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Propagation and Molecular Characterization of Avian Pneumoviruses
(Under the Direction of DR. BRUCE SEAL)

The replication characteristics of Avian pneumovirus (APV) subtype C were studied by generation of a growth curve and by northern blot hybridization of cell-infected RNA with APV N and M genes. APV/C was detected in cell culture media by 24 hours post-infection replicating to a maximum titer of 10^6 by six days post-infection. APV/C transcription could be detected by northern blot hybridization within 24 hours post-infection and continued for 5 days. The N, P, M, F, and M2 genes for the APV subtypes A, B and C were sequenced. Phylogenetic analysis of the APV N, P, M, F and M2 contiguous sequences confirmed the presence of three APV subgroups. Finally, polyacrylamide gel electrophoresis was performed on purified APV/C proteins to determine if the attachment protein, G, was present since it has yet to be cloned. A putative G protein was detected by Coomassie and glycoprotein staining.

INDEX WORDS: Avian pneumovirus, Turkey rhinotracheitis

PROPAGATION AND MOLECULAR CHARACTERIZATION OF AVIAN
PNEUMOVIRUSES

by

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CHAPTER 1

INTRODUCTION

Avian pneumovirus is the etiologic agent of a respiratory disease of poultry, which causes symptoms including coughing, nasal discharge, tracheal rales, foamy conjunctivitis and sinusitis in turkeys of all ages. Laying birds may also experience a drop in egg production. The disease is also known as turkey rhinotracheitis and swollen head syndrome in chickens. Secondary bacterial infections can lead to increased mortality among infected flocks.

Avian pneumovirus is a member of the Paramyxoviridae family, which has two subfamilies. The Paramyxovirinae subfamily encompasses Avian Paramyxovirus types one through nine and Newcastle Disease Virus. The second subfamily is the Pneumovirinae that contains avian pneumovirus and its relatives, the human, bovine, ovine, and caprine respiratory syncytial virus and pneumonia viruses of mice.

Avian pneumovirus, like other Paramyxoviruses, has a nonsegmented, negative-sense RNA genome that, by itself, is not infectious. The genome of most pneumoviruses consists of 8 to 10 genes coding for the two nonstructural proteins, NS1 and NS2; the nucleoprotein, N; the phosphoprotein, P; a matrix protein, M; a fusion protein, F; the second matrix protein, M2; an RNA dependent RNA polymerase, L; a small hydrophobic protein, SH; and a surface glycoprotein, G. Pneumoviruses are distinguished from other Paramyxoviruses in that there is no hemagglutination or neuraminidase activity

associated with its proteins. Unlike its mammalian counterparts, avian pneumovirus lacks the NS1 and NS2 genes.

Avian pneumovirus was discovered during the 1970s in South Africa. It was soon isolated from poultry in Europe, Israel, and Asia. Two distinct subtypes, A and B, were initially characterized. Avian pneumovirus was first isolated in the US during 1996 in Colorado. Subsequently, there have been outbreaks of avian pneumovirus in Minnesota. There was no cross-reactivity using serological reagents from the European A or B subtypes with the Colorado Pneumovirus. Thus, it was characterized as a third, C, subtype.

The first objective of this study was to study the replication characteristics of APV/CO by performing a one-step growth curve and northern blots with viral protein genes. Next, SDS-PAGE electrophoresis was performed with Coomassie staining and glycoprotein staining to determine if the G protein is present in APV/CO. Finally, nucleotide sequencing was completed for the APV/A, APV/B and APV/Co M, N, P, F, and M2 protein genes and phylogenetic analysis was performed on the three subtypes using a contiguous protein sequence.

The hypothesis was that APV/CO would contain the G protein upon protein gel staining as it is present in the APV/A and APV/B subtypes. The APV/A and APV/B N protein gene sequences, which have been published, have shown identical sequences for APV/A and APV/B. The hypothesis was that these two proteins would have differing sequences since all other proteins for the two subtypes which were published, G, M, and F had differing sequences. The APV/B P and M2 protein gene sequences had not been previously published. The phylogenetic analysis was expected to reinforce the different subtypes of APV/A, APV/B and APV/C.

CHAPTER 2

LITERATURE REVIEW

History of Avian Pneumovirus

The first documented outbreak of avian pneumovirus (APV) occurred in South Africa during the late 1970s, although the causative agent was not identified until 1986 (reviewed by Jones, 1996; Alexander, 1997). Since then, APV has been isolated in Europe, Asia, Israel, and South America. Until February 1997 when the National Veterinary Services Laboratory (NVSL, APHIS, USDA) isolated APV from commercial turkeys in Colorado (APV/CO), the United States had been considered APV free (Seal, 1998). Due to the lack of cross-reactivity of the US strain with reagents produced in Europe, APV/CO went undetected during the first ten months of the US outbreak (Seal, 1998). An ELISA test developed by the NVSL using purified APV/CO virus as the antigen demonstrated infection among flocks in the Northern Midwest of the United States, including Minnesota, North Dakota, and South Dakota (Senne, 1998). The lack of serologic activity by APV/CO infected birds with APV from previous isolates demonstrates the emergence of new strains in North America (Seal, 1998).

As discussed by Jones (1996), the initial origin of APV is still unknown. However, the emergence of APV in the most easterly region of Europe lends credence to the hypothesis that the virus was spread by migratory birds, which fly long distances

(Jones, 1996). The role of migratory birds does not necessarily explain the rapid spread of APV throughout Europe or the appearance of APV/CO in the central United States.

Clinical Disease

APV is the causative agent of turkey rhinotracheitis (TRT) and contributes to swollen head syndrome (SHS) in chickens (reviewed in Jones, 1996; Alexander, 1997). The virus may not be the primary pathogen in SHS, because SHS is sometimes detected with *Escherichia coli* and infectious bronchitis virus infections of chickens (reviewed by Cook, 2000). Clinically APV is indistinguishable from *Bordetella avium* infection in turkeys (Jones, 1996; Alexander, 1997). APV infects turkeys of all ages. It has a morbidity rate of 90-100% and a mortality rate of 0-30%, with an increase of mortality up to 90% in the presence of a secondary bacterial infection (Jones, 1996; Randhawa et al., 1996a; Alexander, 1997; Kleven, 1997). Clinical signs of infected turkeys are primarily respiratory and include snicking, rales, sneezing, foamy nasal discharge, conjunctivitis, and submandibular edema (Jones, 1996; Alexander, 1997). In chickens, a subclinical infection may be almost undetectable. Clinical signs of infection in chickens are also respiratory, but may include sinus swelling, torticollis, cerebral disorientation, and depression (Jones, 1996; Alexander, 1997). The detection of APV in the lungs demonstrates that the virus may be capable of causing lower respiratory tract infections, although upper respiratory tract infection is usually observed (Jones, 1996). Virus has been recovered from cilia of turbinate, trachea, and lung epithelial tissues in experimentally infected turkey poults and chickens (Majo et al., 1995; Majo et al., 1996; Majo et al., 1997). Presence of APV in the reproductive tracts of experimentally infected laying turkeys correlates with a drop in egg production observed during APV infections (Majo et al., 1995; Cook et al., 1996). Although viral replication occurs in the mature

oviduct, there is no evidence of egg transmission among turkeys or chickens (Jones, 1996). The replication of APV in the turbinates causes severe rhinitis associated with APV infection, and allows for secondary bacterial infections (Majo et al., 1997; Jones et al., 1988). Reverse transcriptase polymerase chain reaction (RT-PCR) can be used to detect viral genomic RNA from tracheal swabs 14 days post infection (Jing et al., 1993) and antibodies to the virus can be detected by ELISA or virus neutralization several weeks after infection of adult turkeys (Jones et al., 1988).

Diagnosis of Viral Infection

Clinical signs alone cannot be used for diagnosis of APV because these are similar to other respiratory illnesses such as infectious bronchitis virus, avian influenza, *Bordetella avium* and Newcastle disease virus. Diagnosis of APV infections can be made by one of three methods. First, virus can be demonstrated by isolation and viral antigens may be detected by direct fluorescent antibody procedure (Kapur et al., 1998). Virus isolation of APV is difficult because the virus is only present for a short period after infection (Cook et al., 1991). Virus is best isolated from the upper respiratory tract rather than from the trachea since that is where the virus is present in the highest titer (Cook et al., 1991). Primary isolation of virus can be performed by viral replication in tracheal organ cultures from specific pathogen free (SPF) chickens or turkey embryos (Wyeth and Alexander, 1989).

Second, virus specific antibodies in the serum of infected birds, can be demonstrated by indirect fluorescent antibodies (IFA), enzyme-linked immunosorbant assay (ELISA), or virus neutralization (VN) (Grant et al, 1987; O'Loan et al, 1989; Jones, 1996; Kapur et al, 1998). When ELISA is utilized, the antibody must cross-react with APV. ELISA has been used to diagnose APV infections among turkeys in Canada

(Heckert et al., 1994). No APV was found, but the ELISA kit used antigens from European strains of APV and these reagents do not cross react reliably with the North American strain of APV/CO (Senne et al., 1998). ELISA tests are now available for the US strain of APV that utilize inactivated APV/CO as an antigen (Senne et al., 1998) or expressed recombinant matrix protein of APV/CO (Gulati et al., 2000).

A third technique used to diagnose APV infection is by use of polymerase chain reaction (PCR) coupled with reverse transcription (RT) for viral nucleic acid detection (Kapur et al., 1998). RT-PCR, using APV/A fusion gene primers, has been used to detect APV (Jing et al., 1993). Moreover, RT-PCR has been used to differentiate between the A and B strains of APV (Jing et al., 1993; Naylor et al., 1997).

Control of APV Infection

Live-attenuated, oil-adjuvant and inactivated vaccines are currently used for control of APV in turkeys and chickens. Live-attenuated cell-culture adapted APV strains have proven effective as vaccines (Jones et al., 1986; Jones, 1996). Vaccines developed utilizing APV/ A confer protection for turkeys against infection with APV/A and APV/B strains (Cook et al., 1995). Maternal antibodies made in response to APV do not protect turkey poults (Naylor et al., 1997). Vaccination with both the live and inactivated vaccines protected laying turkeys from both respiratory infection and decrease in egg production (Cook et al., 1996). It is not known if these APV vaccines will protect turkeys from infection with APV/CO. Also, prototype live attenuated APV vaccines may cause disease in young poults. This is apparently due to virulent viral subpopulations that must be removed from current vaccine preparations (Naylor and Jones, 1994). A recombinant vaccine made with F protein gene in a fowlpox viral vector gave a partial protective immune response to APV challenge (Qingzong et al., 1994). Treatment of

poultry with cyclophosphamide, used to suppress B-cell responses, prior to vaccination with APV still conferred partial protection (Jones et al., 1992). This infers that cellular immune responses may be more important than humoral responses for protection against APV (Jones et al., 1992). The safety and protective mechanisms of current APV vaccines remains controversial. One of the best methods to control APV is the use of good management practices and biosecurity (reviewed by Cook, 2000). During the early outbreaks of APV/CO in the United States, biosecurity was important in control of APV/CO because a vaccine is currently unavailable. Antibiotics may help to decrease the mortality rate associated with APV when secondary bacterial infections are present (Jones et al., 1992; Cook, 2000).

Classification

APV is classified as a member of the order Mononegalovirales.

Mononegalovirales includes viruses with a single stranded, nonsegmented RNA genome of negative sense. Members of the Mononegalovirales order are enveloped viruses, which derive the envelope from the host cell plasma membrane during assembly. Three viral families make up the order Mononegalovirales: the Filoviridae, the Rhabdoviridae and the Paramyxoviridae (Lamb and Kolakofsky, 1996).

APV is a member of the family Paramyxoviridae, which is comprised of two subfamilies, Paramyxovirinae and Pneumovirinae. Paramyxovirinae is divided into three genera: Rubulavirus, Morbillivirus, and Paramyxoviruses. Included in the Rubulavirus genus is Newcastle Disease virus, which also causes respiratory infections in poultry. Pneumovirinae contains only one genus, Pneumovirus. Members of the Pneumovirus genome include APV, human respiratory syncytial virus (HRSV), bovine respiratory syncytial virus (BRSV), and pneumonia virus of mice (PVM). APV is the only avian

pneumovirus, and due to its differing gene order from the mammalian pneumoviruses, smaller polymerase gene, and the lack of nonstructural proteins it has been proposed to be the type species of a new genus, *Metapneumovirus*, within the subfamily *Pneumovirinae* (Pringle, 1998; Randhawa et al., 1996a).

APV was initially classified as a pneumovirus based on morphological analysis, electrophoretic mobility of its proteins, and the number of mRNA species (Randhawa et al., 1996a). Based on electron microscopy, APV has a pleomorphic shape, contains a nucleocapsid 14 nm in diameter with a helical component, pitch of 7 nm, and well-defined surface projections of 13-14 nm in length (Collins and Gough, 1988). These findings correlate with HRSV, which has nucleocapsid dimensions of 14 nm in diameter with a helical pitch of 6.5 nm (Collins and Gough, 1988). These dimensions are distinct from those of other paramyxoviruses.

Purified APV was found to have electrophoretic mobility of proteins similar to a pneumovirus, and plaques appeared similar to those of RSV and PVM (Ling and Pringle, 1988). APV has 7 viral polypeptides, 2 of which were glycosylated (Collins and Gough, 1988; Ling and Pringle, 1988). RSV has 10 polypeptides, 2 of which are glycosylated (Collins and Gough, 1988). APV lacked the hemagglutination and neuraminidase activities, characteristic of Paramyxoviruses, such as NDV (Alexander, 1997). Further characterization of APV by molecular methods confirmed genomic similarity to *Pneumoviruses* (reviewed in Jones, 1996; Alexander, 1997).

When APV isolates were characterized with monoclonal antibodies and by ELISA, it was found they could be differentiated into two distinct subgroups (Cook et al., 1993; Collins et al., 1993). The UK and French isolates were differentiated from isolates from Hungary, Spain, and Italy (Cook et al., 1993; Collins et al., 1993). The nucleotide and predicted amino acid sequence of the G protein, which is the most variable protein of RSV, confirmed two distinct subgroups analogous to those of RSV (Juhász and Easton,

1994). These subgroups were designated A and B, the same nomenclature used for RSV subgroups (Juhász and Easton, 1994; reviewed in Jones, 1996). APV/A contains isolates from UK and France, while APV/B contains isolates from Hungary, Spain and Italy. APV/B has also been detected in Great Britain more recently (Naylor et al., 1997). Nested PCR developed for A and B strains have shown that APV/A was present in one of the initial outbreaks in Germany during the 1980s (Hafez et al., 2000). APV/A had previously been thought to have not been present in continental Europe. APV/CO does not react serologically with APV/A or APV/B. APV/CO has been shown to be only partially related to APV/A by IFA or VN tests and was not demonstrated to be related to APV/B (Panigrahy et al., 2000). The nucleotide and predicted amino acid sequence of the APV/CO matrix protein gene (M), which is conserved, showed that it was 78% similar to APV/A and 77% similar to APV/B (Seal, 1998). The APV/A and APV/B subtypes had 89% amino acid sequence identity (Seal, 1998). This led to the putative placement of APV/CO into a third subgroup, APV/C.

Two French isolates, Fr/85/1 and Fr/85/2 reacted poorly with APV/A and APV/B in ELISA tests and they were not neutralized by either APV/A or APV/B antisera (Bäyon-Auboyer et al., 2000). In addition, they cannot be detected using PCR primers against the G protein gene for APV/A and APV/B proving that they are potentially non-A and non-B avian pneumoviruses (Bäyon-Auboyer et al., 2000). The nucleotide sequencing of the F, L, and G proteins of Fr/85/1 and Fr/85/2 revealed differences to APV/A, APV/B and APV/C suggesting that they belong to a possible fourth subgroup, APV/D (Bäyon-Auboyer et al., 2000).

Genome Characteristics

Pneumoviruses contain a negative-sense genome of approximately 15kb in length generally encoding 10 genes, versus six or seven as in other paramyxoviruses such as NDV (Alexander, 1997). Genes encoded by pneumoviruses include the nonstructural proteins (NS1 and NS2), the matrix protein (M), the phosphoprotein (P), the nucleoprotein (N), the small hydrophobic protein (SH), the second matrix protein (M2), the fusion protein (F), the attachment protein (G), and the viral RNA dependent RNA polymerase (L). The lack of hemagglutination and neuraminidase activity, associated with the attachment protein, is an important factor in distinguishing pneumoviruses from other paramyxoviruses (Collins et al., 1996).

Genomic sequence analysis has been completed for several European isolates and information has been published for the N (Li et al, 1996), M (Yu et al., 1992a; Randhawa et al., 1996b), M2 (Yu et al., 1992b; Ling et al., 1992), SH (Ling et al., 1992), G (Ling et al., 1992; Juhasz and Easton, 1994), F (Yu et al., 1991; Naylor et al., 1998), P (Ling et al., 1995), and L (Randhawa et al., 1996a) genes. In addition, M (Seal et al., 1998) and F (Seal, 2000) gene sequences of APV/CO have been published. The sequences have correlated with those of other members of the Pneumovirus genus most closely in every case. The gene order of APV is 3'-N-P-M-F-M2-SH-G-L-5' (Ling et al., 1992) which is different from the gene order of mammalian pneumoviruses, whose gene order is 3'-NS1-NS2-N-P-M-SH-G-L-5'. The gene order of APV is similar to that of Rubulavirus, except Rubulavirus lacks the M2 (22K) gene, which is unique to Pneumoviruses (Randhawa et al., 1996a). APV's lack of the nonstructural genes, NS1 and NS2 (Pringle, 1996; Randhawa et al., 1997), and it has a smaller L gene than other members of Pneumovirus (Randhawa et al., 1996a). This accounts for the 13.3 kb genome of APV, compared to the average 15 kb of other pneumoviruses. Since APV has the M2 gene, characteristic of

pneumovirus but lacks the NS1 and NS2 genes, it has been suggested that it be considered a new representative of Pneumovirinae, termed Metapneumovirus (Pringle, 1996; Pringle, 1998).

The Attachment Protein

The attachment, G, protein of pneumoviruses is the major antigenic determinant of pneumoviruses. Based on the sequence variation between different isolates, APV was separated into two subgroups, A and B (Juhasz and Easton, 1994). This subgrouping of A and B is similar to that of HRSV (Mufson et al., 1985; Johnson et al., 1987), based on the variable G protein. The sequence of the G protein gene of APV/A contains 1193 nucleotides, while the APV/B sequence contains 1260 nucleotides. The two subgroups had 98.5-99.7% sequence identity within the group, but only 38% identity between the two groups (Juhasz and Easton, 1994). This correlates with earlier data that could serologically place the isolates into two subgroups (Collins et al., 1993; Cook et al., 1993). This difference in viral proteins could account for the need for reaction differences when using different antigens for serology (Eterradosi et al., 1995).

The APV G proteins of APV/A, APV/B, Fr/85/1 and Fr/85/2 have several common characteristics. First, they are of comparable length and they have orientation typical of type II glycoproteins and they have a mucin-like composition (Bäyon-Auboyer et al., 2000). They have potential for O and N linked glycosylation as well as N myristylation (Bäyon-Auboyer et al., 2000). These proteins have high concentrations of cysteine residues, which possibly contribute to secondary structure by disulphide bridges. Finally, they all have a common hydrophobicity / hydrophilicity profile.

The G protein mediates attachment of the virus to the host cell (Collins et al., 1996). In HRSV, antigenic changes have been shown to be related to mutations of the G

protein (Martinez et al., 1999). Examples of these types of mutations include changes in protein-linked carbohydrates, and amino acid changes resulting from terminal mutations, substitution mutations, A-G transitions and frameshift mutations (Bäyon-Auboyer et al., 2000).

The Fusion Protein

The fusion, F, protein is the second major antigenic protein of the Pneumoviruses. The F protein mediates fusion of the virus envelope to the host cell membrane (Naylor et al., 1998). There are five main characteristics of the fusion protein of Pneumoviruses. First, there is a N-terminal hydrophobic region that functions as a signal sequence for membrane translocation (Yu et al., 1991). Second, there is a stretch of basic amino acids, Arg-Lys rich, where cleavage can occur from F into F1 and F2 subunits (Yu et al., 1991; Naylor et al., 1998). Next, there is a characteristic N-terminal region of the F1 fusion associated subunit. Also, there are approximately 22 hydrophobic residues near the C-terminal of the membrane spanning sequence (Yu et al., 1991). Finally, there is a C-terminal hydrophilic sequence that spans 25 residues. All five of these characteristics are present in the APV F proteins, which further helped to classify it as a Pneumovirus (Yu et al, 1991).

The F gene sequence information for all three subgroups, A (Yu et al., 1991), B (Naylor et al., 1998), and C (Seal et al., 2000), has been published. The APV/A isolate has an F mRNA comprised of 1636 nucleotides with a predicted amino acid sequence 538 residues in length (Yu et al., 1991). The APV/B isolate also has 538 amino acid residues predicted, but has 1640 nucleotides in the mRNA (Naylor et al., 1998). The F1 subunit is composed of 436 amino acids and the smaller F2 subunit is composed of 102 amino acids (Yu et al., 1991). The F gene starts with the sequence GGGACAAGUA,

which is characteristic of all APV genes sequenced (Yu et al., 1991). The US isolates have 98% amino acid sequence identity amongst themselves, but they only have 72% and 71% amino acid sequence identity to APV/A and APV/B respectively (Seal et al., 2000). The APV/CO isolates also differ in their cleavage site with respect to APV/A and APV/B (Seal et al., 2000). This further qualifies that the US isolates belong in a third subgroup, APV/C.

The Fr/85/1 and Fr/85/2 strains of APV have 73.9%, 80.5% and 70.9% identity with the F1 portions of the fusion protein of APV/A, and APV/B and APV/C, respectively (Bayon-Auboyer et al, 2000). This combined with the low sequence similarities of the G protein of the French isolates with those of APV/A and APV/B have led to the proposal that these isolates should form subgroup APV/D (Bäyon-Auboyer et al., 2000).

The Matrix Protein

The matrix, M, protein has been hypothesized to coordinate the assembly of virus components at the cell membrane prior to viral budding, and to help regulate viral gene transcription (Randhawa et al., 1996b). The M protein is an internal protein that is associated between the nucleocapsid and the envelope (Collins et al., 1996). The M protein is the most abundant protein and its contact with the N protein may be the driving force for budding of the virus from the cell (Collins et al., 1996). The M protein of APV is 254 amino acids in length, almost identical to the length of RSV but considerably shorter than that of other paramyxoviruses, which confirms its placement in the family Pneumovirinae (Yu et al., 1992a; Randhawa et al., 1996b). The M protein is very conserved among paramyxoviruses (Rima, 1989) and can be reliably used for molecular epidemiology (Seal, 1996). Nucleotide sequences of the M protein genes of APV/A and

APV/B isolates have 89% identity with each other, but the APV/CO isolates have 78% identity with A and 77% identity with B (Randhawa et al., 1996b; Seal et al., 1998). This agrees with serological data that APV/CO should be placed into a separate subgroup. The amino acid sequences of APV/A, APV/B and APV/CO are more highly conserved in the coding regions than in the noncoding regions, as would be expected (Randhawa et al., 1996b; Seal et al., 1998).

The Nucleoprotein

The nucleoprotein, N, gene is identical in length to that of human, bovine, and ovine respiratory syncytial viruses (Li et al., 1996). This agrees with the high level of sequence conservation observed among nucleoproteins of pneumoviruses. APV N gene is the most promoter proximal of the three major structural proteins (Li et al., 1996). The gene is 1197 nucleotides in length and is composed of a single open reading frame coding for 391 amino acid residues (Li et al., 1996). The nucleoprotein is the major nucleocapsid protein and is complexed with the phosphoprotein, P, and the viral RNA-dependent RNA polymerase, L, around the virion RNA (vRNA) (Randhawa et al., 1996a; Collins et al., 1996).

The Phosphoprotein

The phosphoprotein, P, gene of APV is composed of one open reading frame of 278 amino acids, which potentially encodes two peptides (Ling et al., 1995). The phosphoprotein is part of the nucleocapsid complex consisting of the N, P, and L proteins associated with the genomic RNA (Ling et al., 1995). The phosphoprotein acts as a transcription and replication factor and as a chaperone that binds soluble L and N proteins

(Collins et al., 1996). This complex is responsible for various mechanisms of RNA synthesis such as genome replication accompanied by transcription and therefore vital to virus replication in the host cell (Ling et al., 1995). The highly negative charged C terminus of APV P protein has been hypothesized to possibly interact with the basic part of the N protein as part of the nucleocapsid complex (Ling et al., 1995).

The phosphoprotein genes of Pneumoviruses are distinct from other Paramyxoviruses because they contain one open reading frame while other Paramyxoviruses are polycistronic (Ling et al., 1995). However, the P genes of APV are distinct from its mammalian viral counterparts in that they potentially encode two proteins while the mammalian pneumoviruses only encode one protein (Ling et al., 1995).

The Second Matrix Protein

The second matrix, M2, protein is also designated the 22K protein. The M2 protein is distinct to the Pneumovirinae family of Paramyxoviruses. It is not known what the function of the M2 protein is, but it is hypothesized to possibly carry out the function of some of the larger proteins present in other paramyxoviruses (Yu et al., 1992b; Ling et al., 1992). The M2 protein gene is 787 nucleotides in length and contains two open reading frames (Yu et al., 1992b; Ling et al., 1992). The location of M2 in the virion is unclear, but it may be associated with the same internal structures as N and P in RSV, but in a different location than M (Yu et al., 1992b).

The RNA Dependent RNA Polymerase

The RNA-dependent RNA polymerase protein, (L) gene is the polymerase component for replication and transcription (Randhawa et al., 1996a). The L gene is included in the innercore of the paramyxovirus nucleocapsid and is complexed with viral RNA, the N and the P protein (Randhawa et al., 1996a). Transfection of plasmids encoding the BRSV minigenome under a T7 promoter into vaccinia virus cells with T7 polymerase gave rise to functional progeny proving that N, P, and L are necessary and sufficient for transcription and replication of the BRSV minigenome (Yunus et al., 1998).

The L gene of avian pneumovirus consists of 6099 nucleotides encoding 2004 amino acids in a single open reading frame (Randhawa et al., 1996a). It is the smallest L gene described for any nonsegmented, negative stranded virus (Randhawa et al., 1996a). Avian pneumovirus L gene contains six linear non-contiguous domains, a putative ATP binding site and four polymerase motifs previously described for the L protein of negative stranded viruses (Randhawa et al., 1996a).

CHAPTER 3

MATERIALS AND METHODS

Generation of a One-Step Growth Curve

Primary chicken embryo fibroblast (CEF) cells and VERO cells were cultured using MEM media with 10% fetal bovine serum and antibiotics. APV/CO was propagated in chicken embryo fibroblast cells using standard techniques (Alexander, 1997; Wyeth and Alexander, 1989). Following replication in CEF cells, APV/CO was used to infect seven separate flasks of VERO cells. The flasks were incubated for different amounts of times ranging from one to seven days post-infection. The viral infected cells from each day post-infection were titrated using serial ten-fold dilutions in media. Uninfected VERO cells were used as a negative control. The titer was determined as 1 over the dilution for the last dilution where cytopathic effects (CPE) was determined to be present.

Northern Blot Hybridization

RNA was purified by guanidium extraction (Chirgwin et al., 1979) of infected cells and ultracentrifugation through a 5.5M CsCl gradient (Glisin et al., 1974). The cell-infected RNA was used to perform northern blot hybridization.

Equal amounts (6 ng), as determined by using spectroscopy, of APV/CO cell-infected RNA from one to six days post infection were fractionated by denaturing gel electrophoresis in 1% agarose using MOPS buffer [final concentration of 20mM 3-(N-morpholino)-propane sulfonic acid, 5mM sodium acetate, 10mM EDTA, pH 7.21] (McMaster and Carmichael, 1977; Seal et al., 1991). Integrity of RNA was tested by UV illumination of ethidium bromide stained RNA.

Following gel electrophoresis, RNA was transferred (Seal, 1994) to prewetted Boehringer-Mannheim positively charged nylon membranes. The membrane was baked for 30 minutes at 120°C. The blot was prehybridized and hybridized using DIG easy hybridization buffer (Boehringer Mannheim). Hybridization was accomplished (Seal et al., 1985) using APV/CO M and N gene probes, which were PCR generated, using a reaction mixture containing the specific gene, forward and reverse primers, enzyme, PCR buffer with MgCl₂, dNTPs and DIG labeled dNTPs, as directed in the DIG PCR probe synthesis kit (Boehringer Mannheim). Positive and negative reactions with and without labeled dNTPs were also run. Following overnight hybridization to the probe, the membranes were washed according to the DIG hybridization kit (Boehringer Mannheim) and detection was completed after overnight exposure to NBT (nitroblue tetrazolium salt) / BCIP (5-bromo-4-chloro-3-indolyl phosphate).

Polyacrylamide Gel Electrophoresis

Following infection of cells in cultures with APV/CO, virions were purified by sucrose gradient centrifugation (Collins and Gough, 1988). Detection of APV/CO proteins was accomplished by running proteins diluted in sample buffer (200mM Tris-HCl, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.002% bromophenol blue) and electrophoresed (Hames, 1981) in a discontinuous 4-15% SDS-polyacrylamide

Criterion precast gel manufactured by BioRad (Hercules, CA). The molecular weight markers, 10kDA marker and Kaleidoscope marker were purchased from BIORAD (Hercules, CA). NDV proteins were also electrophoresed by PAGE. Proteins resolved by SDS-PAGE were observed by staining with BIOSAFE Coomassie stain as directed by the manufacturer (BIORAD, Hercules, CA). Avian pneumovirus glycoproteins were observed by staining with GelCode Glycoprotein staining kit utilizing Schiff's reagent (Alexander and Collins, 1977) as directed by the manufacturer (PIERCE, Rockford, IL).

Synthesis of cDNA and Cloning

Isolates used in synthesis of cDNA, cloning, and sequence analysis are listed in Table 1.

Table 1. The avian pneumovirus isolates and subtypes used in nucleotide sequence and phylogenetic analysis.

I S O L A T E	S U B T Y P E
A P V / U K (1 4 . 1) / A	A
A P V / H u n g a r y / B	B
A P V / U S (C O) / C	C

The APV/A, APV/B, and APV/CO isolates were propagated in VERO cells using standard techniques (Wyeth and Alexander, 1989; Alexander, 1997). Following replication in VERO cells, RNA was purified by guanidinium extraction (Chirgwin et al., 1979) of infected cells and ultracentrifugation through CsCl (Glisin et al., 1974). The

cDNA was synthesized by RT-PCR (Belavsky et al., 1989) utilizing an oligo-dT primer (GGGAGGCCCT₁₅) with a conserved APV 5' primer. Specific genes were amplified from cDNA utilizing a PCR elongase reaction (Seal, 1998) using 5' primers designed from the PRIMER2 (Scientific Education Software) program and the oligo-dT primer. The primers used are listed in Table 2.

Table 2. Primers used to clone avian pneumovirus N, P, M2, F, and M protein genes.

PRIMER NAME	PRIMER SEQUENCE	GENE
APVN1	GGGACAAGTIAAAAATGTCTCT	N
APVP1	GGGACAAGTIAIAATGTCITT	P
APVM2-1b	GGACAAGTGAAGATGTCTIG	M2
APVF1	GGGACAAGTGAAAATGTCTTGG	F
APVM1	GGGACAAGTIAAIATGGAGTC	M

Amplified products were cloned using TOPOXL cloning systems (Mead et al., 1991) according to the directions of the manufacturer (Invitrogen, Carlsbad, CA). Ligated plasmids were transformed into *E.coli* using standard transforming buffer and plated on LB media with kanamycin. Colonies were picked and grown in overnight cultures of LB broth with kanamycin. The colonies were purified using miniprep procedure (Zhou et al., 1990) and the QIAGEN miniprep purification system following the directions of the manufacturer. Following purification, colonies were analyzed by digesting purified plasmids with EcoRI and electrophoresis in an agarose gel.

Nucleotide Sequencing Analysis

Double stranded sequencing (Sanger et al., 1977) with *Taq* polymerase (Applied Biosystems Inc.) and fluorescently labeled dideoxynucleotides was performed with an automated sequencer (Smith et al., 1986). Editing of nucleotide sequences, prediction of amino acid sequences, and protein computer structure predictions were completed using the DNASTAR (Madison, WI) and GeneWorks 2.3 (Intelligenetics, Mountain View, CA) programs. Contiguous coding sequences of the N, P, M, F, and M2 genes were aligned (Thompson et al., 1994). Relationships among APV isolates were determined by analysis performed by Phylogenetic Analysis Using Parsimony (PAUP; Swofford, 1989) with BRSV as an outgroup (Bucholz et al., 1999).

CHAPTER 4

RESULTS

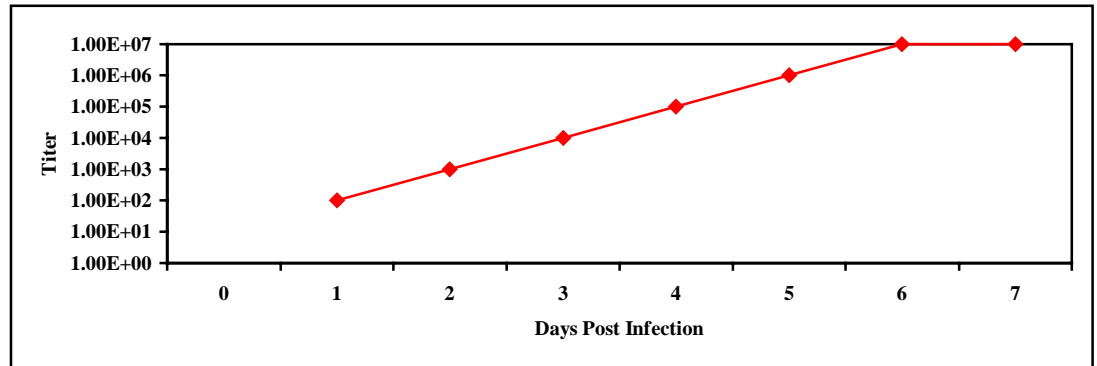
Replication Characteristics of APV/CO

To study the replication characteristics of APV/CO, a one-step growth curve was generated by infecting VERO cells and harvesting media at various times post-infection (Fig. 1A). APV/CO can be detected in cell culture media by 24 hours post-infection. There was no increase in viral titer past six days post infection (data not shown). Viral titer increased linearly reaching a titer of 10^6 by six days post-infection. The transcription of viral genes was studied by performing northern blot hybridization of RNA from APV/CO infected cells, which had been collected one to seven days post-infection. The APV/CO M and N protein genes were utilized as probes to detect transcription. The northern blot for APV/CO M gene is presented in Fig. 1B. Viral transcription is present at one day post-infection in both northern blots and it continues until five days post-infection. The increase in viral transcription can be identified by the greater intensity of the bands present in both northern blots.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed on APV/CO followed by staining to visualize the glycoproteins, which are present in APV/CO. The glycoprotein

A.



B.

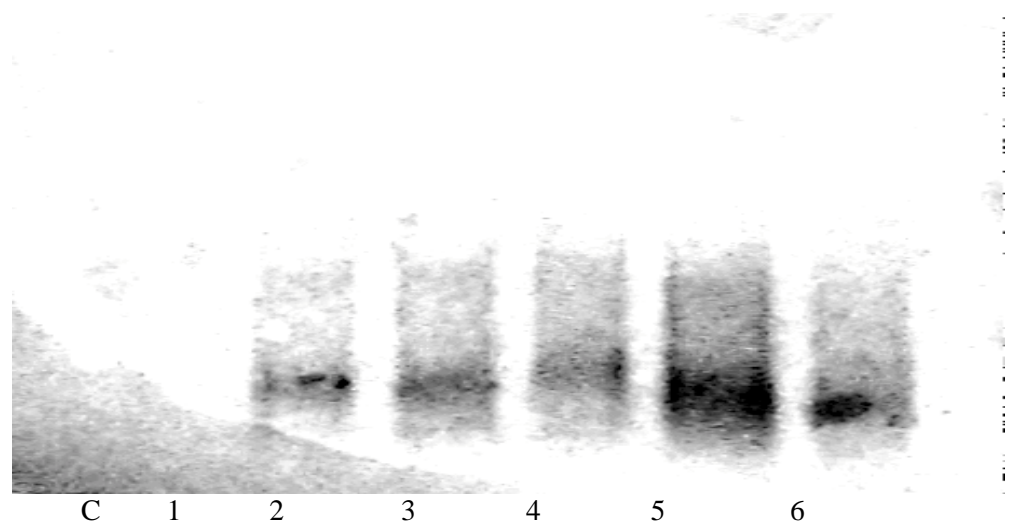


Figure 1. A) The one-step growth curve of APV/CO. Titer is expressed on the y-axis and days post infection on the x-axis. B) Northern blot of APV/CO with M gene. Days post-infection are expressed under the corresponding bands.

stained gel is present in Figure 2A. Two bands are present in APV/CO and have been hypothesized to be the glycoprotein, G, and the fusion protein, F. The APV G protein is predicted to be 43,000 Da and would be the smaller of the two bands. The APV F gene is 58,000 Da prior to cleavage and the F1 particle is 47,000 Da. The large glycoprotein present in NDV is the HN protein, which is 64,000 Da. The F protein in NDV is also 58,000 Da and the F1 protein after cleavage is 47,000 Da. A second protein gel was run on APV/CO and was stained with Coomassie Blue to differentiate all proteins present in APV/CO. The coomassie stained protein gel is presented in Figure 2B.

Nucleotide Sequencing Analysis

Nucleotide sequences were determined for APV/A and APV/B N, P, and M2 protein genes. The APV/B N, P, and M2 protein genes were not previously sequenced and their sequences have been submitted to GenBank. The GenBank accession numbers are included in Table 3.

The APV/A and APV/B N gene are 1186 nucleotides in length. They contain a single ORF, which ranges from position 14 to position 1186. The APV/CO N gene is 1195 nucleotides in length. APV/CO N gene contains a single ORF, which ranges from position 14 to position 1195. The sequence identity amongst APV/CO, APV/A and APV/B N genes is 55%. The sequence identity of APV/CO with APV/A is 54%, APV/CO with APV/B is 67%, and APV/A with APV/B is 74%.

The APV/B N gene nucleotide sequence codes for a protein, which is 391 amino acids in length. The APV/A N protein is also 391 amino acids in length while the APV/CO N protein is 394 amino acids in length. There is 45% identity amongst all three isolates N protein. APV/A and APV/B N proteins share 72% identity, while APV/A and APV/CO N proteins share 53% identity and APV/B and APV/CO N proteins have 51%

2A.

2B.

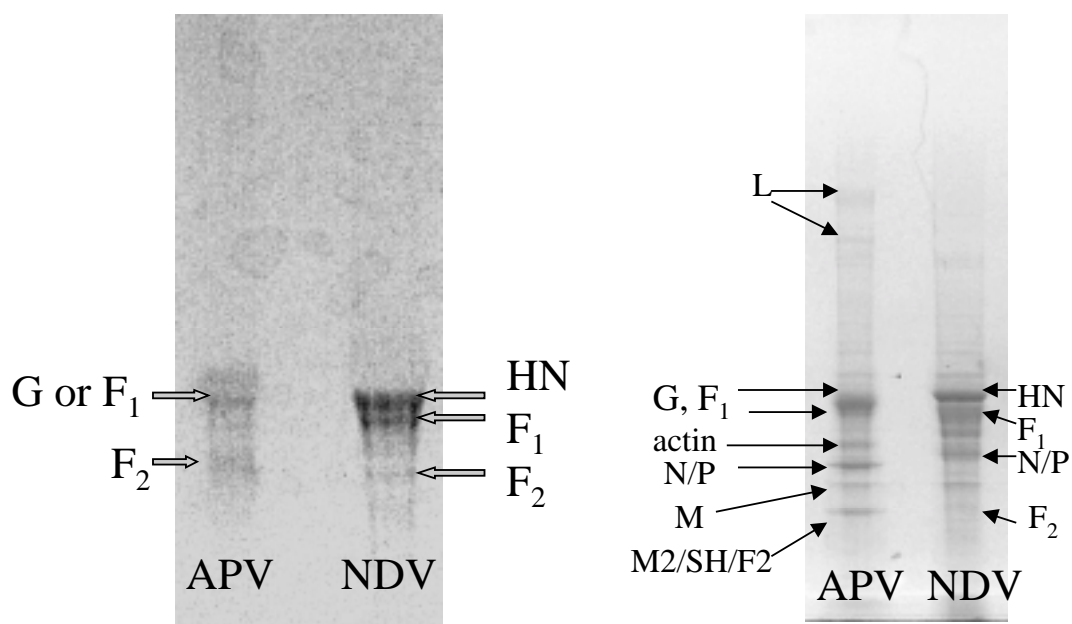


Figure 2. A) The glycoprotein stain of polyacrylamide gel electrophoresis. NDV is on the right and APV/CO is on the left. The proteins are labeled beside their respective bands. B) The coomassie stain of the polyacrylamide gel electrophoresis. NDV is on the right and APV/CO is on the left. The proteins are labeled beside their respective bands.

Table 3. Accession numbers for avian pneumovirus type B sequences which have been submitted to GenBank.

ISOLATE	SUBTYPE	GENE	GenBank ACCESSION NUMBER
Hungary/657/4	APV/B	N	AF325442
Hungary/657/4	APV/B	P	AF325443
Hungary/657/4	APV/B	M2	AF356650

identity. The APV/B N amino acid sequence is presented in Fig. 3 with the alignment of amino acid sequences for APV/A and APV/CO.

The APV/B P gene is 850 nucleotides in length, and it codes for an ORF which ranges from nucleotide position 14 to position 850. The APV/A P gene is 847 nucleotides in length and it contains a single ORF which ranges from position 14 to position 847. The APV/CO P gene contains an ORF which ranges from position 14 to position 894 and the gene is 894 nucleotides in length. The nucleotide sequence identity amongst all three isolates P gene is 32%. The APV/A P gene and the APV/B P gene nucleotide sequences share 51% identity, while APV/CO and APV/A P genes share 45% nucleotide sequence identity. APV/CO and APV/B P genes share 53% nucleotide sequence identity.

The APV/B P gene codes for a protein predicted to be 279 amino acids in length. The APV/A P gene codes for a protein predicted to be 278 amino acids in length and the APV/CO P protein is predicted to be 294 amino acids in length. All three isolates share 45% amino acid sequence identity. APV/A and APV/B share 72% P protein identity while APV/CO and APV/A share 53% P protein identity and APV/CO and APV/B share 51% P protein identity. The APV/B P amino acid sequence was aligned with the predicted amino acid sequences for the P protein APV/A and APV/CO (Fig. 4).

The APV/B M2 gene was 749 nucleotides in length. The M2 gene codes for a major protein with an ORF from position 14 to position 571 with a second minor ORF ranging from position 531 to position 749. The APV/A M2 gene is also 749 nucleotides in length with a major ORF from position 14 to position 571 and a second minor ORF from position 531 to position 749. The APV/CO M2 gene is slightly smaller with a nucleotide sequence 737 nucleotides in length and the major ORF ranging from position 14 to position 565 and the second minor ORF extending from nucleotide position 525 to nucleotide position 737. All three isolates shared 39% nucleotide sequence identity of

the M2 gene. APV/A and APV/B M2 genes shared 68% nucleotide sequence identity while APV/A and APV/CO M2 genes shared 41% nucleotide sequence identity and APV/B and APV/CO M2 genes shared 57% nucleotide sequence identity.

The APV/B M2 gene codes for a major protein predicted to consist of 186 amino acids. The APV/A M2 major protein is also predicted to consist of 186 amino acids, while the APV/CO M2 major protein is predicted to be 184 amino acids in length. The M2 proteins of all three isolates share 68% amino acid sequence identity. The amino acid sequence identity shared by APV/A M2 and APV/B M2 is 89%, while APV/CO M2 has 70% and 72% amino acid sequence identity with APV/A and APV/B, respectively. The APV/B M2 amino acid sequence was aligned with the predicted amino acid sequences for the M2 protein of APV/A and APV/CO (Fig 5).

Phylogenetic analysis was performed on avian pneumovirus isolates using the contiguous nucleotide coding sequences of N, P, M, F and M2 genes. A parsimony-based analysis was performed using 2000 bootstrap replications. APV/CO separates from APV/A and APV/B with 99% confidence, and APV/B separates from APV/A and APV/CO with 100% confidence. The phylogenetic analysis is presented in Figure 6.

APV/A N	EDQ.....	
APV/B N	D.A.....	..K.....	
APV/CO N	..QG.Q...	..S.....	K.K....	T...V...S.	..RE..L...E	
Consensus	MSLESIRLSD	LEYKHAI L.E	SQYTIRRDVG	ATTAITPSEL	QP.VSTLCGM		50
APV/A N	V.....	
APV/B NA...K..S.....	
APV/CO N	..Y.....S	H.....V	..T...E...A.L..TYS.	
Consensus	ILFAKHTDYE	PAAEVGMQYI	STALGADRTQ	QILKNSGSEV	QGVMTKIVTL		100
APV/A N	S...SV....	VG...D....	T..A.GA..R	...V.LT...	
APV/B N	P...PI....I.	PA...N....SL..N	...A.....	
APV/CO N	GKGKNSKGE.	LQM.D..G.E	RS.IEE.DKE	..K..ASATK	DNSGP..Q..	
Consensus	.AEG..RKRE	--VLNIHDVG	..WAD.VERT	ARETM..MV.	EK-.QIPKNQ		150
APV/A N	
APV/B N	
APV/CO N	R..SP..I.I....	..V..N..	..N..LK.F..	
Consensus	KPSALDAPVI	LLCIGALIFT	KLASTVEVGL	ETAIRRASRV	LSDAISRYPR		200
APV/A N	
APV/B NS.....	
APV/CO N	I...K..R..	YD...Q....	..S.....	..S...KA	
Consensus	MDIPRIAKSF	FELFEKKVYY	RNLFIEYGKA	LGSTSTGSRM	ESLFFNIFMQ		250
APV/A N	
APV/B NV.....	
APV/CO NK..T.....E.....	
Consensus	AYGAGQTMLR	WGVIARSSNN	IMLGHVSVQA	ELRQVSEVYD	LVRKMGPESG		300
APV/A N	
APV/B NS.....S.....S.....	
APV/CO NN...A.....S.....L..R..	..V..T.....	
Consensus	LLHLRQSPKA	GLLSLTNCPN	FASVVLGNAA	GLGIIGMYKG	RAPNLELFAA		350
APV/A NT.R. N.....D.....D.R. S...	
APV/B NV.....DK. Q...	
APV/CO NFSS..	..E.K...E	NF.NIN..GQ	NDY.	
Consensus	AESYARSLKE	SNKINLAALG	LTEDEREAAT	SYLGGDEE.S	.KFE		394

Fig 3. Comparative amino acid sequence among nucleocapsid (N) proteins among avian pneumovirus isolates. Amino acid sequence differences are denoted by the single letter code with the consensus sequence below.

APV/A P	M	D	R	T	SAGG	E	N	IA
APV/B P	..L.....	L	Q	I	S	TSV	V	S
APV/CO P	L	N	A	F	KI	GHR	TQ	V
Consensus	MSFPEGKDIL	M	N	GSEAAK	A	EAYQRSLKN	R	RSISG	DPI	TVSEKV	50
APV/A P	NP	S	N	A	PV	KV	I
APV/B P	S	E	S	R	R	TL	PI
APV/CO P	EK	TISK	TK	V	PER	N	WGE	DT	R
Consensus	PLPPLC	S	---	TTP	---	KGA	---	CIKP	---	TKSP
APV/A P	VATD	STST	SA	S	D	P	V
APV/B P	P	AMI	AHPIGA	AQ	R	ES	S	A
APV/CO P	VFAPTS	GK	PAEKGK	T	E	PK	T	K
Consensus	LPTAP	D	T	ET	E	PK	K	KKVKF	N
APV/A P	I	S	T
APV/B P	D	V	N	P	S	S
APV/CO P	..D	DA	S	VL	SA	L	L	S	D
Consensus	EEDNDEKSS	LT	FEEKDTA	SI	EARLEAI	EEKLSMILGM	LKTLNIATAG	200		
APV/A P	I	M	T	D	T
APV/B P	V	A	II	A
APV/CO P	L	AD	IK	A	AK	KS	N
Consensus	PTAARDGIRD	AMVG	REELI	NSIM	EAKGK	IAENMKEED	QRAKIGDGSV	250				
APV/A P	V	S	SG	S	S	E	ES	N	L
APV/B P	R	RM	T	S	T	PDT	G	N
APV/CO P	G	IV	E	T	E	ED	E	ESNPD
Consensus	KLTEKAKELN	K	LEDQSSSG	ESE	EEEE	E	E	D	E	DDI	YSLDM	295

Fig 4. Comparative amino acid sequence alignment of phosphoproteins (P) among avian pneumovirus isolates. Amino acid sequence differences are denoted by the single letter code with the consensus below.

```

APV/A M2 ..... I.....
APV/B M2 ..... T..R.....V...
APV/CO M2 ..RKA..K..V.....E..K.....RY..L.S..L.....
Consensus MSGRNP CRYE .RGKCNRGSS CTFNHNYWSW PDHVLLVRAN YMLNQLLRNT 50

APV/A M2 ..... T.....
APV/B M2 ..... A.....
APV/CO M2 ..S.....D.....T.....DN..EN..AST.....
Consensus DRTDGLSLIS GAGREDRTQD FVLGSANVVQ NYIEGN.TIT KSAACYSLYN 100

APV/A M2 .....TS..S..LE.....I...V.....
APV/B M2 .....S..L.....I.....N.....
APV/CO M2 .....T..RQ...NK...S.....ME...T.....N..K.
Consensus IIKQLQENDV K.ARD.MMD PKHVALHNLV LSY.DMSKNP ASLINSKRL 150

APV/A M2 .R.....L.....P..S....G....N.
APV/B M2 .....S.....GE..NS.....
APV/CO M2 .....L..E....V..N.--TAAM.DSAN.D
Consensus PKEKLLKLAKE IIIQLSAG.E .DNAS.NTLQ KGDSSN 186

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Fig 5. Comparative amino acid sequence of second matrix (M2) proteins among avian pneumoviruses. Amino acid sequence differences are denoted by single-letter code with the consensus below.

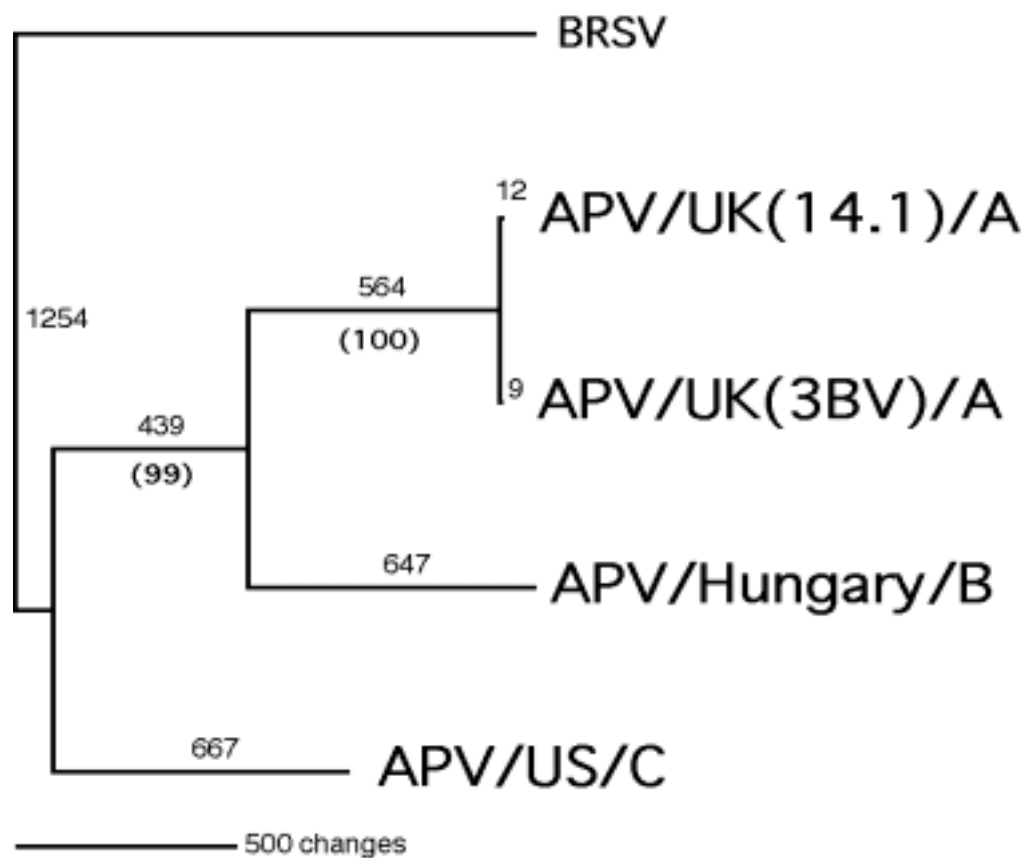


Fig 6. Phylogenetic relationships among avian pneumoviruses. After the contiguous coding sequences of the N, P, M, F, M2 genes had been aligned, a rooted phylogram was generated by maximum parsimony analysis using bovine respiratory syncytial virus as an outgroup. Absolute distances are shown above each branch. The number in parentheses is the bootstrap confidence level after 2000 bootstrap replications.

CHAPTER 6

DISCUSSION

Growth Characteristics of APV/CO

Growth characteristics of APV/CO were studied by determination of a one-step growth curve. The APV/CO was detected in cell culture media (VERO cells) by 24 hours post-infection and it replicated to a peak titer of 10^6 by six days post infection. This corresponds closely to what has been reported for other types of avian pneumovirus in different culture systems. Avian pneumovirus has been noted to replicate to a maximum titer in 3 to 5 days post-infection in the nose and trachea (Jing et al., 1993) and in tracheal organ cultures (Cook, 2000). Cavanagh and Barrett (1988) have reported maximum virus titers at 4 to 5 days post infection for APV/A. These findings also closely correlate with observations of field viruses. APV/CO infected turkeys have been observed to have the intensity of viral symptoms peak during the 4th to 5th day post-infection (Edson, 1997). The slight difference in the time to reach peak viral titer could have been affected by the VERO cells as the culture system or due to prior passaging of the APV/CO isolate.

The transcription of APV/CO mRNA was detected at 24 hours post-infection and continues to five days. This was demonstrated by Northern blot hybridization with the APV/CO M and N genes. In RSV, the transcription of genes follows a 3' to 5' order from a single promoter (Collins et al., 1996), and this would make N and M two of the

first genes to be transcribed separated only by the transcription of the P gene. This is also true for avian pneumovirus. RSV proteins can be detected intracellularly by 6 hrs post-infection with a peak of protein synthesis at 18 to 20 hrs post-infection with quantitative increases based on the increased number of transcribing nucleocapsids (Collins et al., 1996). This corresponds well with the data found for avian pneumovirus which shows that both the M and N genes were being transcribed by 24 hours post-infection. While the increasing band intensity corresponds with greater levels of transcription, it also increases with the linear viral titer increases from days one to six post-infection.

Polyacrylamide Gel Electrophoresis

The polyacrylamide gel electrophoresis, which was performed on APV/CO, showed two glycoproteins when stained with glycoprotein staining reagents. This agrees with the two glycosylated proteins which were found by Collins and Gough (1988) for APV/A. RSV has also been shown to have two envelope glycopolypeptides (Peeples and Levine, 1979). The two proteins found are predicted to be the attachment (G) and the fusion (F) glycoproteins. While it is thought that the smaller band is the G (43kDa) protein, it is not possible to rule out that it is the cleaved F1 (47kDa) protein, which is predicted to be approximately the same size. The NDV stained glycoproteins are HN (68kDa) and F (58kDa). The smaller protein which would correspond to F is the same size as the larger glycoprotein of APV and the F proteins of both NDV and APV are 58,000 Da. The F1 cleaved proteins are both 47,000 and the smaller band in the APV stain is not present in the NDV stain, and it is predicted that the smaller band could indeed be the G protein. To better determine if APV/CO does have the G protein present, mAbs directed against the APV G protein would be needed.

APV/A and APV/B were placed into different subgroups based on their divergent G protein sequence. The G gene has failed to be sequenced for APV/CO and this glycoprotein stain confirms that it is possibly present. The APV/CO G protein is important because it will allow further phylogenetic analysis to be performed to better determine how the APV isolates are related and how they may have evolved.

Nucleotide Sequence Analysis

The avian pneumovirus N gene of APV/A and APV/B both were 1186 nucleotides long and had a predicted sequence of 391 amino acids. The APV/CO N gene was 1195 nucleotides long and had a predicted protein sequence of 394 amino acids. All isolates contained a single open reading frame, and the predicted amino acid sequences were well conserved amongst all three isolates. APV/A and APV/B had the greatest nucleotide sequence similarity of 73%. APV/CO and APV/B shared 67% nucleotide sequence identity while APV/CO and APV/A shared 54% nucleotide sequence identity. APV/A and APV/B N genes are more closely related than APV/CO and APV/A or APV/B. The predicted amino acid sequences of APV/A and APV/B were 91% similar, and APV/CO shared 70% amino acid sequence similarity with APV/A and APV/B. The higher conservation of the amino acid sequence relative to the nucleotide sequence demonstrates that most of the nucleotide changes are in the third position of the amino acids therefore resulting in synonymous changes.

The nucleotide sequence of all three isolates began with GGGACAAGT, which is common for all avian pneumovirus genes (Li et al., 1996). There was greater conservation at the C-terminal end of the N protein predicted amino acid sequence amongst APV/A, APV/B and APV/CO. This conservation of the C-terminal end has been found in all pneumoviruses as well as the very conserved stretch from amino acid

250 to 330 (Li et al., 1996). The conserved region may possibly be important in the nucleocapsid protein's role in binding RNA or another viral component (Collins et al., 1996) or it may be important in maintaining structural integrity (Li et al., 1996).

The phosphoprotein gene of avian pneumovirus is highly variable, and there was only 32% nucleotide sequence identity amongst all three isolates. APV/CO and APV/B, with 53% nucleotide sequence identity, were slightly more related than APV/A and APV/B, which had 51% nucleotide sequence identity. APV/CO and APV/A shared 45% nucleotide sequence identity. The protein sequences amongst all isolates shared 45% identity while APV/A and APV/B shared the highest level of amino acid sequence identity, 72%. This also shows that many of the nucleotide sequence differences occur in the third codon position. APV/CO and APV/A shared 53% amino acid sequence identity, while APV/CO and APV/B shared 51% amino acid sequence identity.

There were only two small conserved regions in the predicted amino acid sequences of APV/A, APV/B, and APV/CO P proteins. The first occurred at the N terminal end for the first 18 amino acids. The second conserved region occurred from amino acid 134 to amino acid 220 with 3 highly divergent spacers at position 160, position 170-171, and position 214. RSV also has two highly conserved regions from position 1 to position 58 and from amino acid 86 to amino acid 241 with a divergent spacer at position 186 (Collins et al., 1996). These conserved regions may be important in the P protein's roles as a transcription and regulation factor and as a chaperone that binds soluble L and N (Collins et al., 1996).

The P gene of other paramyxoviruses has also been found to have a modular organization with two conserved regions (Lamb and Kolakofsky, 1996). The C-terminus has been found to be very important, and the P genes of other paramyxoviruses lose function with deletion of the C-terminal region (Lamb and Kolakofsky, 1996). The C-terminus has also been shown to be very acidic (Lamb and Kolakofsky, 1996), and all

three APV isolates contain acidic amino acids at the C-terminal end despite the high degree of variability. The N-terminus is where most of the phosphorylation occurs (Lamb and Kolakofsky, 1996).

The M2 gene of avian pneumovirus contains two overlapping reading frames. APV/A and APV/B M2 genes contained a major ORF of from nucleotide position 14 to position 571 and a second ORF from nucleotide position 531 to position 749. The APV/CO M2 gene contained a major ORF from nucleotide position 14 to position 565 and from nucleotide position 525 to position 737. This agrees with the two overlapping reading frames reported for APV/A and for RSV (Yu et al., 1992b). The nucleotide sequences amongst all three APV isolates were found to share 39% identity. The nucleotide sequence of APV/A and APV/B were the most closely related sharing 68% identity. APV/CO M2 gene shared 41% and 57% nucleotide sequence identity with APV/A and APV/B, respectively. The predicted amino acid sequences were well conserved amongst all three isolates, they shared 68% protein identity. APV/A and APV/B shared 89% amino acid sequence identity; while APV/CO shared 70% and 72% amino acid sequence identity with APV/A and APV/B, respectively. This was comparable to the amount of conservation present in the N gene.

The M2 gene is only found in pneumoviruses. It is an internal protein, which is consistent with data showing that there are no extensive hydrophobic regions, which are usually present on envelope proteins (Yu et al., 1992b). The N-terminal region of the APV M2 gene was very well conserved with the exception of a divergence at amino acid 11. The N-terminus of the APV M2 gene has also been shown to be well conserved with the RSV M2 gene. The function of the M2 gene is not yet known for pneumoviruses, but it is thought to be associated with the N, P, L and RNA complex which is formed during transcription (Yu et al., 1992b). The high level of conservation, which is observed in the

APV M2 genes, agrees with this and could possibly be important for binding viral proteins or RNA.

The phylogenetic analysis which was performed on the contiguous coding sequences of the N, P, M, F, and M2 genes of APV/A, APV/B, and APV/CO showed that the avian pneumoviruses form a single clade with respect to their mammalian counterparts. There were three distinct types of avian pneumoviruses from this analysis and APV/A and APV/B appear to be more closely related, which was confirmed by the genes, which were sequenced. APV/A and APV/B were placed into two separate subgroups based on their highly divergent sequence of the attachment (G) protein (Juhasz and Easton, 1994). This was consistent with the two subtypes found in HRSV (Johnson et al., 1987). APV/CO was first isolated in the US during 1997, and was first tentatively characterized as a third subtype due to its lack of cross-reactivity with APV/A and APV/B subtypes (Seal, 1998). APV/CO was placed in a third subtype based on its M gene sequence (Seal, 1998). The phylogenetic analysis performed, using APV genes sequenced to date, further confirms that APV/CO is a third subtype of avian pneumoviruses.

REFERENCES

- Alexander, D. J. (1997). Newcastle disease and other avian Paramyxoviridae infections. In *Diseases of Poultry, 10th ed.*, pp. 541-569. Edited by Calnek, B. W., Barnes H. J., McDougall, L. R., Saif, Y. M., & Beard. Ames, Iowa: Iowa State University Press.
- Alexander, D. J. and Collins, M. S. (1977). The purification and polypeptide composition of avian infectious bronchitis virus. *Microbios* 18, 87-98.
- Bayon-Auboyer, M., Arnauld, C., Toquin, D., & Etteradossi, N. (2000). Nucleotide sequences of the F, L, and G protein genes of two non-A/non-B avian pneumoviruses (APV) reveal a novel APV subgroup. *Journal of General Virology* 81, 2723-2733.
- Belyavsky, A. Vinogradova, T., & Rajewsky, K. (1989). PCR-based cDNA library construction: general cDNA libraries at the level of a few cells. *Nucleic Acid Research* 11, 241-256.
- Bucholz, U. J., Finke, S., & Conzelmann, K. -K. (1999). Generation of bovine respiratory syncytial virus (BRSV) from cDNA: BRSV NS2 is not essential for virus replication in tissue culture, and the human RSV leader region acts as a functional BRSV genome promoter. *Journal of Virology* 73, 251-259.
- Cavanagh, D. and Barrett, T. (1988). Pneumovirus-like characteristics of the mRNA and proteins of turkey rhinotracheitis virus. *Virus Research* 11, 241-256.
- Chirgwin, J. M., Przybyla, A. E., McDonald, R. J., & Rutter, W. J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 18, 5294-5299.
- Collins, M. S., Gough, R. E., & Alexander, D. J. (1993). Antigenic differentiation of avian pneumovirus isolates using polyclonal antisera and mouse monoclonal antibodies. *Avian Pathology* 22, 469-479.

Collins, M. S. and Gough, R. E. (1988). Characterization of a Virus Associated with Avian Rhinotracheitis. *Journal of General Virology* 69, 909-916.

Collins, P. L., McIntosh, K., & Chanock, R. M. (1996). Respiratory Syncytial Virus. In *Fields Virology, Third Edition*, pp. 1313-1331. Edited by B. N. Fields, D. M. Knipe, P. M. Howley. Philadelphia: Lippencort-Raven Publishers.

Cook, J. K. A., Orthel, F., Orbell, S., Woods, M. A., & Huggins, M. B. (1996). An experimental turkey rhinotracheitis (TRT) infection in breeding turkeys and the prevention of its clinical effects using live-attenuated and inactivated TRT vaccines. *Avian Pathology* 25, 231-243.

Cook, J. K. A. (2000). Avian rhinotracheitis. *Revue scientifique et technique* 19 (2), 612-613. International Office of Epizootics.

Cook, J. K. A., Jones, B.V., Ellis, M. M., Jing, L., & Cavanagh, D. (1993). Antigenic differentiation of strains of turkey rhinotracheitis virus using monoclonal antibodies. *Avian Pathology* 22, 257-273.

Cook, J. K., Huggins, M. B., Woods, M. A., Orbell, S. J., & Mockett, A. P. (1995). Protection provided by a commercially available vaccine against different strains of turkey rhinotracheitis virus. *The Veterinary Record* 136 (15), 392-393.

Cook, J. K. A., Ellis, M. M., & Huggins, M. B. (1991). The pathogenesis of turkey rhinotracheitis virus in turkey poults inoculated with the virus alone or together with two strains of bacteria. *Avian Pathology* 22, 257-273.

Edson, R. K. (1997). Experience with Avian Pneumoviruses. *Proceedings of the 101st Annual Meeting of the U. S. Animal Health Association* 101, 471-472.

Enterradossi, N., Toquin, D., Guittet, M., & Bennejean, G. (1995). Evaluation of Different Turkey Rhinotracheitis Viruses used as Antigens for Serological Testing following Live Vaccination and Challenge. *Journal of Veterinary Medicine B* 42, 175-186.

Glisin, V., Crkvenjakov, R., & Byus, C. (1974). Ribonucleic acid isolated by cesium chloride centrifugation. *Biochemistry* 13, 2633-1637.

Grant, M., Baxter-Jones, C., & Wilding, G. P. (1987). An enzyme-linked immunosorbent assay for the serodiagnosis of turkey rhinotracheitis infection. *The Veterinary Record* 120 (12), 279-280.

- Gulati, B. R., Cameron, K. T., Seal, B. S., Goyal, S. M., Halvorson, D. A., & Njenga, M. K. (2000). Development of a highly sensitive and specific enzyme-linked immunosorbent assay based on recombinant matrix protein for detection of avian pneumovirus antibodies. *Journal of Clinical Microbiology* 38 (11), 4010-4014.
- Hames, B. D. (1981). An introduction to polyacrylamide gel electrophoresis. In *Gel Electrophoresis of Proteins: A Practical Approach*, pp. 1-91. Edited by B. D. Hames and D. Rickwood. Oxford: IRL Press.
- Hafez, H. M., Hess, M., Prusas, C., Naylor, C. J., & Cavanagh, D. (2000). Presence of Avian Pneumovirus Type A in Continental Europe During the 1980s. *Journal of Veterinary Medicine B* 47, 629-633.
- Heckert, R. A., Meyers, D. J., Afshan, A., & Riva, J. (1994). Development and evaluation of an enzyme-linked immunosorbent assay for the detection of antibodies to avian pneumovirus. *Avian Diseases* 38 (4), 694-700.
- Jing, L., Cook, J. K. A., Brown, T. D. K., Shaw, K., & Cavanagh, D. (1993). Detection of turkey rhinotracheitis virus in turkeys using the polymerase chain reaction. *Avian Pathology* 22, 771-783.
- Johnson, P. R., Spriggs, M. K., Olmstead, R.A., & Collins, P. L. (1987). The G glycoprotein of human respiratory syncytial viruses of subgroup A and B: extensive sequence divergence between antigenically related proteins. *Proceedings of the National Academy of Sciences, USA* 84, 5625-5629.
- Jones, R. C., Baxter-Jones, C., Wilding, G. P., & Kelly, D. F. (1986). Demonstration of a candidate virus for turkey rhinotracheitis in experimentally inoculated turkeys. *The Veterinary Record* 119, 599-600.
- Jones, R. C., Williams, R. A., Baxter-Jones, C., Savage, C. E., & Wilding, G. P. (1988). Experimental infection of laying turkeys with rhinotracheitis virus: distribution of virus in the tissues and serological response. *Avian Pathology* 17, 841-850.
- Jones, R. C., Naylor, C. J., Al-Afaleq, A., Worthington, K. J., & Jones, R. (1992). Effect of cyclophosphamide immunosuppression on the immunity of turkeys to viral tracheitis. *Research in Veterinary Science* 53, 38-41.
- Jones, R. C. (1996). Avian pneumovirus infection: questions still unanswered. *Avian Pathology* 25, 639-648.

Juhasz, K. and Easton, A. J. (1994). Extensive sequence variation in the attachment (G) protein gene of avian pneumovirus: evidence for two distinct subgroups. *Journal of General Virology* 75, 2873-2880.

Kapur, V., Dar, A., Tune, K., & Goyal, S. (1998). Molecular Diagnostic Tests for Avian Pneumovirus. Proceedings and Technical Supplement of The Roche Avian Pneumovirus Workshop, 54-55.

Kleven, S. H. (1997). Report of the Committee. Transmissible Diseases of Poultry and Other Avian Species. Proceedings of the 101st Annual Meeting of the U. S. Animal Health Association 101, 486-491.

Lamb, R. A. and Kolakofsky, D. (1996). *Paramyxoviridae: the viruses and their replication*. In *Fields Virology, Third Edition*, pp. 1177-1204. Edited by B. N. Fields, D. M. Knipe, P. M. Howley. Philadelphia: Lippencott-Raven Publishers.

Li, J., Ling, R., Randhawa, J. S., Shaw, K., Davis, P. J., Juhasz, K., Pringle, C.R., Easton, A. J., & Cavanagh, D. (1996). Sequence of the nucleocapsid protein gene of subgroup A and B avian pneumoviruses. *Virus Research* 41, 185-191.

Ling, R., Davis, P. J., Qingzhong, Y., Wood, C. M., Pringle, C. R., Cavanagh, D., & Easton, A. J. (1995). Sequence and in vitro expression of the phosphoprotein gene of avian pneumovirus. *Virus Research* 36, 247-257.

Ling, R. and Pringle, C. R. (1988). Turkey Rhinotracheitis Virus: in vivo and in vitro Polypeptide Synthesis. *Journal of General Virology* 69, 917-923.

Ling, R., Easton, A. J., & Pringle, C. R. (1992). Sequence analysis of the 22K, SH and G genes of turkey rhinotracheitis virus and their intergenic regions reveals a gene order different from that of other pneumoviruses. *Journal of General Virology* 73, 1709-1715.

Majo, N., Allan, G. M., O'Loan, C. J., Pages, A., & Ramis, A. J. (1995). A sequential histopathologic and immunocytochemical study of chickens, turkey poults, and broiler breeders experimentally infected with turkey rhinotracheitis virus. *Avian Diseases* 39, 887-896.

Majo, N., Marti, M., O'Loan, C. J., Pages, A., & Ramis, A. J. (1996). Ultrastructural study of turkey rhinotracheitis virus infection in turbinates of experimentally infected chickens. *Veterinary Microbiology* 52, 37-48.

Majo, N., Gibert, X., Vilafranca, M., O'Loan, C. J., Allan, G. M., Costa, L., Pages, A., & Ramis, A. (1997). Turkey rhinotracheitis virus and *Escherichia coli* experimental infection in chickens: histopathological, immunocytochemical and microbiological study. *Veterinary Microbiology* 57, 29-40.

Martínez, I., Valdés, O., Delfraro, A., Arbiza, J., Russi, J., & Melero, J. (1999). Evolutionary pattern of the G glycoprotein of human respiratory syncytial viruses from antigenic group B: the use of alternative termination codons and lineage diversification. *Journal of General Virology* 80, 125-130.

McMaster, G. K. and Carmichael, G. G. (1977). Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. *Proceedings of the National Academy of Sciences, USA* 74 (11), 4835-4838.

Mead, D. A., Pey, N. K., Herrnstadt, C., Marcil, R. A., Smith, L. M. (1991). A universal method for the direct cloning of PCR amplified nucleic acid. *Biotechnology* 9, 657-663.

Mufson, M. A., Örvell, C., Rafnar, B., & Norrby, E. (1985). Two distinct subtypes of human respiratory syncytial virus. *Journal of General Virology* 66, 2111-2124.

Naylor, C. J., Britton, P., & Cavanagh, D. (1998). The ectodomains but not the transmembrane domains of the fusion proteins of subtypes A and B avian pneumoviruses are conserved to a similar extent as those of human respiratory syncytial virus. *Journal of General Virology* 79, 1393-1398.

Naylor, C. J., Shaw, K., Britton, P., & Cavanagh, D. (1997). Appearance of type B avian pneumovirus in Great Britain. *Avian Pathology* 26, 327-338.

Naylor, C. J. and Jones, R. C. (1994). Demonstration of a virulent subpopulation in a prototype live attenuated turkey rhinotracheitis vaccine. *Vaccine* 12 (13), 1225-1230.

O'Loan, C. J., Allan, G., Baxter-Jones, C., McNulty, M. S. (1989). An improved ELISA and serum neutralization test for the detection of turkey rhinotracheitis virus antibodies. *Journal of Virological Methods* 25 (3), 271-282.

Panigrahy, B., Senne, D. A., Pedersen, J. C., Gidlewski, T., Edson, R. K. (2000). Experimental and serological observations on avian pneumovirus (APV/turkey/Colorado/97) infection in turkeys. *Avian Diseases* 44 (1), 17-22.

Peebles, M. and Levine, S. (1979). Respiratory syncytial virus polypeptides and their location in the virion. *Virology* 95 (1), 137-145.

- Pringle, C. R. (1996). Virus Taxonomy 1996. A bulletin from the 10th International Congress of Virology in Jerusalem. Archives of Virology 141, 2251-2256.
- Pringle, C. R. (1998). Virus Taxonomy, San Diego, 1998. Archives of Virology 143, 1449-1459.
- Qingzong, Y., Barrett, T., Brown, T. D., Cook, J. K., Green, P., Skinner, M. A., Cavanagh, D. (1994). Protection against turkey rhinotracheitis pneumovirus (TRTV) induced by a fowlpox virus recombinant expressing the TRTV fusion glycoprotein (F). Vaccine 12 (6), 569-573.
- Randhawa, J. S., Marriott, A. C., Pringle, C. R., & Easton, A. J. (1997). Rescue of Synthetic Minireplicons Establishes the Absence of the NS1 and NS2 Genes from Avian Pneumovirus. Journal of Virology 71 (12), 9849-9854.
- Randhawa, J. S., Pringle, C. J., & Easton, A. J. (1996b) Nucleotide sequence of the Matrix Protein Gene of a Subgroup B Avian Pneumovirus.
- Randhawa, J. S., Wilson, S. D., Tolley, K. P., Cavanagh, D., Pringle, C. R., & Easton, A. J. (1996a). Nucleotide sequence of the gene encoding the viral polymerase of avian pneumovirus. Journal of General Virology 77, 3047-3051.
- Rima, B. (1989). Comparison of amino acid sequences of the major structural proteins of the paramyxo- and morbilliviruses. In *Genetics and Pathogenicity of Negative Strand Viruses*, pp. 254-263. Edited by D. Kolakofsky & B. M. J. Mahy. Elsevier, Amsterdam.
- Sanger, F., Nicklen, S., & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. Proceedings of the National Academy of Science USA 74, 5463-5467.
- Seal, B. S., Sellers, H. S., & Meinersmann, R. J. (2000). Fusion protein predicted amino acid sequence of the first US avian pneumovirus isolate and lack of heterogeneity among other US isolates. Virus Research 66, 139-147.
- Seal, B. S. (1998). Matrix protein gene nucleotide and predicted amino acid sequence demonstrate that the first US avian pneumovirus isolate is distinct from European strains. Virus Research 58, 45-52.

Seal, B. S. (1996). Analysis of matrix protein gene nucleotide sequence diversity among Newcastle disease virus isolates demonstrates that recent disease outbreaks are caused by viruses of psittacine origin. *Virus Genes* 11, 217-224.

Seal, B. S. and Whetstone C. A. (1994). Immediate-early gene expression of gene mapping comparisons among isolates of bovine herpesvirus 1 and 5. *Veterinary Microbiology* 38, 369-384.

Seal, B. S., Irving, J. M., & Whetstone, C. A. (1991). Transcriptional analysis of the bovine herpesvirus 1 Cooper isolate. Temporal analysis and characterization of immediate-early, early, and late RNA. *Archives of Virology* 121 (1-4), 55-73.

Seal, B. S., St. Jeor, S.C., & Taylor, R. E. L. (1985). Restriction endonuclease analysis of bovine herpesvirus 1 DNA and nucleic acid homology between isolates. *Journal of General Virology* 66, 2787-2792.

Senne, D. A, Pedersen, J. C., & Panigrahy, B. (1998). Avian Pneumovirus in Turkeys: Preliminary Findings. *Proceedings and Technical Supplement of: The Roche Avian Pneumovirus Workshop*, 47-49.

Smith, L. M., Sanders, J. Z., Kaiser, R. J., Hughs, P., Dodd, C., Connell, C. R., Heiner, C., Kent, S. B., & Hood, L. E. (1986). Fluorescence detection in automated DNA sequence analysis. *Nature* 321, 673-681.

Swofford, D. (1989). PAUP: phylogenetic analysis using parsimony. Version 3.1, Champaign, IL, Illinois Natural History Survey.

Thompson, J. D., Higgins, D. G., & Gibson, T. J. (1994). CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acid Research* 22, 4673-3680.

Wyeth, P. J. and Alexander, D. J. (1989). Pneumovirus infections. In *A Laboratory Manual for the Isolation and Identification of Avian Pathogens, Third Edition*, pp. 121-123. Edited by H. G. Purchase, L. H. Arp, C. H. Domermuth, & J. E. Pearson. Kennett Square, PA: American Association of Avian Pathologists.

Yu, Q., Davis, P. J., Li, J., & Cavanagh, D. (1992a). Cloning and Sequencing of the Matrix Protein (M) Gene of Turkey Rhinotracheitis Virus Reveal a Gene Order Different from That of Respiratory Syncytial Virus. *Virology* 186, 426-434.

Yu, Q., Davis, P. J., Brown, T. D. K., & Cavanagh, D. (1992b). Sequence and in vitro expression of the M2 gene of turkey rhinotracheitis pneumovirus. *Journal of General Virology* 73, 1355-1363.

Yu, Q., Davis, P. J., Barrett, T., Binns, M. M., Boursnell, M. E. G., & Cavanagh, D. (1991). Deduced amino acid sequence of the fusion glycoprotein of turkey rhinotracheitis virus has greater identity with that of human respiratory syncytial virus, a pneumovirus, than that of paramyxovirus and morbilliviruses. *Journal of General Virology* 72, 75-81.

Yunus, A. S., Collins, P. L., & Samal, S. U. (1998). Sequence analysis of a functional polymerase (L) gene of bovine respiratory syncytial virus: determination of minimal transacting requirements for RNA replication. *Journal of General Virology* 79, 2231-2238.

Zhou, C., Yang, Y., & Jong, A. Y. (1990). Mini-prep in ten minutes. *Biotechniques* 8 (2), 172-173.