 days post inoculation (DPI). Two turkeys from the aMPV-inoculated group were sacrificed on days 1, 2, and 5 post inoculation, and sterile lung and trachea samples collected. Serum was collected from control and aMPV inoculated turkeys at 14 DPI.

**Experiment 2.** Ten two-week-old Pekin ducks received $10^{3.4}$ TCID$_{50}$ of aMPV/CO or 200 µl of PBS via intranasal inoculation. Animals were monitored daily for clinical signs of disease. The ducks were orally swabbed 2, 4, and 14 days post inoculation (DPI). On 2, 4, and 14 DPI two ducks from each group were sacrificed and lung and trachea samples collected. Serum was collected from control and aMPV inoculated ducks at 14 DPI.

**Virus isolation.** Samples of lung and trachea, along with oral swabs were taken to detect virus replication in experimental studies. Oral swabs were placed in 1.0 ml of PBS containing antibiotics (1000 units/ml of penicillin, 10 µg/ml of gentamicin, and 5 µg/ml of amphotericin B) (Sigma, St. Louis, MO). Lung and trachea tissues were ground.
using mortal and pestle and prepared with a final suspension of 20% (w/v). Vero cells (an African green monkey kidney cell line) were inoculated with 200 µl of these experimental samples, incubated for 45 minutes, and the media replaced (9). Cells were examined daily for cytopathic effect (CPE), characterized by scattered focal areas of cell rounding and syncytia formation (5, 8). At seven days post inoculation, cells were recorded as positive or negative for CPE, subjected to three rounds of freeze thawing, and 200 µl of fluid passed onto fresh Vero cells. All samples were passed through Vero cells three times before considered negative. All samples were also evaluated by RT-PCR to confirm the presence or absence of aMPV in cell culture.

**RNA extraction.** RNA was extracted from oral swabs or tissues using TRIZOL® reagent (Invitrogen; Carlsbad, California). 200 µl of the swab material was added to 1 ml of TRIZOL®. Vero cells inoculated with experimental samples were subjected to freeze thawing and 200 µl of material added to 1 ml of TRIZOL®. RNA was extracted following the manufacture’s protocol. RNA was resuspended in 30 µl of RNase free water and stored at -70ºC.

**RT-PCR.** RT-PCR was used to detect aMPV viral nucleic acid in swab and tissue samples, as well as to confirm results from virus isolation. RT-PCR was carried out using the One-Step RT-PCR kit (Qiagen, Valencia, CA). 5 µl of sample RNA was added to the RT-PCR mixture with 0.6 µM of each primer. Primers were developed to detect viruses isolated in the United States and amplify a 650 base pair (bp) product of the matrix (M) gene (25). RT-PCR was carried out following the manufacture’s directions in a 25 µl reaction. An additional PCR step was included to amplify low levels of viral nucleic acid not detected with the initial RT-PCR reaction (2). For the second
PCR reaction, 5 µl of the first reaction was added to Qiagen master mix PCR reagents in a total volume of 25 µl and PCR carried out following manufacture’s directions. RNA extracted from viral stocks was used as a positive control, and no template and no RT reactions served as negative controls. PCR products were visualized by ethidium bromide staining of 1% TBE agarose gels.

**Blocking ELISA.** A blocking enzyme linked immunosorbent assay (bELISA) was developed using sucrose-purified aMPV/CO as an antigen and a polyclonal rabbit anti-aMPV for detection (24). Purified virus (2 µg/ml) in a bicarbonate coating buffer was used to coat ELISA plates. Heat inactivated serum was tested at a 1:3 dilution. A polyclonal rabbit antiserum to aMPV/CO was IgG purified, HRP conjugated, and added to the ELISA for detection (11, 21), and o-phenylenediameine dihydrochloride served as substrate. Substrate development was stopped with sulfuric acid following the manufacture’s protocol and read at 490 nm (Sigma). Positive and negative serum samples are determined by differences in absorbance compared to negative controls. Samples are considered positive if they are 3 standard deviations (SD) lower than negative control (3).

**IFA.** Indirect fluorescent antibody staining was performed on Vero cells grown on cover slips inoculated with swab and tissue samples described above to detect aMPV antigen. Vero cells were infected with 200 µl of the third pass of tissues or swabs were incubated for 48 hours and fixed with 80% acetone. For staining cover slips were washed in PBS, incubated at 37°C for 30 min with a rabbit anti-aMPV antibody (1:3,000 dilution), and detected with a fluorescein-isothiocyanate-conjugated goat anti-rabbit IgG
(Vector, Burlingame, CA). Cover slips were inoculated with aMPV/CO (MOI 1) or PBS and incubated for 48 hours to generate positive and negative controls (4).

**Histopathology.** Birds were euthanized by intravenous injection of sodium pentobarbital (100 mg/kg body weight) on sampling days. Tissues taken for histopathology included upper and lower trachea, lung, bursa, spleen, intestines, and nasal cavity. The tissues were fixed in 10% buffered formalin solution, routinely processed in paraffin blocks, sectioned, and stained with hematoxylin and eosin.

**RESULTS**

**Clinical signs.** Clinical signs were mild in both the turkeys and ducks. Three of the aMPV inoculated turkey poults had nasal discharge at days 2, 3, and 5 PI with two turkeys having a mucous plug in the infraorbital sinus at 5 DPI. Increased mucous in the sinus cavity was visible in one out of two of the aMPV ducks necropsied on days 2 and 4 post inoculation. The control groups remained free from clinical signs of infection throughout the experiment.

**Virus isolation.** aMPV was detected in oral swabs from most turkeys, 77.8% 1 DPI and 100% 2 DPI (Table 4.1). By day 3 and 5 PI, fewer turkeys were shedding virus (40%). Virus could also be detected in the trachea and lung samples from the aMPV infected turkeys (Table 4.1). Virus was also detected in 50% of oral swabs from ducks tested on 2, 4, and 14 DPI (Table 4.2). Virus was recovered from lung and trachea samples of ducks sampled on days 2, 4, and 14 DPI (Table 4.2). Control birds were negative throughout the experiment (Table 4.1 and 4.2).

**RT-PCR.** Samples positive for aMPV by RT-PCR had the predicted amplification product of 650 bp. Not all samples positive by virus isolation were
confirmed by RT-PCR. RT-PCR detected aMPV/CO nucleic acids in half of the turkey samples (swabs and tissues) on day 3-post inoculation (Tables 4.1). For the duck experiment, aMPV/CO was detected in oral swabs on days 2, 5, and 14 post infection (Table 4.2). Tracheas and lungs were positive for virus on day 3 in the turkeys. In the ducks, tracheas were positive on days 2, 4, 14, and lung samples were positive at days 2 and 4 post inoculation (Table 4.1 and 4.2). Control birds remained negative throughout the experiment (Table 4.1 and 4.2).

**IFA.** Indirect fluorescent antibody tests for aMPV antigen in Vero cell cultures confirmed CPE and RT-PCR results of aMPV/CO infected turkeys (Table 4.1). The IFA assay results were similar to results of CPE and RT-PCR, with sensitivity being similar to CPE and slightly less than RT-PCR.

**Blocking ELISA.** Antibodies are shown as absorbance values in Figure 4.1. For the turkey serum, a sample with an absorbance less than 0.236 would be considered positive, while samples with an optical density (O.D.) above 0.236 are negative. Serum samples collected from control turkeys and ducks and did not have antibodies for aMPV, while experimentally inoculated birds had detectable antibodies at 14 DPI.

**Histopathology.** Histological lesions were more severe in the turkeys as compared to the Pekin ducks. Two of six turkeys developed mild epithelial hyperplasia of the ventral turbinates (2 and 5 DPI), and one of these turkeys also had mild lymphoplasmacytic inflammation of the infraorbital sinus (5 DPI). One turkey (5 DPI) had severe heterophilic to lymphocytic rhinitis, which was accompanied by deciliation and mild necrosis to early squamous metaplasia of the respiratory epithelium. Similar to the two other affected turkeys, lesions in this bird were most severe in the ventral
turbinates and infraorbital sinuses. In addition to lesions in the nasal cavity, mild heterophilic air sacculitis (2/6), mild focal heterophilic conjunctivitis (2/6), and mild hyperplasia of the bronchiolar-associated lymphoid tissue (BALT) of the lung (3/6) also were observed in the turkeys with lesions in the upper respiratory tract. In contrast to the turkeys, lesions induced by pneumovirus inoculation of Pekin ducks were not observed.

DISCUSSION

The origin of avian metapneumovirus in South Africa and its emergence in Europe suggests a role for waterfowl or other migratory birds in the transmission of the virus (5, 13, 27, 28). aMPV has been identified in wild birds captured on aMPV infected turkey farms in Minnesota, indicating these birds can support at least limited virus replication (20). Experimental studies with Pekin ducks inoculated with a subgroup B virus failed to result in seroconversion, virus isolation, or clinical signs of infection (10). This differs from similar experiments with ducks and an aMPV isolated from Minnesota, which resulted in virus isolation and seroconversion (19). Additionally an aMPV was isolated from field cases among Muscovy ducks in France. This virus is more closely related to the subtype C viruses found in the US than to the A and B subtypes found in Europe based of immunofluorescence staining and ELISA assays (7, 23). The differences between the duck experiments may be explained by differences in virus strains. The aMPV isolates in the United States differ from European and other aMPV isolates in sequence of their M and F genes, lack of ability to cause cilia stasis, and lack of neutralization by monospecific antibodies to A and B isolates (7, 8, 17, 18). These differences may also result in differing species susceptibility. Theoretically, if aMPV
infection in the United States was the result of a direct introduction from wild birds, the first virus isolated in the US should be more capable of infecting wild birds.

In this study, Pekin ducks were inoculated with the first aMPV isolated in the US, aMPV/CO/97, and compared to infected turkeys. The resulting clinical signs and lesions of the aMPV infection were limited to the respiratory tract in both turkeys and ducks. The aMPV-inoculated turkeys had an increase of mucous in the nasal cavity, with two turkeys having mucous plugs in the infraorbital sinus early during infection. Similar lesions were observed in the Pekin ducks and histological lesions were more severe in the turkeys than in the Pekin ducks. These lesions were limited to epithelial cells of the respiratory tracts along with mild inflammation in the sinus cavity. Air sacculitis, conjunctivitis, and rhinitis, accompanied by deciliation and mild necrosis was observed in the respiratory epithelium of the turkeys. In contrast, no lesions were induced by aMPV/CO inoculation of the Pekin ducks.

Virus was detected in oral swabs, trachea, and lung samples of the aMPV/CO inoculated Pekin ducks and turkeys by virus isolation, RT-PCR, and IFA of Vero cell cultures throughout the experiment. The VI and IFA assays were included to validate the RT-PCR test. The RT-PCR assay was more sensitive than either the VI or IFA assays as previously reported (7). Along with increased sensitivity, the RT-PCR assay was needed to determine the specificity of the CPE in cell culture. The blocking ELISA demonstrated aMPV/CO specific antibodies in both the ducks and turkeys at 14 DPI. The presence of virus and antibodies in both the ducks and turkeys demonstrates ducks can be infected with the aMPV Colorado isolate, support virus replication, and shed virus.
similar to turkeys. This data demonstrates that waterfowl are capable of acting as a potential reservoir for aMPV, helping to explain the appearance of aMPV worldwide.

ACKNOWLEDGMENTS

The authors would like to acknowledge University of Georgia Polyclonal Antibody Production Service for the production of the rabbit polyclonal antiserum and Cam Green for purification and HRP labeling of the antibody, and Joan Beck for assistance in the laboratory. This research was supported by USDA/ARS CRIS project 6612-32000-022.

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17. Seal BS. Matrix protein gene nucleotide and predicted amino acid sequence demonstrate that the first US avian pneumovirus isolate is distinct from European strains. Virus Res 1998; 58:45-52.


## Table 4.1. Results from the virus isolation, RT-PCR, and IFA assays from the aMPV/CO inoculated turkeys.

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## Table 4.2. Results from the virus isolation, RT-PCR, and IFA assays from the aMPV/CO experimentally inoculated ducks.

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Figure 4.1. Blocking ELISA analysis of 14-day serum samples from ducks and turkeys infected with aMPV/CO. Control birds (horizontal lines), aMPV inoculated birds (vertical lines), negative control serum (diagonal lines), and positive control serum (dashed lines) were determined to be positive for aMPV antibodies if the OD was below 0.236 and negative for aMPV antibodies if the OD was above 0.236.
CHAPTER 5
BINDING OF AVIAN METAPNEUMOVIRUS COLORADO TO VERO CELLS:
POTENTIAL ROLE FOR HEPARAN SULFATE AND CX3CR1

1Turpin, E.A. and D.E. Swayne. To be submitted to Virus Research.
SUMMARY

Avian metapneumoviruses (aMPV) are responsible for an upper respiratory infection in poultry. Although aMPV have been studied since their identification in 1986, little is known about receptor binding to host cells in initiation of the infection process. For this study, a variety of enzymatic treatments and compounds were tested in an attempt to identify host proteins involved in virus attachment. Heparinase I and III reduced aMPV binding to host cells in a dose dependent manner. The incubation of aMPV with heparin or heparan sulfate also reduced virus binding in a dose dependent manner. These results indicate that heparan sulfate was involved in aMPV attachment to host cells. CX3CR1 also appears to be involved in aMPV attachment, but to a lesser extent. The addition of free fractalkine (a CX3C chemokine), an anti-CX3CR1 antibody, and an anti-fractalkine antibody are all capable of reducing aMPV binding. This indicates that heparan sulfate and CX3CR1 are both responsible for aMPV attachment to Vero cells.

INTRODUCTION

Avian metapneumoviruses (aMPV) were first isolated and identified as pneumoviruses in 1986 (3, 8, 7, 16, 26). aMPV are responsible for upper respiratory infections of poultry, primarily turkeys, characterized by nasal discharge, tracheal rales, foamy conjunctivitis, and sinusitis (4, 12). aMPVs are in the family Paramyxoviridae, subfamily Pneumovirinae, and are members of the new genus Metapneumovirus (MPV) (4, 20). The MPVs differ from the members of the Pneumovirinae subfamily by lacking the nonstructural proteins, NS1 and NS2, and in gene order (27). The only other member
of the *Metapneumovirus* subfamily is the newly isolated human metapneumovirus (hMPV) (25).

The original aMPVs isolated in Europe were designated subtypes A and B. During 1997, subtype C aMPVs were identified in the United States, and subtype D viruses were subsequently identified among Muscovy ducks in Europe (2, 4, 21, 22). The hMPVs were first isolated from children in the Netherlands during 2001 and have recently been identified in Australia, Canada, and the United States. However, serologic evidence of hMPV infection can be found in serum archives as far back as the 1950s (11, 17, 19, 25). Surprisingly the hMPVs found around the world, appear to be more closely related to the subtype C aMPV found only in the United States, than to aMPV A and B subtypes found in Europe (25).

An important aspect in viral pathogenesis is identifying the steps required for attachment and infection of target cells. aMPVs have three surface proteins, the glycoprotein (G), fusion (F) protein, and short hydrophobic (SH) proteins. The G protein is believed to be the major attachment protein because antibodies specific for the G protein neutralize the virus (13). The F protein is involved in cell fusion, but there is no identified function for the SH protein (15, 23). If the aMPVs behave like other members of the *Paramyxoviridae* family, both G and F proteins are most likely involved in attachment to target cells. In order to better understand the virus-cellular interactions, this research has focused on treating aMPV or Vero cells and determining the effect of virus binding to identify cellular proteins involved in attachment. A broad range of methods was tested to determine the effects on virus attachment. Subsequent experiments later focused on treatments that reduced binding of virus to cells.
MATERIALS AND METHODS

**Virus and Cells.** The aMPV subtype C isolate, aMPV/turkey/Colorado/97 (aMPV/CO/97) was obtained from the National Veterinary Service Laboratory, Ames, Iowa. The virus was originally isolated from commercial turkeys in Colorado with respiratory disease (18). Vero cells were inoculated with aMPV and frozen at –70°C at 72 hr post infection. Infected cells were subjected to three rounds of freeze thaws. Cell debris was removed by clarification at 10,000x g for 30 minutes at 4°C. The virus was pelleted by ultra centrifugation at 80,000x g for 2 hr and resuspended in phosphate buffered saline (PBS) and then centrifuged through a 20/60% (wt/vol) sucrose cushion (76,000x g for 2 hr). The virus band was collected and diluted in PBS and overlaid on a sucrose gradient (20-70%) for 76,000x g for 2 hr (9). The virus band was collected and resuspended in a 1:6 volume of PBS and pelleted as described above. The purified virus was rinsed to remove sucrose and resuspended in PBS to a concentration of approximately 1 mg/ml. The protein concentration was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL), and TCID<sub>50</sub> performed to determine infectivity. Biotin labeled aMPV (biotin-aMPV) was generated by incubating the sucrose-purified aMPV with EZ-Link Sulfo-NHS-Biotin (Pierce) following the manufacture’s protocol. Free biotin was removed by dialysis and the amount of biotin labeled virus was determined by western blot analysis. The sucrose-purified aMPV was titrated in Vero cells before and after incubation with biotin. The biotin labeling did result in the reduction of virus infectivity, from 2 x 10<sup>8</sup> TCID<sub>50</sub>/ml to 1 x 10<sup>5</sup> TCID<sub>50</sub>/ml, but the
biotin-aMPV was able to replicate in Vero cells and produce cytopathic effect (data not shown).

**Treatments.** Vero cells were treated with the following enzymes at 37 C for 1 hr; heparinase I, heparinase III, neuraminidase, trypsin (Sigma, St Louis MO), and chondroitinase ABC (Calbiochem, La Jolla, CA). Vero cells were incubated with the following reagents at 4 C for 1 hr; anti-CX3CR1 antibody (Sigma), recombinant human fractalkine (R&D Systems, Minneapolis, MN), EDTA, or EGTA (Sigma). aMPV was incubated with the following reagents for 1 hr at 4 C; heparan sulfate, heparin (Sigma), anti-human fractalkine antibody (R&D). Vero cells were stained with both the anti-CX3CR1 and anti-fractalkine antibodies to determine expression. Both antibodies identified cells expressing both fractalkine and its receptor as compared to isotype controls. Vero cells bound to virus were detected with streptavidin-FITC (Pierce, Rockford, IL) or FITC anti-rabbit IgG antibody (Vector Laboratories, Burlingame, CA).

**Binding Studies.** Vero cells were washed with PBS and detached from the cell culture flask by mechanical disruption. After counting, 500,000 cells were washed with cold MEM, incubated with treatments for 1 hr, and washed with cold MEM. Virus was then added and incubated for 1 hr at 4 C followed by washing with cold MEM. For reduction of binding studies, 25 µg of biotin-aMPV was incubated at 4 C for 1 hr (25 µg of virus = 2.5 x 10⁶ TCID₅₀). Virus binding was detected by streptavidin-FITC or rabbit anti-aMPV antibody (1:500) followed by FITC-anti-rabbit IgG antibodies. Cells were incubated and washed with cold MEM without serum.

**Flow Cytometry.** Flow cytometry analysis was done with a Beckman Coulter Epics XL flow cytometry. 500,000 cells were treated as described above, and
resuspended in 1 ml of cold MEM for analysis. The flow rate was set to approximately
150 cells/second and at least 10,000 cells analyzed. To determine the effects of
treatments, no virus and virus alone groups were included as negative and positive
controls in all experiments. All data presented is representative of at least three
experiments.

RESULTS

**aMPV binding to Vero cells.** Binding of aMPV to Vero cells was measured
using unlabeled aMPV, as well as biotin-linked virus. Vero cells were first treated with
sucrose-purified aMPV ranging from 0 to 392 µg and presence of bound virus was
detected using an anti-aMPV antibody. In this assay, aMPV bound to Vero cells was
detected, and the amount of binding increased with increasing amounts of virus (Figure
5.1A). This binding was then compared with that of the directly labeled biotin-aMPV.
The results of these assays were comparable with those using unlabeled virus (Figure
5.1B). In both assays, increasing concentrations of virus resulted in an increased
percentage of fluorescent cells (Figure 5.1). Although high levels of fluorescent cells
were detected with the biotin-aMPV, saturation was not achieved even at highest virus
congen~trations as can be seen with the increasing mean intensity of fluorescence (Figure
5.1). To verify that binding was specific, unlabeled aMPV was used to block the binding
of biotin-aMPV to Vero cells (Figure 5.2). The addition of the unlabeled virus reduced
biotin-aMPV binding to the Vero cells from 95% to 50%.

**Effect of treatments on biotin-aMPV binding to Vero cells.** A variety of
enzymes and compounds were incubated with virus or cells and the effect on biotin-
aMPV binding was determined. No virus controls resulted in 5% fluorescent cells while
the addition of 25 µg of biotin-aMPV resulted in 86% fluorescent cells. Vero cells were subjected to different treatments to measure their effect on virus binding (Figure 5.3). The addition of neuraminidase, which cleaves sialic acid, resulted in increased binding of biotin-aMPV to the surface of cells by approximately 10%. Chondroitinase ABC, which cleaves chondroitin sulfate and derrmantin sulfate, and EDTA had little effect on virus binding (less than 5%). While EGTA (26%), heparinase I (62%) and III (30%), as well as trypsin (38%), all resulted in decreased binding of aMPV to the cell surface (Figure 5.3).

**Dose dependent effects of treatments on biotin-aMPV binding to Vero cells.** The effect of concentration was tested with chondroitinase ABC, heparinase I and heparinase III. Increasing concentrations of heparinase I and III resulted in decreasing percentages of fluorescent cells as measured by cytometry (Figure 5.4A). No effect was noted with the chondroitinase ABC. Due to the effects of heparinase I and III on reduction of binding, heparin and heparan sulfate were tested for their ability to reduce virus binding. Incubation of biotin-aMPV with either heparin or haparan sulfate resulted in decrease percentage of fluorescent cells in a concentration dependent manner (Figure 5.4B).

**Effect of fractalkine, anti-fractalkine antibody, and anti-CX3CR1 antibody on biotin-aMPV binding to Vero cells.** The results obtained with the panel of cellular receptor inhibitors above suggest a role for heparin and heparan sulfate in the binding of aMPV to susceptible cells, but all binding was not inhibited indicating a role for a second binding site. This is similar to what has been reported for pneumoviruses, specifically RSV, which has been reported to bind to both heparan sulfate and CX3CR1 (5, 6, 10, 24).
Consequently, Vero cells were incubated with fractalkine or anti-CX3CR1 antibody. This resulted in an 18% or 33% reduction in aMPV binding to Vero cells, respectively (Figure 5.5). The addition of the anti-fractalkine antibody to the aMPV also resulted in a 21% reduction of the percentage of fluorescent cells detected by flow cytometry (Figure 5.5).

**DISCUSSION**

aMPV are an emerging virus that belongs to the *Paramyxoviridae* family, subfamily *Pneumovirinae*, and genus *Metapneumovirus*. Although much is known about the binding events of other members of this family and subfamily, little is known about the binding events involved in aMPV infections. To better understand the receptors on the surface of the cell that interacts with the virus, Vero cells were subjected to a variety of treatments and virus binding was assayed.

The presence of viral receptors on Vero cells was tested. Initially sucrose purified virus was examined by flow cytometry for its ability to bind to Vero cells. This was accomplished by incubating Vero cells with increasing concentrations of aMPV at 4°C followed by the addition of a rabbit polyclonal anti-aMPV antibody and then FITC-anti-rabbit IgG. This procedure did result in concentration dependent binding of aMPV to Vero cells, but required a large amount of virus to achieve 50% positive staining of cells. Biotin labeled aMPV was detected by western blot and determined to remain infectious for Vero cells indicating that the biotin-aMPV was still able to bind to cells and replicate in cell culture. Incubating cells with increasing concentrations of biotin-aMPV resulted in an increased percentage of fluorescent cells, indicating virus binding in a dose dependent manner. Although 90% staining was achieved utilizing 25 µg of virus with a
mean intensity of fluorescence (MIF) of 40%, increasing virus concentrations up to 188 µg only resulted in 98% fluorescent cells but with an increase in MIF to 90%. This indicates that saturation of cellular receptors was not achieved even with 188 µg of biotin-aMPV. This could be due to multiple cellular and viral receptors.

Vero cells were treated with various enzymes and compounds to help determine what cellular proteins may be involved in virus binding. The effects of neuraminidase on cell binding were examined due to the utilization of sialic acid receptor by members of the paramyxovirus family (14). Neuraminidase treatment of Vero cells resulted in increased binding indicating that the cleavage of sialic acid potentially unmasked a binding site, which resulted in the increased aMPV binding. Other treatments utilized in this study cleave major glycosaminoglycans that can be found on the surface of many cells and have been reported in virus binding (5). EDTA and chondroitinase ABC had no effect on virus binding, suggesting that chondroitin sulfate and dermatan sulfate are not involved with aMPV binding. To determine if heparin or heparan sulfate are involved, Vero cells were incubated with heparinase I and III. This incubation resulted in a concentration dependent reduction in aMPV binding. The cleavage of heparin and heparan sulfate resulted in an inhibition of aMPV binding up to approximately 60% with both enzymes. Since, heparin and heparan sulfate appeared to be involved in aMPV binding, heparin and heparan sulfate were added to block receptors and prevent binding. Binding inhibition was noted in a concentration dependent manner with both reagents. The ability of free heparin, heparan sulfate, and the heparinase enzymes to reduce aMPV binding indicates that heparan sulfate residues on the surface of the cell are involved in virus binding.
Although binding was reduced by as much as 60% by incubation with heparinase I, heparinase III, heparin, or heparan sulfate, complete inhibition was not achieved. This indicates a role for a second receptor in aMPV binding to Vero cells. A CX3C motif has been identified in the glycoprotein of aMPV (1). To determine if this motif functions in aMPV binding to Vero cells, antibodies to the CX3C motif and receptor were examined. Vero cells were first examined for the expression of the CX3CR1 and fractalkine, a CX3C chemokine. Both the receptor and chemokine are present on the cells as detected by flow cytometry (data not shown). The addition of the CX3CR1 antibody to Vero cells prior to biotin-aMPV resulted in a decrease in the percentage of fluorescent cells, indicating that CX3CR1 has a role in aMPV attachment. To further determine the role of this motif in binding, cells were incubated with recombinant human fractalkine, and biotin-aMPV was incubated with an antibody to fractalkine. The addition of these treatments reduced the percentage of fluorescent cells to levels seen with the CX3CR1 antibody, again, indicating a role for this receptor in aMPV binding.

Heparan sulfate was suspected to be involved in aMPV binding due to studies with RSV where heparin-binding domains have been identified in the G protein (5, 6, 10). Similar domains appear to be in the G protein of the aMPV although their function has not been shown (1). Along with the similarities seen with the heparin binding motifs in the aMPV G protein to those described in RSV, similarities also exists in the CX3C motifs (1, 24). This study indicates that like other viruses, aMPV utilizes more than one component of the cells surface for attachment. Both heparan sulfate and CX3CR1 appear to function in aMPV attachment to the Vero cells. This study only begins to understand the complex interactions between the virus and the cells. More studies are needed to
determine which viral proteins and which specific regions or the proteins are involved in attachment. Although this study has identified two cell surface components involved in attachment, other things may be involved in attachment.

REFERENCES


Figure 5.1. Dose response of aMPV binding to Vero cells measured by flow cytometry. (A) Vero cells were incubated with increasing concentrations (0, 19, 39, 76, 196, and 392 µg) of sucrose purified aMPV at 4°C for 1 hr. Vero cells bound to aMPV were detected using a rabbit polyclonal anti-aMPV antibody followed by a FITC-anti-rabbit IgG antibody. (B) Vero cells were incubated with increasing concentrations (1, 6, 12, 25, 62, 100, and 188 µg) of biotin-aMPV at 4°C for 1 hr. Vero cells bound to biotin-aMPV were detected using FITC-streptavidin. Percentage of fluorescent cells and mean intensity of fluorescence were measured.
Figure 5.2. Specificity of the biotin-aMPV binding to Vero cells. (A) 24 µg of biotin-aMPV (gray) resulted in 95% fluorescent cells compared to the no virus control (white). (B) The addition of 39 µg of unlabeled virus (gray) prior to incubation with biotin-aMPV resulted in 50% fluorescent cells.

Figure 5.3. Effect of treatments on biotin-aMPV binding to Vero cells. Cells were incubated at 4 C (EDTA and EGTA) or 37 C (trypsin, neuraminidase, heparinase I, heparinase III, and chondroitinase ABC) with various treatments for 1 hr, incubated with biotin-aMPV, and detected with FITC-streptavidin.
Figure 5.4. Enzymatic treatment of Vero cells results in a decrease in aMPV binding. (A) Vero cells were treated with 1, 0.3, or 0.6 U of heparinase I, heparinase III, or chondroitinase ABC at 37 C for 1 hr. Cells were then washed and incubated with biotin-aMPV and fluorescent cells detected using FITC-streptavidin. (B) Vero cells were treated with increasing concentrations of heparin or heparan sulfate at 4 C for 1 hr. Cells were then washed and incubated with biotin-aMPV and fluorescent cells detected using FITC-streptavidin.
Figure 5.5. Effect of fractalkine, anti-fractalkine, and anti-CX3CR1 on biotin-aMPV binding. Vero cells were incubated with anti-CX3CR1 or fractalkine at 4 C for 1 hr prior to the addition of biotin-aMPV. The anti-fractalkine antibody was incubated with the aMPV prior to incubation with the Vero cells. The percent of fluorescent cells were detected by the addition of FITC-streptavidin.
CHAPTER 6
DEVELOPMENT AND EVALUATION OF A BLOCKING ELISA FOR THE
DETECTION OF SUBTYPE C AVIAN METAPNEUMOVIRUS ANTIBODIES IN
MULTIPLE DOMESTIC AVIAN SPECIES

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ABSTRACT

The first cases of infection by avian metapneumoviruses (aMPV) were described in turkeys with respiratory disease in South Africa during 1978. The causative agent was isolated and identified as a pneumovirus in 1986. aMPV antibodies have been detected in domestic non-poultry species in Europe, but such tests are currently not available in the United States. To understand the potential role of domestic ducks, geese, and wild waterfowl in aMPV epidemiology, we developed a blocking enzyme-linked immunosorbent assay (bELISA) for the detection of anti-subtype C aMPV (aMPV-C) antibodies. This assay method overcomes the species-specific platform of indirect ELISAs to allow detection of aMPV-C-specific antibodies from potentially any avian or mammalian species. Evaluation of the bELISA was initially done with experimental turkey serum samples and found to correlate with virus neutralization assays and indirect enzyme-linked immunosorbent assay (iELISA). One thousand serum samples from turkey flocks in Minnesota were evaluated by the bELISA, and found to agree 94.9% with the results obtained by the iELISA. In addition, the bELISA could detect anti-aMPV-C antibodies from experimentally infected ducks, indicating its usefulness in screening serum samples from multiple avian species. This is the first diagnostic assay for the detection of aMPV subtype C antibodies from domestic turkeys, geese, and ducks in the United States.

INTRODUCTION

Avian metapneumoviruses (aMPV) belong to the family Paramyxoviridae, subfamily Pneumovirinae, and genus Metapneumovirus (20). aMPV causes an acute upper respiratory disease characterized by coughing, nasal discharge, tracheal rales,
foamy conjunctivitis, and sinusitis that have been reported principally in turkeys (3, 7, 22). Cases have also been identified in chickens, ducks, pheasants, and guinea fowl (10). The first cases were described among turkeys in South Africa during 1978, and the causative agent was isolated and identified as a pneumovirus during 1986 in Europe (4, 15, 18). Since its initial identification in Europe, the virus has been detected throughout most of Europe, Japan and South America (1, 7, 26). In 1997, the first avian metapneumovirus was isolated from commercial turkeys in Colorado suffering from a respiratory disease, and this strain was found to differ from previously reported aMPV isolates (9, 21).

Identification of aMPV infection of turkey flocks routinely involves serology, reverse transcriptase PCR (RT-PCR) and virus isolation assays (7, 11). RT-PCR and virus isolation are generally labor intensive, expensive, and are dependent on duration of virus replication in the animal, which usually terminates before clinical signs develop (17). Viral serologic evidence is present long after infection (7). The ability to use serum antibodies to determine present or past infection increases the possibility of discovering if birds have been exposed to aMPV while adding the ease and low cost of serologic testing.

Avian metapneumoviruses have been tentatively designated as subtype A, B, C or D based on virus neutralization and genomic sequence analysis (6, 7, 16). Subtype A and B viruses currently are found in Europe, Japan, and South and Central America, subtype D in France and subtype C only in the United States (1, 3, 7, 9, 26). Due to the differences in amino acid sequences of the viral protein, serologic tests are not cross-reactive for all subtypes (7).
There have been many ELISA assays developed for the detection of antibodies to aMPV. ELISAs for aMPV-C currently available in the United States use whole virus prepared from lysed cell culture as an antigen and depends on anti-turkey or anti-chicken secondary antibodies for detection (5). Based on this assay, aMPV infections in the United States are currently detected only in Minnesota. In 1999, 37% of turkey flocks in Minnesota were positive for aMPV antibodies by ELISA, while 48.7% were positive in 2000 (5, 13). Additionally, Gulati, et al, (13, 14) have developed two recombinant ELISAs using matrix or nucleocapsid protein as antigen for the detection of antibodies to aMPV-C. Although these ELISAs are sensitive and specific they can only detect antibodies from turkey and chicken samples.

aMPV has been reported in farm-reared pheasants, ducks, and guinea fowl outside of the United States (7, 10, 28, 25). Much of the research in the US has focused on turkeys and wild birds, with little emphasis on farm raised ducks and geese. The presence of the virus in experimentally infected ducks and the resent isolation from wild geese demonstrate that these birds may also harbor the virus (22, 23). A quick and inexpensive test is necessary to determine the infection status of domestic geese and ducks, which could also be used with other poultry or wild birds. To address this shortfall and to better understand the epidemiology of aMPV, we developed a blocking ELISA (bELISA) for the detection of aMPV-C antibodies with greater host diversity.

**MATERIALS AND METHODS**

**Virus Purification.** The aMPV-C isolate, aMPV/turkey/Colorado/97 (aMPV/CO) was obtained from the National Veterinary Services Laboratories, Ames, Iowa. The virus was originally isolated from commercial turkeys in Colorado with
respiratory signs (19). Vero cells were inoculated with aMPV/CO and frozen at –70°C at 72 hours post infection. Infected cells were subjected to three rounds of freeze-thaws. The virus was inactivated by β-propiolactone (BPL) treatment (0.1%) (Sigma, St. Louis, MO) at room temperature for two hours, followed by overnight refrigeration. Cell debris was removed by clarification at 10,000x g for 30 minutes at 4°C. The virus was pelleted by ultra centrifugation at 80,000x g for 2 hours and resuspended in 2M Tris buffer, pH 8.8 and then centrifuged through a 20/60% (wt/vol) sucrose cushion (76,000x g for 2 hours). The band was collected and diluted in Tris buffer and overlaid on a sucrose gradient (20-70%) for 76,000x g for 2 hours (12). The virus band was collected and resuspended in a 1:6 volume of Tris buffer and pelleted as described above. The purified virus was rinsed to remove sucrose and resuspended in Tris buffer to a concentration of approximately 1 mg/ml. The protein concentration was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL). This purified product was used as antigen in antibody production, and as antigen for both indirect and blocking ELISAs.

**Anti-aMPV antibody production.** The Polyclonal Antibody Production Service, University of Georgia, Athens, GA prepared aMPV antiserum. New Zealand white rabbits received 0.5 ml (225 µg) of inactivated purified aMPV antigen with Freund’s complete adjuvant. Subsequent immunizations were given at 21 and 58 days with Freund’s incomplete adjuvant and 225 µg of antigen. Rabbits were bled at 91 days after the initial immunization. Immunoglobulins were purified from total serum proteins using T-Gel Purification Kit (Pierce). The purified rabbit polyclonal samples were then directly conjugated to horseradish peroxidase (HRP) using EZ-Link Activated Peroxidase Antibody Labeling Kit (Pierce). The HRP conjugated rabbit anti-aMPV antibody has
been tested by ELISA and western blot analysis and does not react with non-infected Vero cells or other avian viruses.

**Serum samples.** *Turkey hyperimmune serum:* Eleven specific pathogen free four-week-old Small Beltsville White turkeys, obtained from an *in house* flock, received a subcutaneous injection of BPL inactivated aMPV/CO (40 µg) in an oil emulsion vaccine or sham control in a 1 ml dose behind the neck. The turkeys were vaccinated again at 2 weeks and bled at 28 days after the initial inoculation.

*Turkey convalescent serum:* Turkeys were obtained from British United Turkeys of America (BUTA), (Lewisburg, WV). At two weeks of age, the turkey poultls were inoculated via an intranasal route with live aMPV/CO (10^{4.5} TCID_{50}/ml), *E. coli* (10^7 colony forming units/ml), Newcastle disease virus (NDV) (10^5 50% egg infectious dose/ml), or sham inoculated as previously described (27). Serum samples were collected at day 0, 8, and 14 post inoculation.

*Field serum samples:* 1000 individually labeled turkey serum samples were obtained from the Minnesota Poultry Testing Laboratory (MPTL), Willmar, MN. The serum samples were individually labeled and tested with both the bELISA and diagnostic iELISA (described below).

**Experimental infection of Pekin ducks.** SPF Pekin duck eggs were obtained from Cornell University, Ithaca, NY. Once hatched, the ducks were housed in stainless steel isolation cabinets in negative pressure isolation rooms within a Biosafety Level 3 Agriculture facility at SEPRL, Athens, GA. Ducks were inoculated at two weeks of age with 10^{3.4} TCID_{50} of aMPV/CO in a 0.2 ml dose via intranasal route. Serum samples were taken at 0 and 14 days post inoculation.
**Virus neutralization test.** Virus neutralization (VN) assay was preformed in replicates of four. Each serum sample was assayed on Vero cells in a 96-well flat-bottomed tissue culture plate. Test serum, diluted 1:2 to 1:512, was added to 100 TCID$_{50}$ of aMPV/CO and incubated for 30 min at 37 C. After incubation, 50 µl was inoculated onto Vero cell monolayers and incubated for 30 minutes, followed by the addition of 150 µl of minimum essential media (MEM) with 3% fetal bovine sera and monitored for cytopathic effect (24).

**Indirect ELISA.** Two indirect ELISA tests were used during this study. An indirect ELISA (iELISA) was developed at Southeast Poultry Research Laboratory to test experimental samples as previously described (27). Briefly, ELISA plates were coated with 2 µg/ml of purified aMPV/CO and incubated overnight. After blocking, test serum was added at a starting dilution of 1:25 followed by 5-fold dilutions. A HRP-conjugated goat anti-turkey IgG antibody (Southern Biotech, Birmingham, Alabama) was added to detect bound antibodies. Serum samples with an optical densities (OD) of three standard deviations above the negative controls were considered positive for aMPV antibodies. Each plate contained multiple positive and negative serum samples to serve as controls. The second iELISA used in this study was the diagnostic iELISA currently used at the Minnesota Poultry Testing Laboratory (5).

**Blocking ELISA.** Immulon 4HBX (Dynex, Chantilly, VA) plates were coated with 2 µg/ml of purified aMPV/CO and incubated overnight at 4 C in 0.1 M bicarbonate coating buffer, pH 9.6. Plates were blocked with 300 µl of 1% polyvinylpyrrolidone (PVP) for 1 hour at room temperature (Sigma). Test serum was added at a 1:5 dilution in a 100 µl volume and incubated for 1 hour at room temperature. Plates were washed with
PBS-Tween 20 (0.05%). After washing, HRP-conjugated rabbit anti-aMPV/CO was added (100 µl of 1:500 dilution) and detected using an O-phenylenediamine dihydrochloride substrate. Substrate development was stopped with the addition of 2M sulfuric acid and OD determined at 490 nm. Serum samples with an OD three standard deviations below the negative controls were considered positive. Each plate contained multiple positive and negative serum samples to serve as controls. Non-inoculated Vero cells, avian influenza virus (AIV) serum samples, NDV serum samples, and bovine respiratory syncytial virus (bRSV) serum samples were included as negative controls. Optimal antigen and rabbit anti-aMPV/CO-HRP-antibody concentrations were determined using standard checkerboard titration (8).

**Western blot analysis.** Purified aMPV/CO was separated by SDS-PAGE using a 10% polyacrylamide gel (BioRad, Hercules, California) under reducing conditions, and transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline with 0.01% Tween 20 (TBST) for 1 hr (2). Serum samples were added at a 1:50 dilution and incubated for 1 hour at room temperature or 4 C overnight. The membrane was washed in TBST, incubated with goat anti-turkey IgG-HRP or goat anti-rabbit IgG (Southern Biotech) and detected by enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Piscataway, NJ).

**Statistics.** Results from the iELISA and the bELISA were tested for correlation using Pearson Product Moment Correlation with PC-based software (SigmaStat, Jandel Scientific, San Rafael, CA).
RESULTS

Experimental turkey samples.

Turkey hyperimmune serum. Eleven turkey serum samples from the sham and aMPV-C vaccinated birds were tested for aMPV antibodies using a VN assay and iELISA. Eight of the nine aMPV-vaccinated turkeys were found to have neutralizing antibodies to aMPV/CO as detected by the VN test (Figure 6.1). All nine of the aMPV vaccinated turkeys had detectible antibodies to aMPV/CO as determined by both the iELISA and bELISAs. No aMPV antibodies were detected in the two sham vaccinated turkeys with any of the assays.

Turkey convalescent serum. All BUTA turkeys receiving live aMPV/CO inoculation developed antibodies to the virus by 15 days post inoculation (Figure 6.2). Serum samples from E. coli, AIV, or NDV inoculated turkeys showed no VN activity, and were negative by both the iELISA and bELISA. Although some of the aMPV/CO inoculated turkey serum samples taken at 8 DPI did have detectable antibodies with the iELISA, the bELISA and VN assay did not detect antibodies (data not shown). aMPV infected birds had detectable antibodies by 14 DPI (Figure 6.2).

Field turkey samples.

Comparison of ELISA tests: Turkey field serum samples (1000) collected during 2001 in Minnesota, were tested using a diagnostic iELISA and a bELISA (Table 6.1). Of the 1000 turkey serum samples tested, 235 were positive for aMPV-C antibodies with both our bELISA and the iELISA while 714 samples were negative with both tests. The diagnostic iELISA detected 280 positive samples and 720 negative while the bELISA identified 244 positive and 756 negative serum samples. When the results from
the individual samples were compared, 51 of the serum samples were not in agreement, resulting in a 94.9% agreement between the two ELISA tests, which is significant ($R=0.871$, $P<2.1\times10^{-310}$).

**Western blot analysis:** The 51 turkey serum discrepancies from direct comparison were further analyzed by western blot (Figure 6.3). Western blots were used to determine the presence or absence of anti-aMPV antibodies in the disputed serum samples. Lanes with bands specific for aMPV proteins were considered positive for anti-aMPV antibodies. It was determined that 27 of the 50 serum samples tested were positive for anti-aMPV-C antibodies and 23 were negative by western blot analysis. One serum sample had insufficient volume for the western blot analysis. Based on the western blot analysis, the bELISA had 3 false positives and 24 false negatives (Table 6.2). Multiple banding patterns were noted in the field serum samples, indicating varying immunological responses to individual viral proteins (Figure 6.3).

**Experimental infection of Pekin ducks.** Serum samples from experimentally infected ducks were tested for the presence of aMPV-C antibodies using a VN assay. The serum samples were then tested in the bELISA. All inoculated ducks had detectable antibodies to aMPV/CO by 15 days post inoculation, while control ducks remained negative throughout the experiment (Figure 6.4). Clinical signs of aMPV infection observed in the ducks inoculated with live aMPV/CO were limited to increased mucus in the sinus cavity at 2-6 days post inoculation (data not shown). The bELISA was able to detect all of the positive duck serum samples as confirmed by VN assays (Figure 6.4).
DISCUSSION

Since the appearance of aMPV in the United States during 1997, investigators have speculated that wild birds were a reservoir, harboring and disseminating the virus. Shin, et al., (23) detected viral nucleic acids in wild birds captured on or near aMPV infected turkey farms in Minnesota. Although the origin of the virus is unknown, their research indicated that viruses found in turkeys can be isolated from other bird species. This was also demonstrated by Shin, et al., (22) in which experimentally inoculated domestic ducks had signs of aMPV infection, detection of virus in oral swabs, and neutralizing antibodies in the serum, indicating that aMPV can replicate in ducks. Experimental findings from this study also demonstrated that ducks were able to replicate the virus as determined by virus reisolation from choanal swabs (data not shown), increased mucus in sinus cavities (data not shown), and production of measurable antibody response (Figure 6.4). To more thoroughly identify and understand potential aMPV reservoirs, such as farm reared geese and ducks, as well as wild waterfowl we developed a bELISA that can be used to detect antibodies to aMPV-C infection regardless of species.

Our bELISA was tested and optimized using sera from different experimentally infected turkeys (Figures 6.1 and 6.2). The bELISA did not detect any antibodies in NDV, AIV or E. coli infected turkeys or chickens. The aMPV antibody status of the experimental serum samples was first determined using virus neutralization tests and an iELISA. All serum samples from infected birds that were positive by VN were also detected with the bELISA. The iELISA was able to detect serum samples from experimentally infected birds collected at 8 DPI that were not detected by the bELISA or VN assays. By day 14, antibodies were detected using all assays. This was not
surprising since bELISAs are generally less sensitive than iELISAs (8). To compensate for reduced sensitivity of the bELISA, we were able to reduce the dilution of the test serum from 1:25 to 1:5 without increasing background. Even more concentrated serum samples can be tested using the bELISA with minimal increases in background, however we chose 1:5 due to limited amount of total sample volume. When tested for specificity, the bELISA did not recognize serum antibodies to AIV, NDV, or bRSV.

After testing experimental serum samples, the bELISA was used to analyze 1,000-field turkey serum samples collected during 2001 at the MPTL. The results from the bELISA and the diagnostic iELISA had a high level of agreement (94.9%). When the results from the two tests were compared, only 51 of the 1,000 samples differed. The serum samples that differed between the two ELISA assays were analyzed by western blot. Of these, 27 were positive for aMPV antibodies and 23 were negative. When compared with the diagnostic iELISA and western blots, the bELISA was able to correctly identify the presence or absence of aMPV antibodies in 97.3% of the samples demonstrating a high level of sensitivity comparable to the current diagnostic assay. The bELISA did have a slight increase in false negatives rates, as compared with the diagnostic iELISA assay, but the bELISA has the advantage of being able to test multiple avian species.

To demonstrate that this assay can effectively and accurately detect anti-aMPV antibodies from a non-turkey serum sample we used the bELISA to test serum from experimentally infected Pekin ducks. We found that the bELISA was just as effective at detecting anti-aMPV-C antibodies from duck sera as it was with turkey sera (Figures 6.1, 6.2, and 6.4). The bELISA was slightly less sensitive than the iELISA at detecting
aMPV-C antibodies, but more sensitive than the virus neutralization assay. The ability of the bELISA to detect the presence of aMPV antibodies in both turkeys and ducks, along with its demonstrated sensitivity, specificity, supports the potential usefulness of the bELISA for the detection of aMPV antibodies in any avian serum sample. Because aMPV continues to cause problems in the United States, research is needed to determine the extent of aMPV infection in domestic poultry, domestic geese and ducks, as well as the extent of infection in wild birds.

ACKNOWLEDGEMENTS

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REFERENCES


Table 6.1. Comparison of bELISA results to those of the diagnostic iELISA using 1000 turkey field serum samples.

<table>
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<tr>
<td></td>
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<tr>
<td>iELISA</td>
<td></td>
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<tr>
<td>Positive</td>
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</tr>
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<td>Negative</td>
<td>9</td>
</tr>
<tr>
<td>Total</td>
<td>244</td>
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Table 6.2. Analysis of turkey field sera samples in disagreement between the bELISA and the iELISA. The table shows the results from the western blot analysis of turkey field sera samples compared to the bELISA.

<table>
<thead>
<tr>
<th>bELISA</th>
<th>Western Blot</th>
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<td></td>
</tr>
<tr>
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<tr>
<td>Total</td>
<td>27</td>
<td>23</td>
<td>27/50 (54%)</td>
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Figure 6.1. Antibody titers from turkey hyperimmunne serum. The graph contains OD values from the bELISA. Values below 0.143 are positive for antibodies to aMPV. Gray bars represent the vaccinated turkeys, while white bars represent control turkeys, positive (gray) and negative (white) controls are dotted. Results from VN and iELISA assays are listed below the graph. Serum samples were taken 4 weeks post initial vaccination.
Figure 6.2. Antibody titers from convalescent turkey serum samples. The graph contains OD values from bELISA. Values below 0.284 are positive for antibodies to aMPV. Tested groups included control (white), *E. coli* (horizontal stripes), NDV (diagonal stripes), and aMPV inoculated (gray), positive (gray) and negative (white) controls are dotted. Results from VN and iELISA assays are listed below the graph. Serum samples were taken 14 days post inoculation.
Figure 6.3. Western blot of field turkey serum samples. Purified aMPV/CO was separated by SDS-PAGE and transferred to a nitrocellulose membrane. Serum samples were added at a 1:50 dilution. Lane 3 contains negative control and lane 1 contains positive control sera.
Figure 6.4. Antibody titers from experimental infection of ducks. The graph contains OD values from the bELISA. Values below 0.34 are positive for antibodies to aMPV. Gray bars represent the vaccinated turkeys, while white bars represent control turkeys, positive (gray) and negative (white) controls are dotted. Results from VN and iELISA assays are listed below the graph. Serum samples were taken 14 days post inoculation.
CHAPTER 7

DETECTION OF AVIAN METAPNEUMOVIRUS (aMPV) AND aMPV SPECIFIC ANTIBODIES IN WILD BIRDS IN THE SOUTHERN AND MIDWESTERN UNITED STATES

SUMMARY

Avian metapneumoviruses (aMPV) are responsible for upper respiratory infections primarily in poultry. Since the emergence of aMPV in the United States during 1996, wild birds have been implicated as a source and reservoir of the virus. To examine the potential role of migratory waterfowl and other wild birds in aMPV ecology, these studies focused on determining which wild bird species, outside of states with aMPV infected domestic turkeys, are infected with aMPV. To determine the at risk wild bird populations, serum samples from multiple species were initially screened using a blocking enzyme linked immunosorbent assay (bELISA) for the detection of aMPV antibodies. The presence of antibodies was confirmed by virus neutralization and western blot assays. Antibodies to aMPV were identified in five species; American coots, American crows, Canada geese, cattle egrets, and pigeons. In subsequent sampling, oral swabs were collected from wild bird populations at highest risk for aMPV; the American coots and Canada geese. From these swabs, 12 aMPV isolates were identified, six from coots and six from geese. The matrix and glycoprotein genes from these isolates were sequenced and compared to the previously described aMPV subtype C viruses. Sequence analysis revealed 82-98% nucleotide sequence identity with aMPV/CO/97, a subtype C virus. This suggests that wild birds can be a reservoir of subtype C aMPVs and serve as a potential mechanism to spread aMPV to other poultry raising areas in the United States.

INTRODUCTION

Avian matapneumoviruses (aMPVs) belong to the family Paramyxoviridae, subfamily Pneumovirinae, and the genus Metapneumovirus. The genus
Metapneumovirus contains the human (hMPV) and avian metapneumoviruses. These viruses are nonsegmented, single stranded, negative sense RNA viruses that result in upper respiratory infections. aMPV disease in turkeys is characterized by catarrhal inflammation of the upper respiratory tract, nasal and ocular discharge, foamy conjunctivitis, sneezing, tracheal rales, swollen infraorbital sinuses, and sinusitis (4, 6, 10, 11). The disease caused by aMPV was first described in 1978 in South Africa and shown to be a pneumovirus in 1986 (3, 5, 9, 23, 24). Until 1996, the turkeys in the United States were not known to be infected with aMPV (8).

The first reports of aMPV infection in the United States occurred in Colorado during 1996, and the virus was subsequently isolated and characterized in 1997 (8). Currently, subtype C aMPVs are known to be a recurring problem among turkey flocks in Minnesota, and differ from the aMPVs found in other parts of the world, i.e. subtypes A, B, and D. The sudden emergence and sporadic occurrence of aMPV disease in the US has lead to speculation that wild birds may be involved in its spread. Wild birds are an important reservoir for other viruses; e.g. avian influenza and Newcastle disease viruses (12, 17). In an attempt to examine this possibility, research was focused on virus recovery from wild birds found in MN. It was demonstrated that aMPV can be isolated from sentinel or wild birds placed or captured on infected turkey farms in areas endemic for aMPV (16). However, it has not been possible to determine if the sampled wild birds already harbored virus, or if they acquired aMPV from the infected turkeys in the area.

To better address the role of wild birds in aMPV epidemiology, it is important that we determine the presence and distribution of aMPV in wild bird populations outside endemic areas. The current study focuses on determining if wild bird species have aMPV
infections in the Southern and Midwestern USA, states outside the area having aMPV infections in domestic poultry. Our initial objective was to identify species potentially involved in the epidemiology of aMPV through serologic testing. Once aMPV antibodies were detected, the second objective was to collect oral swabs from birds in the at risk populations for virus isolation and sequence analysis. Focusing on wild bird species with high prevalence of antibodies in the subsequent sampling year should increase chances for virus isolation. We report several different species of wild birds in several geographically distinct areas, which were found to be serologically positive for aMPV antibodies which subsequently yielded aMPV isolates for phylogenetic characterization

MATERIALS AND METHODS

**Serum Samples.** Species of wild birds at risk for aMPV infection were initially identified by detecting aMPV-specific antibodies using a blocking enzyme linked immunosorbent assay (bELISA). Serum samples from multiple wild bird species from Georgia, South Carolina, and Arkansas were obtained from archived serum samples collected in 2000 and held at the Southeastern Cooperative Wildlife Disease Study, located in Athens, GA. Additionally serum samples were collected from Canada geese in Ohio during the spring of 2002. Table 7.1 lists the species and number of serum samples tested.

**bELISA.** Serum samples were tested in a blocking enzyme linked immunosorbent assay (bELISA) as previously described (21). Briefly, microtiter plates were coated with sucrose-purified aMPV Colorado (aMPV/CO) isolate originally isolated in 1997 from domestic turkeys (8). Serum samples were added at 1:5 dilution, followed by a rabbit anti-aMPV/CO polyclonal antibody conjugated to horseradish peroxidase
The threshold for positive samples was an optical density three standard deviations below that of the negative controls.

**Virus Neutralization.** Serum samples that were determined to contain antibodies to aMPV by the bELISA assay were further tested for the presence of neutralizing antibodies by virus neutralization (VN) assays. Briefly, serum samples were tested at a starting dilution of 1:10 followed by serial two-fold dilutions. The 1:10 dilution was included to eliminate toxicity due to high serum concentrations. Diluted serum samples were incubated with 100 50% tissue culture infectious dose (TCID\textsubscript{50}) of aMPV/CO for 30 min at 37 C. After incubation, the mixture of serum and virus was added to monolayers of Vero cells and incubated. Plates were read at 7 days post inoculation and neutralizing antibodies were detected by the absence of cytopathic effect (CPE) in the cell monolayer.

**Western Blot.** Purified aMPV/CO was separated by SDS-PAGE using a 10% polyacrylamide gel (BioRad, Hercules, California) under reducing conditions, and transferred to a nitrocellulose membrane. The membrane was blocked with 1% polyvinylpyrrolidone (PVP) for 1 hr (Sigma, St. Louis, MO). Serum samples were tested at a 1:50 dilution. Either a HRP conjugated goat anti-turkey IgG or goat anti-rabbit IgG antibody (Southern Biotech) was added and detected by enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Piscataway, NJ). Positive and negative turkey and polyclonal rabbit serum samples were included as controls.

**Swab Samples.** Oral swab samples were collected from populations of wild birds previously determined to have anti-aMPV antibodies. Oral swabs were placed in 1.5 ml of phosphate buffered saline (PBS) with antibiotics (1000 units/ml of penicillin, 10 µg/ml of gentamicin, and 5 µg/ml of amphotericin B) (Sigma) and held at 4 C until facilities
were available for freezing and storage at – 70 C. Numbers of swabs, species, locations, and years collected are listed in Table 7.2. Oral swabs were tested by RT-PCR and virus isolation for detection of aMPV.

**Virus Isolation.** Oral swabs from species from which aMPV antibodies were detected were passed three times in chicken embryo fibroblasts (CEF) to detect the presence of viable aMPV. CEF cells were prepared as previously described (14) and inoculated with 200 µl of swab material. After a 30-minute incubation period, the cells were refed and monitored for CPE for one week. CEF cells inoculated with swab samples were subjected to freeze thawing 7 days post inoculation and the material used for subsequent cell passes or RNA extractions. After the final passage, RNA was extracted from a 200 µl aliquot and examined for the presence of aMPV nucleic acids.

**RNA Extractions.** RNA was extracted from oral swab samples with TRIzol® reagent (Invitrogen, Carlsbad, California). 200 µl of the original swab samples or CEF cells inoculated with swab samples was added to 750 µl of TRIzol®. RNA was extracted following the manufacturer’s protocol, resuspended in 30 µl of RNase-free water, and stored at –70 C.

**RT-PCR.** Reverse transcriptase polymerase chain reaction (RT-PCR) was used to detect the presence of aMPV nucleic acids in the original oral swabs or in cell culture extracts from isolation attempts from oral swab samples. Primers to the matrix (M) gene of aMPV were created that recognize subtype C aMPV and also detect some subtype A and B aMPV were used to screen swab samples. The primers aMPV4 5’-ACAAGTRASGATGGAGTCCT-3’ and aMPV681 5’-ATTACCTGAACTCCTGCACC-3’ amplify a 677 base pair (bp) product. Additionally,
aMPV subtype C specific primers were also included (22). RT-PCR was performed with the One-Step RT-PCR kit (Qiagen, Valencia, CA). Five microliters of sample RNA was added to the RT-PCR mixture with 10 units of ribonuclease inhibitor (Invitrogen) and 0.6 µM of each primer in a final volume of 25 µl. The RT reaction was carried out at 50 C for 30 min with a 15 min initial PCR activation step at 95 C. PCR parameters consisted of 94 C for 3 min, 30 cycles of 94 C for 45 sec, 55 C for 30 sec, 72 C for 1 min, followed by an additional extension step of 72 C for 7 min. An additional PCR reaction was included to amplify low levels of viral nucleic acid not detected with the initial RT-PCR reaction (2). For the second PCR reaction, 5 µl of the first reaction was added to a Taq PCR Master Mix (Qiagen) along with 0.5 µM of each primer in a total volume of 25 µl. Samples that were positive for the M gene were subsequently tested using primers specific for the glycoprotein (G) gene, which amplify a 1.3 KB product, as previously described (1). In all experiments RNA extracted from viral stocks was used as a positive control, noninfected CEF cells, and no template reactions served as negative controls. RT-PCR products were visualized by ethidium bromide staining of 1% Tris borate (0.045M) EDTA (0.001M) electrophoresis buffer (TBE) agarose gels.

**Sequencing and Analysis.** Bands were cut from agarose gels and extracted using Qiaquick gel extraction kit (Qiagen). Double stranded sequencing with Taq polymerase and fluorescently labeled dideoxyxucleotides was performed using an automated sequencer (13, 18). Nucleotide sequence editing and alignments of sequences were completed using DNASTAR (Madison, WI). Alignments were performed using CLUSTALW method (20). To determine the relationship among the aMPV isolates phylogenetic analysis using parsimony (PAUP) was performed (19). Analysis of the M
and G genes included all sequenced aMPV isolates available along with a hMPV isolate. Isolates from GenBank used in the alignments for the generation of the M gene phylogenetic tree included, subtype C isolates AF262571, AF298638, AF298636, AF298633, AF187149, subtype A isolate X58639, and subtype B U37586. Also included in the phylogenetic tree for the M gene were the hMPVs, NP690065, AF371354, AF371352, AF371357, and AF371350. Sequence used in the generation of the G tree include subtype D AJ288946 and AJ251085, subtype A L34034 and L340030, subtype B L34034 and L34033, hMPV isolate NC004148 and subtype C sequences provided by Alvarez, et al (1). Sequencing aMPV isolates identified from wild birds in this study have been submitted to GenBank.

RESULTS

Serology. 725 serum samples were tested representing 15 species of wild birds (Table 7.1). From these samples, antibodies to aMPV were detected in 131 samples representing 5 species. American crows, American coots, Canada geese, cattle egrets, and pigeons had serum samples that tested positive by bELISA for aMPV antibodies. The Canada geese (49%) sampled in Ohio had the highest percentage of positive serum samples by bELISA, followed by Canada geese (25%) and American coots (17%) from Georgia and Arkansas USA.

Although crows, coots, geese, cattle egrets, and pigeons all had detectable antibodies to aMPV using the bELISA, only the Canada geese and American coots had neutralizing antibodies to aMPV as detected by the VN assay (Table 7.1). All of the serum samples from the crows, egrets, and pigeons determined to be positive by the bELISA assay were tested by western blots. Of these serum samples all species had
detectable antibodies by western blot analysis. Representative serum samples from the coots and geese were also analyzed via western blot, and antibodies to aMPV were detected in both species (Figure 7.1).

**aMPV Detection.** Of the 181 oral swabs collected from the American coots, six of the swab samples resulted in the amplification of the correct size band with RT-PCR primers to the M gene (Table 7.2). 525 oral swabs were collected from Canada geese. In four swabs obtained from geese in Ohio and two swabs obtained from Georgia/South Carolina, aMPV was detected by M gene RT-PCR (Table 7.2). RT-PCR to the G gene resulted in amplification products in one swab from geese in OH.

**Sequence Analysis.** Sequence analysis of the goose and coot swabs identified aMPVs related to the subtype C aMPVs found in the US. Nucleotide phylogenetic analysis of the M gene results in a phylogenetic tree where the newly isolated wild birds aMPVs cluster with the other sequenced subtype C aMPVs (Figure 7.2). The goose and coot samples have 82-98% nucleotide identity with the subtype C virus aMPV/CO/97, 57-65% with subtype A, 57-67% with subtype B, and 54-74% with hMPV. RT-PCR with the G gene only resulted in amplification products in 1 of the 12 isolates identified as positive by M gene RT-PCR. The construction of a phylogenetic tree with the G gene also resulted in grouping of the wild bird isolate with the subtype C viruses (Figure 7.3). The G gene of the goose isolate has 97% nucleotide identity with subtype C aMPV, 20% with subtype A, 20-22% with subtype B, 22% with subtype D, and 25% with hMPVs.

**DISCUSSION**

aMPV appeared suddenly in domestic turkeys in the United States in 1996 and the source is unknown. Screening of wild birds for aMPV has been ongoing in Minnesota,
where aMPV is currently a problem in the US. aMPV has been isolated from a few wild birds that were associated with aMPV infected turkey farms, and these isolates were closely related to the aMPVs that were recovered from turkeys (15). This does support the hypothesis that wild birds can be involved in the local ecology of aMPV. However, these data does not address if wild birds functioned as a reservoir or if they were responsible for the first introduction into domestic poultry in an affected area. A key component to the wild bird transmission hypothesis is the idea that wild birds are able to carry the virus from one area to another. This has been reported with other respiratory viruses that affect poultry (12, 17). Therefore, it is critical to determine if wild birds outside of the aMPV-infected turkey area can harbor aMPV. To specifically address this question our research has focused on determining if wild birds outside of Minnesota, specifically in the Southern and Midwestern US are infected with aMPV.

When aMPV has been isolated from wild birds in areas with endemic aMPV infections in domestic turkeys, the recovery rate has been very low (15). We hoped to improve our chances at recovering aMPV from wild birds by first identifying populations of birds with anti-aMPV antibodies and then attempting virus isolation from these identified species. This was accomplished by screening serum samples collected during 2002 in the Midwest and archived serum samples collected in 2000 from the southern United States using a bELISA. The bELISA was utilized to identify five species of wild birds with antibodies to aMPV; American crows (12%), American coots (18%), Canada geese (25-49%), pigeons (0.5%), and cattle egrets (5%). Neutralizing antibodies were detected only in the goose and coot samples, confirming the presence of aMPV specific antibodies in these two species. This does not rule out the possibility of aMPV specific
antibodies in the other species sampled. The VN assay is less sensitive than the ELISA assay due to its limitation of only detecting neutralizing antibodies, which often occur in low levels and are not always generated in low-grade infections. When serum samples from all five species were analyzed by western blot assays, antibodies to aMPV were detected in goose, coot, crow, pigeon, and egrets. This indicates that all five species have been exposed to aMPV and develop a measurable humoral immune response. Although all five species did have detectable antibodies to aMPV, virus isolation attempts focused on the Canada geese and American coots due to higher levels of seropositive birds in these species.

Oral swabs were collected in the fall 2001 from American coots and in the spring 2002 for the Canada geese. Sampling times were determined by the availability and ease of capture for each species. RT-PCR with primers specific to the M gene was used for an initial screen of the wild bird oral swabs due to its high level of conservation among other aMPV isolates. This lead to the identification of 12 swabs with amplification products specific to aMPV, this included six swabs from coots in GA/SC, four swabs from geese in OH, and two swabs from the geese in GA/SC. The aMPV sequences identified in wild birds have high nucleotide identity (82-98%) to subtype C viruses. The American coot isolates, 26 and 144, have the lowest identity with the subtype C viruses. This high degree of nucleotide identity with the subtype C viruses indicates that the newly isolated coot and goose isolates belong to the subtype C aMPVs, as do all aMPVs isolated in the US to date. Once swab samples were determined to be positive by the initial screening, the swabs were then analyzed by RT-PCR to the G gene. The G gene was chosen due to the high levels of nonidentity seen both among the four subgroups of aMPV. Analysis of
this gene could help to determine the relationship of aMPV isolates from wild birds to those collected from turkeys. RT-PCR with the G gene resulted in the generation of sequence from 1 isolate from a goose in Ohio. This isolate was found to be 97% similar to subtype C aMPV, and approximately 20% identical to subtype A, B, D aMPV, and hMPV. The lack of amplification products in the other swab samples is most likely the result of high levels of sequence divergence seen within the aMPV G genes.

These data demonstrates the presence of aMPV and anti-aMPV antibodies in wild birds outside of Minnesota. This indicates that aMPV is able to replicate and induce antibodies in wild birds. The presence of the antibodies to aMPV in the wild birds sampled during 2000, demonstrate the presence of the virus outside of endemic areas for at least 2 years. Older serum samples and more extensive testing are necessary to determine the extent and duration of aMPV infection in wild bird populations. The high number of lakes and migratory birds in close proximity to turkey farms in Minnesota has been implicated in the continuing circulation of aMPV infection in this area (16). Although the theory of repeated introductions of aMPV from wild birds has been suggested, sequence analysis of the F gene of aMPV isolates from Minnesota suggests that there has been only a single recent introduction of aMPV into turkey populations in Minnesota (7). This suggests that only one introduction of aMPV has occurred in MN and the virus has been spread from farm-to-farm and not though repeated new introductions from wild birds. However, wild birds have been infected on aMPV infected turkey farms and may serve as local transfer host. The infrequent introduction of virus from wild birds into the poultry populations does help explain the lack of infection in the southern states where aMPV can be found in wild birds. The presence of the virus
among wild birds in the Southern and Midwestern states could eventually result in the introduction of the virus into poultry flocks if a breakdown in biosecurity occurs.

ACKNOWLEDGEMENTS

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Table 7.1. Serum samples screened for the presence of aMPV antibodies by bELISA, virus neutralization, and western blot assays. Serum samples were collected from wild birds in Georgia, South Carolina, and Arkansas except for the Canada geese, which were also sampled in Ohio. The species common and scientific names, along with number of positive serum samples, total number tested, percent positive, virus neutralization and western blot results are listed.

<table>
<thead>
<tr>
<th>Species*</th>
<th>Positive/Total (Percent)</th>
<th>VN Pos/Total</th>
<th>Western</th>
</tr>
</thead>
<tbody>
<tr>
<td>American crow (<em>Corvus brachyrhynchos</em>)</td>
<td>6/51 (12%)</td>
<td>0/6</td>
<td>4/6</td>
</tr>
<tr>
<td>American coot (<em>Fulica americana</em>)</td>
<td>20/114 (18%)</td>
<td>10/20</td>
<td>10/10</td>
</tr>
<tr>
<td>Canada goose (<em>Branta canadensis</em>)</td>
<td>52/205 (25%)</td>
<td>7/52</td>
<td>6/6</td>
</tr>
<tr>
<td>Canada goose (<em>Branta canadensis</em>) - OH</td>
<td>51/105 (49%)</td>
<td>5/51</td>
<td>4/4</td>
</tr>
<tr>
<td>Cattle egret (<em>Bubulcus ibis</em>)</td>
<td>1/22 (5%)</td>
<td>0/1</td>
<td>1/1</td>
</tr>
<tr>
<td>Pigeon (<em>Columba livia</em>)</td>
<td>1/194 (1%)</td>
<td>0/1</td>
<td>1/1</td>
</tr>
<tr>
<td><strong>Total Positive Species</strong></td>
<td><strong>131/691 (19%)</strong></td>
<td><strong>22/80</strong></td>
<td><strong>26/28</strong></td>
</tr>
</tbody>
</table>

* Species of wild birds lacking antibodies to aMPV and number of serum samples tested; Eurasian collared dove (*Streptopelia decaocto*) 1, fish crow (*Corvus ossifragus*) 3, grackle (*Quiscalus quiscula*) 14, hearing gull (*Larus argentatus*) 1, heron (*Ardea herodias*) 1, kingfisher (*Ceryle alcyon*) 1, laughing gull (*Larus atricilla*) 3, ringbill gull (*Larus delawarensis*) 13, robin (*Turdus migratorius*) 1, and owl (*Bubo virginianus*) 3.
Table 7.2. Species tested for the presence of avian metapneumovirus nucleic acids by RT-PCR	extsuperscript{a}. Species, location, and total number of swabs collected are listed below.

<table>
<thead>
<tr>
<th>Species</th>
<th>Location</th>
<th>Date Collected</th>
<th>Number</th>
<th>RT-PCR	extsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>American coot</td>
<td>GA/SC</td>
<td>Winter 2001</td>
<td>181</td>
<td>6</td>
</tr>
<tr>
<td>Canada goose</td>
<td>GA/SC</td>
<td>Spring 2002</td>
<td>227</td>
<td>2</td>
</tr>
<tr>
<td>Canada goose</td>
<td>Ohio</td>
<td>Spring 2002</td>
<td>298</td>
<td>4</td>
</tr>
</tbody>
</table>

	extsuperscript{a}RT-PCR with primers to matrix gene of aMPV.
Figure 7.1. Western blot analysis of wild bird serum samples utilizing avian metapneumovirus (aMPV) antigen. aMPV antigen was electrophoresis through 10% SDS-PAGE gels and transferred to nitrocellulose. Serum samples were assayed at 1:50 dilutions and detected with an HRP-anti-bird antibody and chemiluminescence. Lane 1 pigeon, lane 2 egret, lane 3, 11, 12, 13 and 14 Canada goose, lane 4, 5, and 6 crow, lane 7, 9, 10 and 15 coot, lane 8 negative control turkey serum, lanes 15 and 16 positive turkey serum samples, and lane 17 standard.
Figure 7.2. Phylogenetic relationship of the newly isolated wild birds isolates based on 640 nucleotides from the matrix gene. The phylogenetic tree was constructed with all the wild bird isolates, representative isolates from subtype C, including turkey and wild bird viruses, hMPV, and subtype A and B viruses based on nucleotide sequences of the M gene. Following alignments, rooted phylograms were generated by maximum parsimony and 100 bootstraps.
Figure 7.3. Phylogenetic relationship of the newly isolated wild birds isolate based on the glycoprotein gene of aMPV. A phylogenetic tree was constructed using the wild bird isolate, all subtype C isolates, all subtype A and B isolates, and a hMPV 1020 nucleotides from the G gene. Following alignments, rooted phylograms were generated by maximum parsimony, with subtype A as an outgroup and 100 bootstraps.
CHAPTER 8

CONCLUSIONS

This study focused on understanding the pathobiology and ecology of subtype C aMPV. Specifically the pathobiology of the aMPV Colorado isolate identified in 1997 (aMPV/CO/97) was utilized during these investigation. These two, very different yet related, aspects of virology were addressed by dividing the project into two main objectives. This first was to assess the ability of aMPV/CO/97 to replicate and cause disease in turkeys and ducks. This understanding of aMPV disease was then expanded upon to determine the initial events involved in aMPV attachment by determining binding properties during infection of cells in culture. The second objective was to determine the potential role of wild birds in aMPV ecology and epidemiology. Serum and oral swabs were collected from wild bird populations and assayed for the presence of aMPV specific antibodies and viral nucleic acids, respectively.

Turkeys inoculated with aMPV/CO/97 alone resulted in little to no clinical disease. These results are not unexpected since clinical signs reported in the field vary vastly, from snicking, nasal discharge, to swelling of the infraorbital sinuses and mortality. This suggests the involvement of secondary agents in the production of disease in natural infections. In fact, field veterinarians have reported increased incidence of aMPV infections concurrent with Newcastle disease virus (NDV) vaccinations. This observation lead to the development of a dual aMPV/NDV infection model. In these experiments, the inoculation of turkeys with aMPV/CO/97 three days
prior to Newcastle disease virus (NDV) or *E. coli* resulted in the manifestation of clinical signs in the NDV/aMPV dual infected group similar to what has been reported in the field. Nasal discharge, decreased feed consumption, swollen infraorbital sinuses and lethargy were noted in the dual infected birds. This was not noted in the turkeys receiving either agent alone. Milder clinical signs were noted in the aMPV/ *E. coli* dual infected turkeys, but were more severe than in either single infection groups of aMPV or *E. coli*. Increased frequency and length of aMPV recovery was also noted in the turkeys with aMPV/NDV dual infection. Similarly, more severe histopathological changes were noted in the aMPV/NDV group compared to single pathogen inoculum and negative control groups. Clinical signs and histopathological changes were limited to the upper respiratory tract in all aMPV-infected groups. The increased severity of clinical signs, virus recovery, and histopathological changes observed in the aMPV/NDV dual infected group indicates that the additional stress of NDV infection resulted in the exacerbation of clinical signs from aMPV infection, mimicking natural outbreaks of aMPV infections among commercial turkeys.

Once the pathogenicity of aMPV/CO/97 was assessed in turkeys, the effect of infection was measured in ducks. The inoculation of Pekin ducks resulted in virus infection of the upper respiratory tract as evident by increased mucous in the sinus cavity as early as two days post inoculation (DPI). Although aMPV was detected in oral swabs and respiratory tissues at early time points and seroconversion did occur, no clinical signs were evident. Antibodies were detected with virus neutralization assays and a blocking enzyme linked immunosorbent assay (bELISA). The inoculation of the ducks with aMPV/CO/97 resulted in infection with no overt clinical signs, suggesting a subclinical
infection; and was similar to what was observed with the inoculation of turkeys with aMPV/CO/97 alone. The ability of the ducks to support virus replication suggests further investigation of wild waterfowl as carriers of the virus.

To further assess aMPV’s ability and potential to cause disease it is imperative that we understand the initial events involved in viral binding and resulting infection. These events could be major determinants in understanding tissue tropism and host range. To investigate this aspect of aMPV biology an assay was developed where biotin linked aMPV/CO/97 binding to Vero cells was measured by flow cytometry using streptavidin-FITC. Vero cells or aMPV was treated with various compounds and enzymes and their effect on aMPV binding determined by comparison to untreated cells and virus. Neuraminidase treatment of cells resulted in increased binding, suggesting sialic acid may mask or interfere with aMPV attachment. The addition of heparinase I or III, which cleave heparin and heparan sulfate, resulted in a dose dependent decrease in aMPV binding. This was confirmed by the ability of heparin or heparan sulfate incubation with the virus to prevent virus binding. These results indicate that heparan sulfate, found on the surface of most cells, is involved with aMPV attachment to Vero cells. Although treatments did result in decreased binding, no treatment completely prevented binding, implicating a second attachment event, not yet identified. A similar phenomenon has been described with members of the mammalian pneumoviruses, specifically the respiratory syncytial viruses (RSVs), where a heparin motif has been identified in the G protein. Also, a CX3C receptor (CX3CR1) on the cell surface and a CX3C motif on the glycoprotein of respiratory syncytial virus (RSV) are important for binding. The involvement of heparan sulfate and CX3C motifs was identified in the G protein of the
aMPV Colorado isolate. In experiments with aMPV and fractalkine (a CX3C chemokine), anti-fractalkine, or anti-CX3CR1 were capable of reducing aMPV binding. The involvement of heparan sulfate and CX3CR1 in aMPV binding suggests that the virus has the potential to bind a wide variety of cells due to the distribution of heparan sulfate on the surface of most cells.

The second objective of this study involved determining the role of wild birds in the ecology of aMPV in the United States. The isolation of aMPV from sentinel domestic ducks or wild birds trapped on farms with aMPV-infected turkeys within endemic areas of Minnesota. The goal was to determine if wild birds outside of Minnesota have been exposed to aMPV. Virus replication among aMPV-infected turkeys is limited to 3-5 days post infection. Because of this limited replication period in turkeys, initial screening was completed for aMPV antibodies, which are known to persist longer than virus. A rapid serologic test for aMPV, which would detect anti-aMPV antibodies from any host was developed. Subsequently, archived serum samples were screened to identify infected populations of wild birds and bird populations with anti-aMPV antibodies were sampled for virus in subsequent years.

Enzyme linked immunosorbent assays (ELISA) have been developed for the detection of aMPV that rely on an anti-turkey IgG or anti-chicken IgG antibody for the detection of positive samples. To overcome this limitation, a blocking ELISA (bELISA) was developed. This assay utilized sucrose purified aMPV/CO/97 as an antigen followed by the addition of test serum samples, and detected by a rabbit polyclonal aMPV antibody conjugated to HRP. Serum samples from ducks and turkeys experimentally inoculated with aMPV/CO/97 were initially tested with an indirect ELISA (iELISA) or
virus neutralization (VN) assay to determine the presence of aMPV specific antibodies before testing with the bELISA. Undiluted serum samples could be detected as positive or negative using the bELISA assay. This was an improvement over the iELISAs where samples below 1:25 have high background. However, a dilution of 1:5 was chosen due to limited volume of wild bird serum samples. This dilution would require less serum while not reducing sensitivity of testing. Once the bELISA was standardized using experimental samples, 1000 field turkey serum samples were tested. A 96% correlation was seen with the results from the bELISA and the standard iELISA utilized by diagnostic labs. Serum samples that did not agree between the two tests were analyzed by western blots and the sensitivity of the bELISA was determined to be slightly less than that of the iELISA. However, the iELISA could not be utilized for testing multiple avian species. The results confirmed that the bELISA could be utilized to detect antibodies to aMPV among turkey and duck serum samples.

Serum samples from fifteen species of wild birds from the Southeast and Canada geese from Ohio were screened by the bELISA for antibodies to aMPV. The bELISA detected antibodies to aMPV in five species of wild birds; American coots, American crows, cattle egrets, Canada geese, and pigeons. Seropositives varied from 1% in pigeons to 49% in Canada geese from Ohio. Serum samples with antibodies as detected by the bELISA were then screened by VN assays for the presence of neutralizing antibodies. Neutralizing antibodies were detected only in the Canada geese and American coots. To confirm the presence of the aMPV specific antibodies in the egrets, crows and coots, identified serum samples were tested by western blot assays, to confirm the presence of aMPV specific antibodies.
Although antibodies were detected in five species, the subsequent study focused on virus isolation of aMPV from Canada geese and American coots only, because of higher seroprevalence and ease of sampling. Oral swabs were collected from American coots in the fall of 2001 and Canada geese in the spring of 2002. The American coots sampled on the Georgia-South Carolina border, were migratory birds that over-winter in the southern United States. The Canada geese sampled in Ohio were migratory, while the geese sampled in Georgia represented resident species. Oral swabs were screened by RT-PCR with primers to the matrix gene, specific to aMPV subtype C which also have some cross reaction to subtype A and B viruses. From the 181 swabs collected from the coots, six viruses were detected. Swabs were collected from the geese (525), resulting in two viruses detected from GA and four from OH. The nucleotide sequence of the M gene of the wild bird viruses had 80-98% identity as compared to the other aMPV isolates found in the United States. The glycoprotein of these isolates was examined using primers specific to aMPV subtype C, and only one sequence was amplified. Nucleotide sequence for this of the G gene had 98% identity to the Colorado and Minnesota isolates previously sequenced. This indicates that the aMPVs identified among wild birds in the southeastern US and Ohio were very similar to virus isolated from turkey flocks in Minnesota. Therefore migratory wild birds as well as resident geese in the Southeast may serve as a reservoir for aMPV in the United States.

These studies have helped to better define aMPV disease in the United states, by focusing on the effects of dual infections, the effects of infection in ducks, and identifying potential cell surface proteins important for virus attachment. This has lead to a better understanding of which cells aMPV can infect and the disease process, eventually
leading to more effective treatments and preventions. The identification of a reservoir in wild birds for aMPV will also lead to improved biosecurity and prevent further spread of aMPV. Since the presence of aMPV has now been detected in wild birds from the Southern and Midwestern United States, a further emphasis on biosecurity is needed if aMPV infection outside of MN is to be prevented.