A comparison of various methods for the detection of the U.S. avian pneumovirus

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A comparison of various methods for the detection
of the U.S. avian pneumovirus

by

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A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

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This is to certify that the Master’s thesis of

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has met the thesis requirements of Iowa State University.

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LIST OF ABBREVIATIONS AND ACRONYMS

AAF - amniotic/allantoic fluid
ABI - Applied Biosystems, Inc.
APV - avian pneumovirus
AR - allantoic route of inoculation
BL-3 - biosafety level 3
BRSV - bovine respiratory syncytial virus
cDNA - complementary deoxyribonucleic acid
CEF - chicken fibroblast cells
DEP - diethlypyrocarbonate
DNA - deoxyribonucleic acid
DNTP - deoxyribonucleotide triphosphates
DPI - days post inoculation
E. coli - Escherichia coli
ELISA - enzyme-linked immunosorbent assay
EM - electron microscopy
F - fusion protein gene
FAM - 6-carboxyfluorescein
G - glycopolypeptide protein gene
HRSV - human respiratory syncytial virus
IBD - infectious bursal disease
IBV - infectious bronchitis virus
IF - immunofluorescence
IFA - indirect fluorescent antibody
IPC - internal positive control
K - kilobase
L - large polymerase protein
M - matrix protein gene
MEM - minimum essential medium
MG - Mycoplasma gallispticum
MPV - murine pneumovirus
N - nucleoprotein protein gene
NAC - no amplification control
NDV - Newcastle disease virus
NS - nonstructural protein
NTC - no template control
OD - optical density
ORSV - ovine respiratory syncytial virus
ORT - Ornithobacterium rhinotracheale
P - phosphorylated protein gene
PAGE - polyacrylamide gel electrophoresis
PCR - polymerase chain reaction
ΔRn - relative normalized fluorescence

RNA - ribonucleic acid

RSV - respiratory syncytial virus

RT-PCR - reverse-transcription polymerase chain reaction

SH - small hydrophobic protein gene

SHS - swollen head syndrome

SPF - specific-pathogen free

TAMRA - 6-carboxy-tetramethylrhodamine

TBTB - tris buffered tryptose broth

TCID\textsubscript{50} - tissue culture infective dose - 50

TOC - tracheal organ cultures

TRT - turkey rhinotracheitis

U.S. - United States of America

UK - United Kingdom

US/APV - U.S. subgroup of avian pneumovirus

US/CO - US/Colorado strain of avian pneumovirus

VI - virus isolation

VN - virus neutralization

YS - yolk sac route of inoculation
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THE SENSITIVITY AND SPECIFICITY OF A RT-PCR ASSAY FOR THE AVIAN PNEUMOVIRUS (COLORADO STRAIN)

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GENERAL INTRODUCTION

Introduction

Avian pneumovirus (APV) is the etiological agent of turkey rhinotracheitis (TRT), an upper respiratory disease of turkeys, and swollen head syndrome (SHS) in chickens (1,2,17,18). Avian pneumovirus, a non-segmented negative sense single-stranded RNA virus, is a member of the family Paramyxoviridae, subfamily Pneumovirinae and genus Metapneumovirus (19). Of the 8 proteins encoded by the APV genome, the matrix (M) and polymerase (L) proteins are two of the more conserved (20). The fusion (F) protein promotes cell fusion while the glycopolypeptide (G) protein is involved in cell attachment. The F and G proteins are major antigenic epitopes for the virus (12).

Avian pneumovirus first appeared in the United States (U.S.) in commercial meat turkeys in Colorado during May 1996 and persisted until March 1997. Condemnation losses due to airsacculitis were escalated by as much as 1.5% and mortality losses were as high as 20% in some flocks. The isolation of the APV/turkey/Colorado/97 (US/CO APV) represents the first isolation of APV in the U.S. In April 1997 antibodies to APV were detected in turkeys in Minnesota. APV continues to be a disease problem in Minnesota causing much financial loss to the industry since 1997 with high mortality rates and condemnation losses reaching 10% in some flocks (7). Henceforth, the term US APV will denote the antigenically similar APVs isolated in Minnesota and Colorado. Where the US subgroup of APV originated from or how it appeared in Colorado remains a mystery; however, APV is now in the US and a diagnostic test is needed to quickly detect the disease.
The U.S. APV is antigenically different from previously characterized APVs (20). Prior to the isolation of the US/CO APV, most APVs have been classified as either subgroup A or B viruses, based on cross neutralization studies and glycopolypeptide gene sequence analysis (8,12). European subgroup A and B viruses have limited cross reactivity with the U.S. subgroup of APV. Experimental studies have demonstrated that turkeys infected/vaccinated with the Colorado APV were not protected against challenge with subgroup A or B virus. However, turkeys infected/vaccinated with subgroup A or B virus were protected against challenge with Colorado APV (21). Serological and immunofluorescent reagents used in the detection of APV and APV antibody are subgroup specific. In view of the limited cross reactivity and the recent matrix (M) gene sequence analysis (20), confirming the unique antigenic characteristic of the US/CO APV, diagnostic tests for the detection of the US/APV are needed. The European strains of subgroup A and B APV are still considered exotic to the US and need to be distinguished diagnostically from the US subgroup.

APV is a fastidious agent that passes very quickly through the respiratory tract. A brief incubation period of 1-3 days precedes a short clinical phase, 3-7 days (10,16). High morbidity rates (90-100%) and mortality rates as high as 30% with exacerbating factors, such as secondary bacterial pathogens and poor husbandry, establish TRT as a economically significant disease (22). APV infects the ciliated epithelial cells of the upper respiratory tract causing severe rhinitis and tracheitis. Focal deciliation incapacitates the mucociliary clearance mechanism allowing opportunistic bacterial pathogens to invade the upper respiratory tract. *E. coli*, and other secondary pathogens, increase mortality rates and carcass
condemnation due to airsacculitis (11,14). APV infection is characterized by rapid onset and is quickly transmitted by contact. Other modes of transmission have been theorized, but are not substantiated as yet. APV spread quickly throughout most of England and Wales in the mid 1980's. The speed with which this disease can be transmitted through susceptible flocks under favorable conditions further emphasizes the need for a rapid and sensitive assay for the detection of APV.

Traditional isolation techniques for the detection of APV are performed in tracheal organ cultures (TOC) or embryonating chicken or turkey eggs. Both TOC and embryo inoculation techniques require several blind passages followed by immunofluorescence staining to identify the virus. These procedures are slow and time consuming and may take a period of 3 to 6 weeks to detect the virus. Virus isolation techniques are insensitive and contamination by bacterial pathogens remains a problem.

Presence of antibody to APV is considered evidence of exposure to infection (21). Enzyme-linked immunosorbent assays (ELISA) using anti-species conjugates and subgroup specific inactivated antigens can detect antibodies as early as 7-10 days after infection (3,21). A reverse-transcription polymerase chain reaction assay (RT-PCR) has been successfully used for the detection of APV (5,13,15). RT-PCR which involves isolation of viral RNA, production of cDNA and amplification of cDNA by the PCR can be performed in 24-48 hours. RT-PCR saves considerable time compared to standard isolation procedures.

RT-PCR has been demonstrated to be a sensitive diagnostic tool in detecting avian pathogens (5). Nested RT-PCR techniques capable of detecting APV as early as 1 day post inoculation (DPI) and as late as 19 and 20 DPI have been reported (15). Therefore RT-PCR
based techniques could extend detection of APV to as long as 19 DPI, whereas the window of opportunity for detection of the virus by virus isolation is 3-7 DPI, approximately 4 days. This expanded window of opportunity would improve the capability of detecting APV.

RT-PCR assays have been used to detect APV from tracheal and turbinate tissues (15) and tracheal swabs (4,5,13). Tracheal and turbinate tissues yield large quantities of APV while virus retrieval from lung specimens has been difficult and inconsistent (9). The capability of detecting viral RNA using the RT-PCR procedure in swab specimens would allow detection of the virus in live birds. A swab pool representing several birds would increase the likelihood of detection of viral nucleic acid or virus from field samples and reduce the number of PCR tests required. Swab pools will increase the likelihood of virus detection by compensating for RNA degradation with an enlarged viral RNA load and increase the chance that birds in the 3-7 DPI window are included in the sample pool.

A RT-PCR that is capable of detecting all subgroups of APV would be a useful diagnostic tool. However, detection of the US subgroup of APV is the immediate goal of this project. It was demonstrated by Dar et al. (6) that the US/CO APV can be detected using primers directed to the fusion gene of APV subgroup A. These primers will detect both subgroup A and the US/APV. The purpose of this study is to compare the sensitivity of the RT-PCR assay with traditional virus isolation. Commercial turkeys were experimentally inoculated with the US/CO APV and respiratory and swab specimens collected periodically for 21 days to determine which tissues or swabs are productive for detection of the disease.
Thesis organization

This thesis consists of a general abstract; a general introduction; a review of the literature; two manuscripts titled respectively: “Detection of avian pneumovirus in tissue and swab specimens from infected turkeys”, and “The sensitivity and specificity of a RT-PCR assay for the detection of avian pneumovirus (Colorado strain)”; a general conclusion. The master’s candidate, Janice Stanley Pedersen, is the senior author and principal investigator for the two manuscripts in this thesis.

References


Avian pneumovirus (APV) is the etiological agent of turkey rhinotracheitis (TRT), an acute upper respiratory tract disease of turkeys, which was first described in South Africa in 1978 (13). Initial characterization of APV was based on virion morphology as well as biochemical and molecular data (27). APV also infects chickens and causes swollen head syndrome (SHS) (14,75,97). Avian pneumovirus is a member of the family Paramyxoviridae which has five genera: Rubulavirus, composed of Newcastle disease virus (NDV) and other avian paramyxoviruses, Morbillivirus, Respirovirus, Pneumovirus and Metapneumovirus (99). APV is a member of the subfamily Pneumovirinae and genus Metapneumovirus (20,83,99). APV was recently separated from the genus Pneumovirus because it has no nonstructural NS1 and NS2 proteins (102) and its genome organization differs from that of other pneumoviruses (64,110,124). APV has been designated as type species for the newly named Metapneumovirus genus (99). Members of the genus Pneumovirus are bovine respiratory syncytial virus (BRSV), ovine respiratory syncytial virus (ORSV), human respiratory syncytial virus (HRSV) and murine pneumovirus (MPV).

Morphology

Electron microscopy (EM) studies have shown APV to be pleomorphic ranging between 80-200 nm in diameter, but occasionally as large as 500 nm (74,89,118,121). Viral particles are spherical at times, but they can be bizarre in shape. Elongated filaments range from 70 nm to 2 um in length, averaging 100-200 nm, with an average diameter of 80-100 nm (3). The membranous envelope is coated with a fringe of spikes 12-15 nm long. The
surface projections of APV are well defined and resemble those found on the human and bovine respiratory syncytial virus and not the shorter and less distinct fringe of MPV (23).

The helical nucleocapsid, 14 nm in diameter with a 7 nm pitch, differentiates APV from other viruses of the genera Respirovirus and Morbillivirus. Respirovirus and morbillivirus nucleocapsids have a smaller pitch and are 18 nm in diameter (15,23,74,121). APV’s nucleocapsid is less rigid than that of bovine or human respiratory syncytial virus (RSV). The helical nucleocapsid is stretched and extended, resembling MPV. APV is a non-segmented negative sense single-stranded RNA virus that is sensitive to lipid solvents and stable at pH 3.0 to 9.0. The virus is inactivated at 56°C after 30 min. and has a buoyant density of 1.1 g/ml (22). Unlike NDV and other avian paramyxoviruses, APV does not hemagglutinate turkey, chicken, human O or guinea pig erythrocytes (20). Away from the host, the virus has poor survivability and probably does not last for longer than 3-4 days (52).

History

Even though clinical signs for TRT were initially described in 1978, it was not until 1986 that a viral etiology was established for the disease (25,74,118,121). In March of 1978, Buys et al. (13,15) isolated a viral agent from a flock of turkeys experiencing severe respiratory symptoms with 90% morbidity, and in many instances, mortality of up to 30%. Most of the turkeys died from Escherichia coli (E.coli) associated airsacculitis, which followed the initial respiratory symptoms. No specific causative agent was identified.

In 1978-79, following the outbreak in South Africa, a respiratory disease spread through turkey flocks in Israel (67). Clinical signs included nasal discharge, swelling of the infraorbital sinuses and conjunctivitis. High morbidity and lower mortality were reported in
uncomplicated infections; when the disease was accompanied by colisepticaemia mortality increased considerably. Pathogenic agents isolated included Yucaipa virus a paramyxovirus, lentogenic NDV, Alcaligenes faecalis and Mycoplasma gallisepticum (MG) (67). Heller (48) concluded that these three agents in concert could reproduce the disease and called the infection turkey coryza, while others identified the causative agent as Alcaligenes faecalis or Bordetella avium (15).

In 1981 an explosive outbreak of an upper respiratory disease in fattening poults was observed in Brittany, France. Morbidity peaked to 100% quickly with mortality reaching 30-40% in 3- to 12-week-old poults. Following the initial outbreak, regular epizootic waves followed at roughly 6-month intervals (6). The overall epidemiology, clinical signs and post-mortem findings were suggestive of chlamydia (6,7). More recent opinion suggest that, Chlamydia psittaci may have been the main agent responsible for the first epizootic waves of rhinotracheitis; however, virus predisposed the poults to rhinotracheitis, while secondary agents produced the full clinical respiratory disease (67). A clinically similar disease occurred throughout Europe during this time period (2).

In June of 1985, an acute and rapidly spreading respiratory disease of turkeys appeared in Norfolk, England. The disease was recognized as a new clinical entity in the United Kingdom (UK). It was believed that this was the same disease seen previously in South Africa, Italy, Israel, Spain, and France (9). The disease first entered the UK in East Anglia, the region closest to continental Europe, and within 6 months had spread throughout England and Wales (53). The age of onset was variable with the initial outbreak affecting birds over 15 weeks of age; however, the majority of the time, the disease started in poults
1-5 weeks of age and then spread to older birds (9). Few incidents were seen in birds younger than 2 weeks of age, suggesting that vertical transmission was not a factor. Morbidity reached 100% with a variable mortality rate peaking at 30%. The higher levels of mortality were reported when the disease was associated with *E. coli* infection and husbandry problems. The method of transmission was not identified but assumed to be connected with mechanical carriage and feed lorries (9, 110). A viral etiology was determined following the isolation of a ciliostatic virus in tracheal organ cultures (TOC) (55, 74, 118, 121). McDougall & Cook (61) suggested that an orthomyxovirus or a coronavirus was the causative agent. Following EM studies Wyeth *et al.* (121) proposed the agent to be a member of the family *Paramyxoviridae*. Collins & Gough (23) concluded in 1988 the agent associated with TRT was APV, a member of the family *Paramyxoviridae* and genus *Pneumovirus*. The list of proposed causative agents for TRT included adenovirus, bordetella-like bacteria, Yucaipa virus, infectious bronchitis virus, Newcastle disease virus, and chlamydia (83). Early on the disease was referred to by some as turkey coryza. Stuart (111) differentiated APV from turkey coryza; improved management was successful in eradicating turkey coryza but not APV.

**SHS**, as well as TRT, was initially described in South Africa in the 1970s. SHS was originally assumed to be a secondary problem associated with NDV since it was first reported in broilers in South Africa during 1971/72 when most poultry producing areas around the world were experiencing a NDV pandemic. However, with the intensive use of live vaccines, NDV diminished in incidence, yet SHS seemed to be on the increase (64). Picault *et al.* (97) isolated a TRT-like virus from chickens by inoculating specific-pathogen-free (SPF) turkeys...
with suspensions of chicken organs. Both SPF turkeys and SPF chickens were inoculated with tissue suspensions; however the disease was only reproduced in turkeys. A TRT-like agent was observed by EM in nasal exudates and in virus isolated from infected TOC. SHS was observed for the first time in Spain in 1984. The disease spread progressively over the entire country reaching a peak in the late 1980s when almost 100% of the broiler and breeder farms were effected. APV was isolated from broilers in 1988; prior to this time serological testing for APV had been conducted in an effort to determine a cause. Enzyme-linked immunosorbent assay (ELISA) testing for APV produced mixed results with sporadic positive sera. This problem was later attributed to a lack of antigenic specificity. ELISA antigen used to test sera in Spain was received from Central Veterinary Laboratory (CVL), Weybridge, England (92). Monoclonal antibody studies have since revealed a definite antigenic difference between the Spanish and British APV (25).

APV was first isolated in the United States in 1997 from turkeys in Colorado experiencing a severe respiratory disease (108). Several months after the initial isolation, APV was detected in turkeys in Minnesota. APV has not been eliminated from Minnesota where it remains a disease problem. Only turkeys, and not chickens, have been effected in the U.S. APV is distributed world wide with the exception of Canada and Australia (106).

Subgroup identification

Monoclonal antibodies of the IgG1 or IgG2a isotype, which recognize the G glycoprotein of APV, and polyclonal sera were used to study antigenic relatedness between UK, French, South African (S.A.), Hungarian, Italian, and Dutch isolates. Multiple S.A. and UK viruses isolated over several years, as well as the other European viruses, were included
in the study. Cross-neutralization tests using polyclonal antiserum showed some diversity between the APV isolates, yet, all isolates examined had some degree of relatedness and belong to one serotype (25). Cross-neutralization studies using neutralizing monoclonal antibodies (mAbs) were used to identify two subgroups (A and B) within the single serotype of APV. Studies showed that the 1978 South African isolate is antigenically related to 1985-1990 UK isolates, but distinctly different from the French virus isolated in 1986 (25). It was concluded that the UK and French viruses originated from different sources. UK strains isolated over a 6-year period were closely related, indicating antigenic stability. APVs have been separated into distinct subgroups, following the nomenclature used for HRSV. The UK and South African viruses formed subgroup A, while the remaining European viruses (French, Spanish, Dutch, and Hungarian strains), formed subgroup B (25). The formation of two subgroups is supported by G glycoprotein genome analysis (60). Subgroup A and B can be distinguished on the basis of restriction enzyme digestion of PCR-generated G protein products. Geographic separation of subgroup A and B viruses no longer exists. Subgroup A viruses have now been identified in Europe and subgroup B viruses are now predominant in the UK (53). Cross-neutralization studies with the recently isolated APV/turkey/Colorado/97 have shown that the virus has a unique antigenic characteristic compared to subgroup A and B APVs (108). Initial attempts to detect APV antibody in turkey sera collected in Colorado in 1997 was complicated by the use of subgroup A and B ELISA reagents (106). Sequencing analysis of the M protein gene has separated the US/CO APV from European APV type A and B strains (105). The classification of this virus as a
third subgroup or as a second serogroup within the Metapneumovirus genus has been proposed. (106).

Disease description and distribution

APV clinical disease in turkeys, primarily young poults, is characterized by rhinitis, tracheitis, submaxillary edema, and swollen infraorbital sinuses. Clinical signs include snicking, rales, nasal and ocular discharge, depression, head shaking and sneezing (67,83). One of the most characteristic features of the disease is rapid onset. Morbidity within a house or a pen may approach 100% within 24 hours of the first symptoms (9). The initial symptoms of sneezing, coughing and submaxillary edema are followed by rales and a watery nasal discharge. As the disease progresses the discharge becomes thicker and eventually blocks the nares causing the birds to breathe with an open mouth. The infraorbital sinuses become distended and filled with fluid which is clear at first but becomes caseous later (9,15). Infection of the infraorbital sinuses can be unilateral or bilateral. Approximately 5 days post infection (p.i.) a frothy ocular discharge may appear (83). Normally most flocks make a complete recovery within 10-12 days (9,15,55,74,121). Secondary bacterial pathogens and poor husbandry complicate the disease by extending recovery time and increasing intensity of the infection.

In laying turkeys, clinical signs are generally less severe (83). Older birds often experience airsacculitis which is associated with accumulation of grayish fluid and later caseous material in the air sacs. Reduction in feed consumption is common in layers and adult breeding flocks with a possible rapid drop in egg production of up to 70% (3,26,110). Reduced egg production may last for up to 2-3 weeks with full recovery by 4 weeks (26).
Stuart (110) reported, following field observations, that many of the eggs laid during the recovery period are white and have thin shells thus reducing the number of settable eggs (9,110). Experimental inoculation of layers with APV has not shown an increase incidence of poor quality or pale-shelled eggs during the recovery period (26). Based on European outbreaks, the first birds to suffer are poults, followed by layers (38).

Turkeys of all ages are susceptible, though not equally so. There is considerable variation in mortality rates, ranging from as low as 0.4% to as high as 30% and generally occurring before 13 weeks of age (9,15,110). When disease is seen, morbidity is often as high as 100-90% (9,15). Young turkey poults experience higher mortality than older birds and layers (1,110). Stuart (111) demonstrated that APV infection is more severe in 1-day-old poults than 6-week-old birds. Mortality occurs in three phases; the first phase is concurrent with the initial signs. The second phase is 1-3 weeks later when secondary agents appear to be the main cause. The third phase develops in houses that have had high mortalities in the earlier phases, there is a need for culling and birds die due to problems such as swollen hocks or adhesions between the pericardial sac and the epicardium (83,111).

SHS is a term commonly used to refer to avian pneumovirus infections in chickens. SHS is a condition where gelatinous deposits collect under the skin. Edema of the head is a characteristic sign of APV in chickens; however, swollen heads are also associated with other diseases such as Bordetellosis and Colibacillosis. SHS is considered an inappropriate term by some, as SHS may occur in the absence of pneumovirus infection. Swollen heads in chickens have never been reproduced with APV infection alone (52), but have been reported to occur with infectious bronchitis virus (IBV) infection (77). Avian pneumovirus in chickens
will be referred to as SHS and is considered a disease of chickens that occurs when APV interacts in synergism with another agent.

SHS usually occurs in broilers and broiler breeders (59). Primary upper respiratory lesions include rhinitis, tracheitis and sinusitis; secondary lesions which may develop are pneumonia, and airsacculitis (80). Other clinical signs include depression, lack of locomotion, sneezing, coughing, head shaking, head scratching and periorbital edema which is often unilateral but may extend over the whole skull (14,83). Swelling of the head is due to a gelatinous exudate under the skin. Experimental infection with APV alone does not cause SHS, but results in very mild clinical signs or asymptomatic infection (14,54,57). E. coli has been shown to be involved with this disease and can be isolated from the purulent exudate in the nasal cavity (59). Similar to TRT in turkeys, SHS has a short incubation period of 4-5 days (14) and is exacerbated by secondary bacterial infection and poor husbandry. Nunoya (85) reported a gradual spread of SHS in contrast to TRT, which is a rapidly spreading disease in turkeys. Flock mortality and morbidity are low in uncomplicated infections ranging from 1 to 7% (83,84).

A discrete respiratory phase with a nasal sneeze or “snick,” slight nasal discharge and at times a loss of appetite is seen initially. As the disease quickly progresses, there is reddening of the conjunctivae and swelling of the lacrimal gland, followed 12-24 hours later by submandibular edema starting around the eyes, progressing over the head, and descending to the intermandibular tissues and wattles (14,38,77,110). Conjunctivitis may cause the birds to scratch around the eyes (38). The eyes of severely affected chickens close due to swollen eyelids and facial skin (84). Sneezing and nasal discharge is experienced by more than 50%
of the birds, while swelling of the infraorbital sinuses occurs in a lower percentage of the
birds with fewer than 1% developing classical swollen heads (14). Nervous system
involvement consisting of mild equilibrium disturbance to severe postural abnormalities of
the head and neck may accompany the respiratory disease. Inner ear infection leads to loss of
balance and incoordination (59). In contrast to turkeys, the more severe disease often occurs
in mature breeders rather than young chickens. Necropsy may reveal ulcers in the cornea,
swelling of the skin of the head due to gelatinous fluid, inspissated exudate in the
subcutaneous tissues, caseous exudate in the bones of the head, middle and inner ears, and
meninges, and inflamed or ruptured ovarian follicles with egg yolk peritonitis. Loss of egg
production can be a problem in chicken breeders (84).

In addition to the isolation of APV in South Africa (13) and the European countries of
France (40), Israel (117), Spain, Germany, Hungary (83) and the UK (9); seroconversion to
APV has been observed in poultry in Asia (69) and South and Central America (113). Other
countries reporting evidence of the disease are the Netherlands, Greece (2), Taiwan (69),
Japan (112), Mexico (53), Brazil (10) and the Dominican Republic (96). Major poultry
producing countries which have not shown evidence of APV virus infection are Canada and
Australia (53). Bell and Alexander conducted an extensive serological study of Australian
turkey flocks in 1990 and failed to find any evidence of the disease (83). The United States
was free of APV infection until 1997 when APV was isolated from commercial turkeys in
Colorado experiencing respiratory disease (108). An antigenically similar APV was later
isolated from commercial turkeys in Minnesota where eradication of the disease has been
difficult. Serological surveys have shown evidence of the disease in Minnesota, South Dakota, and North Dakota (108).

Following the explosive UK outbreak, when TRT spread across England and Wales within a 9-month period, studies were conducted to determine the mode of transmission. Contaminated water, movement of affected or recovered poults, personnel, equipment, feed trucks, etc. have all been implicated. Stuart suggested the involvement of feed lorries in the spread of the disease throughout England (110). Because of the speed by which the disease spread, it has frequently been assumed to be transmitted by air (83). Following experimental inoculation studies, Giraud reported (83) that APV could be transmitted through the air while Cook (31) reported that APV is not transmitted by air. At present, only contact spread has been confirmed (3). Transmission studies conducted on waterfowl have demonstrated that ducks can be experimentally infected with the US/APV. Virus was recovered from experimentally exposed ducklings, but no clinical signs were observed (78). The role of wild birds in the US is currently under review. Sparrows in close proximity to grower facilities have been found to be positive for APV by molecular techniques (104). Human RSV was shown by Hall, (44,43,45) to be transmitted between patients during contact with infected individuals or their secretions. Even though APV has been found to infect the oviduct epithelium of laying turkeys, there have been no reports of vertical transmission from layers to progeny (58,83). Observation does not implicate vertical transmission as the disease has a short incubation period and poults are usually not affected until several weeks of age.
Monoclonal antibody studies conducted with APV isolates collected over a period of ten years demonstrated that APV isolated from South Africa in 1978 is more closely related to UK viruses isolated between 1985 and 1990 than a 1986 French isolate, suggesting different sources of the virus despite the close time period for isolation and proximate geographical location (35). Stuart purposed that the UK APV could have originated in South Africa and was possibly transmitted by migratory birds (110). German workers have recently reported the detection of antibodies to APV in the sera of herring gulls near the Baltic coast (46). It is possible that long-distance migratory birds such as the arctic tern or swallow were responsible for the transmission of APV from South Africa to the UK; however, there is no evidence to confirm the involvement of migratory birds. Further transmission studies are warranted.

APV has been isolated from both turkeys (74, 121) and chickens (13, 97) following natural infections. Gough et al. (42) inoculated chickens, turkeys, Pekin ducklings, goslings, pheasants, guinea fowl and pigeons with turkey APV CVL 14/1, a subgroup A virus. All birds were observed daily and sampled periodically for virus isolation (VI) and antibody detection. Only turkeys displayed overt clinical signs typical of APV (42). A transient conjunctivitis was seen in the pheasants 2-4 days post inoculation (DPI). Antibodies were detected by ELISA in turkey, chicken, pheasant, and guinea fowl sera while virus neutralizing (VN) antibodies were detected only in chicken and turkey sera. Pheasants and guinea fowl appear to be susceptible to APV as demonstrated with an antibody response; however, no virus was recovered from either species (42). Cook (32) demonstrated that the biological behavior of APV may at least be partially species dependent. For this reason, the
lack of virus isolation and low antibody titers in other avian species should be viewed cautiously. APV CVL 14/1 is a turkey isolate, and turkeys were shown to be the most susceptible to APV in this study.

Secondary bacterial infections

Bacteria are important secondary pathogens in TRT as seen in both field and experimental infections of turkeys. No one bacterial pathogen has been identified to be the sole problem, although investigations have identified several candidates: *E. coli*, *Ornithobacterium rhinotracheale* (ORT), *MG*, *Bordetella avium* (*B. avium*) and *Pasteurella* (17, 52, 81). Several researchers have noted that complications caused by *E. coli* are frequent (31, 38). Infection was slightly more severe when *Bordetella avium* and *Pasteurella*-like organisms were coinfected with APV. At necropsy, the poults inoculated with both bacterial and viral inoculums had thickened air sacs while those inoculated with virus only showed no air sac abnormalities (31). Virulent APV was recovered from the internal organs or intestinal tract of birds infected with both virus and bacteria. The involvement of bacterial infections increases the length and severity of infection by causing the virus to persist longer and penetrate deeper into the tissues. *B. avium* colonized in the upper respiratory tract to some degree but did not invade internal organs unless virus was included in the inoculum (31).

*MG*, a natural respiratory pathogen of turkeys, is recognized as an exacerbating factor in respiratory diseases of poultry caused by viruses such as ND and IBV. Coinfection of APV and *MG* results in a more severe respiratory disease than with either agent alone. Dual infection leads to a higher incidence of gross lesions but did not cause an increased persistence or invasiveness of the virus (83). Field outbreaks of TRT in turkeys can be
complicated by intercurrent infection with any one of several bacterial pathogens: *E. coli*, *Bordetella* or *Pasteurella* sp., ORT and/or mycoplasmas (59).

Initial laboratory pathogenicity studies of SHS in chickens inoculated with APV alone failed to reproduce the clinical disease, leading researchers to hypothesize a mixed infection. Two facts support such a hypothesis: first, microbiological studies of SHS clinical cases demonstrate the presence of several bacteria, including *E. coli*, (69,84,95); second, pathogenesis studies of APV infection in chickens suggest that the lesions observed in the respiratory epithelium of the nasal cavity can favor the colonization of secondary agents (72). Picault (97) and Catelli (16) observed that *E. coli* intensified clinical signs of SHS. In experimentally inoculated chickens, concurrent APV and *E. coli* infection results in a more severe clinical and pathological disease. All dual infected chickens sacrificed on 5 and 7 DPI showed lethargy, bristled feathers, purulent nasal discharge and conjunctival congestion. At 9 and 11 DPI chickens exhibited a poor general condition along with mucopurulent nasal discharge and congestion of the conjunctiva. At 14 and 18 DPI only a slight nasal discharge was present and the birds were in good general condition. In contrast, chickens inoculated with APV alone showed lethargy, bristled feathers and slight mucoid nasal discharge at 5 and 7 DPI; at 10 DPI the birds showed no evidence of clinical signs or gross lesions (72). These studies demonstrate that secondary pathogens can increase the severity and length of APV disease in chickens. There have been only two reports on the successful reproduction of SHS in chickens using homogenized upper respiratory organs (95) and sinus exudates (14).

Two basic conditions are responsible for exacerbated multiplication of bacteria: alteration of nonspecific defense mechanisms in the respiratory tract and
immunosuppression. APV infection causes rhinitis and sinusitis which are responsible for the destruction of respiratory epithelium allowing the colonization and proliferation of bacteria in the subcutaneous tissue (80). It has been demonstrated that viruses causing respiratory tract lesions, such as IBV or avian adenovirus, can facilitate dissemination and multiplication of *E. coli* in several organs (79). Histopathological and immunocytochemical studies support the hypothesis that APV acts as a primary agent, inducing respiratory epithelial lesions that allow entrance and colonization of the epithelium by *E. coli* or other microorganisms which are the common flora of the respiratory tract (72).

An extensive study of the ultrastructural changes in chicken turbinates is helpful in understanding the pathogenesis of both SHS and TRT. Viral antigen was observed using IF techniques in the cytoplasm and associated cilia of the turbinate epithelial cells on 3 and 5 DPI. Clumping and loss of cilia were observed in the apical cell membrane of many infected cells. On day 7 p.i. regenerative changes were observed in the ciliated epithelium. It has been suggested that epithelial damage decreases the efficiency of the mucociliary clearance mechanism thus predisposing the bird to secondary bacterial infections (71).

**Virus isolation**

APV is an elusive agent as isolation of the virus followed reports of the disease by several years. The use of conventional virus isolation techniques, e.g. culture in chicken cell monolayers or embryonated chicken eggs, despite various attempts were not initially successful in isolation of the virus (12). Initial isolation and identification of APV was conducted in chicken embryo TOC. APV replicates well in TOCs causing complete ciliostasis (27). However, the US/CO does not cause ciliostasis. With APV, ciliostasis is
first observed 4-5 DPI in turkey TOC and at 6-8 DPI in chicken TOC, but is not complete until 10-11 DPI. Several blind passages may be necessary before complete ciliostasis is achieved (12). Low to moderate virus titers are typically reported. Initial isolation attempts yielded viral stocks with $10^3$ to $10^4$ median ciliostatic doses (CD$_{50}$/ml) (12). The major advantage of TOC is the availability of a suitable marker, ciliostasis, indicating the presence of the virus. The US/APV does not produce ciliostasis in TOCs (33). The identity of the agent for both TOC and embryo propagated virus needs to be confirmed by virus neutralization or immunofluorescence.

After initial propagation in TOCs the virus can be adapted to cell culture monolayers prepared from several chicken tissues, e.g. chicken embryo liver cells and chicken embryo fibroblast cells (CEF), MDBK cells, MA104 cells (34) as well as VERO cells, but a period of adaptation is required before cytopathic changes become evident. The adaptation to cell culture is associated with a loss of virulence of the virus for turkeys and chickens (27). Syncytial cell formation can be initially observed within 48-72 hr., depending on virus titer, with complete infection of the monolayer by 5-7 days. These same cytopathic changes do not generally become apparent during routine blind passage of respiratory tissues collected from the field. Immunofluorescence techniques can detect evidence of virus infection in cell culture despite the lack of observable cytopathic effect (12). Cultivation of the virus in cell culture has facilitated the development of a number of serological tests, i.e. ELISA and virus neutralization (VN).

APV has also been shown to grow in 6- to 8-day-old embryonated eggs inoculated via the yolk sac (YS) route. Embryo death, occurring between 7-14 DPI is common but not seen
in all cases. Embryo lesions resulting from viral infection include stunting, hemorrhagic embryos, “clubbed down” and enlarged necrotic livers (93,121). Even though these embryonic lesions are a result of infection, they are not unique to APV and therefore the agent needs to be identified. Prior to embryo inoculation, it is often necessary to remove bacteria from clinical respiratory specimens by passing the sample through membrane filters (0.2 to 0.45 um), thus preventing nonspecific embryo death (12,121). Several serial passages may be necessary before a sufficient titer of the virus is achieved and specific embryo death occurs (121). Chicken embryos inoculated with tissue suspensions by chorioallantoic membrane and/or allantoic sac routes (AR) have been unsuccessful for APV propagation despite numerous serial passages (15). Neither CAM or AR routes produced specific embryo mortality or macroscopic pathology in the embryos (18). Embryo YS material subsequently prepared and inoculated into VERO or chicken cell culture will allow identification of the virus either by EM or immunofluorescence (15).

APV can be recovered from tissues for only a relatively short period following infection, adding to the difficulty of isolation. Virus is shed for a short period from the upper respiratory tract and specimens for virus isolation (VI) need to be collected between 1-5 days p.i. Once obvious nasal discharge or swollen sinuses are seen, attempts at virus isolation may be unsuccessful. This small window of opportunity decreases the success of virus isolation. APV is more difficult to isolate from chickens than from turkeys, reasons for this are unknown (27).

In a comparative study, Jones found that turkey turbinates and trachea collected 2-5 DPI were the only respiratory tissues from which virus could be recovered. Virus was also
recovered from the middle magnum and vagina of laying turkeys at 9 DPI. Jones concluded that APV has an affinity for ciliated epithelial surfaces. Sequential studies conducted by Majo et al. (72) determined the main site of APV replication to be the ciliated cells of turbinates (1-5 DPI), trachea (3-6 DPI), and lung. No viral antigen was detected in tissue samples from conjunctiva. An *in vivo* study of APV conducted in chickens and turkeys reports virus recovery from lung, air sacs, trachea and turbinates using TOCs. The highest titer of virus was recovered from turbinates at 3-5 DPI; thereafter, the amount recovered fell quickly with no recovery after 7 DPI. Both turbinates and trachea yielded virus very early (3 DPI) and consistently till 7 DPI, while a sporadic recovery pattern was seen with the lung. In several birds, virus was recovered from the lung both early and late in the infection, but not during the middle phase. In other birds, virus was recovered only from 10-12 DPI or from 3-7 DPI. Both chicken and turkey air sacs between 3-12 DPI yielded virus. In the case of both the lung and air sacs, the quantity of virus recovered was small and virus recovery was inconsistent from bird to bird (32,70). Immunoperoxidase staining has shown that APV can replicate in the oviduct epithelium (61). *In vivo* attempts to recover virus from the oviduct failed to demonstrate APV replication in the hen oviduct, even after intravenous inoculation. At the same time, *in vitro* experiments revealed an intrinsic susceptibility of oviduct epithelium to APV infection (61). Despite the effect APV has on the reproductive tract, as demonstrated by a drop in egg production, oviduct epithelium is not a preferred tissue for recovery of the virus.
Histopathology

A serous rhinitis develops 1-2 DPI in the turbinates with an increase in glandular activity, epithelial exfoliation, focal loss of cilia, hyperemia, and mild mononuclear inflammatory infiltrate in the submucosa (70). Mucopurulent exudate blocking the turbinates 3 to 5 DPI contains heterophils, mucus, erythrocytes, and epithelial cells. By 6-8 DPI, the lesions are more advanced with mucus and epithelial cells in the lumen. Watery eyes can be seen in some birds (9,15,58,70). Clinical signs subside in poults by days 9-12 DPI (9,58).

Viral antigen can be detected in the turbinate epithelium by immunofluorescence (IF) between 1 and 5 DPI and in the trachea between 1 and 7 DPI with the maximum number of fluorescing cells seen on days 3-5 (27,70). Majo found that of all the tissues examined (trachea, turbinate, lung, air sac) the most consistent and severe lesions were associated with turbinates. A severe tracheitis was seen between 3 and 6 DPI with inflammatory exudate in the tracheal lumen, epithelial exfoliation, edema, hyperemia, and mononuclear inflammatory infiltrate in the submucosa. The viral antigen was associated with the ciliated epithelial cells (15,70). Viral antigen was also detected in epithelial cilia of the infraorbital sinuses and lacrimal duct between days 3 and 5 p.i. (70). APV appears to be directed to ciliated epithelial cells of the turbinates and trachea.

Lung lesions include inflammatory exudate in the bronchial lumen, hyperplasia of the bronchial epithelium, and the presence of abundant mononuclear inflammatory infiltrate in the submucosa of the bronchi that considerably decreases the diameter of the bronchial lumen (70). Jones (58) was unable to detect viral antigen by IF in the lungs and air sacs demonstrating that the disease primarily infects upper respiratory tissues.
Few studies have investigated APV infection in the reproductive system despite reports of significant drops in egg production. The presence of whitish masses of inspissated albumen scattered throughout various regions of the oviduct have been found to be common in 30-week-old turkey layers. Egg peritonitis and the presence of egg material in the abdominal cavity suggest damage to the oviduct and/or ovaries (58). Viral antigens have been detected by IF staining in the uterus epithelium on 7 DPI and in the surface epithelium of the oviduct including the vagina on 9 DPI (58). The quantity of epithelium containing virus is very small relative to the total volume of oviduct tissue. It was hypothesized that the virus could possibly travel to the reproductive organs via the bloodstream after primary replication in the upper respiratory tissues. However, this hypothesis was not confirmed by virus isolation studies (58). APV has been demonstrated in the reproductive tract of experimentally infected laying turkeys, but it has not been demonstrated in the oviduct of broiler breeders (57,70,88).

The pathology of SHS is similar to TRT yet unique. Virus persistence and tissue distribution have been studied using histological and immunochemical methods because virus isolation is difficult. On 1-2 DPI there is a mild mononuclear cell infiltration and edema seen in the lamina propria that leads to thickening of the mucosa, focal deciliation, and congestion by day 4 to 5 p.i. (16). Petechiasis of the turbinate mucosa progresses to a generalized red-to-purple discoloration. Mucoid exudate production is increased concurrently with petechiasis. Turbinate epithelial cells flatten, and there is a progressive sloughing of cilia starting at 6 DPI (16,77). Intraorbital swelling and nasal discharge is cleared by 9 DPI (15). Viral antigen is consistently demonstrated in the nasal turbinate.
ciliated border between 1 and 5 DPI and in the trachea and sinus on 4 DPI. The trachea
generally remains unaffected in the acute phase, although slight petechiation and reduced
ciliary activity may be observed in the upper trachea (16). Catelli concluded that the
infraorbital sinuses and trachea are infected, but the degree of tissue damage and
inflammatory changes are less evident and lasts for a short time compared to those of the
nasal turbinate, especially the internal side to the turbinate spiral (16).

Congestion and lymphoid hyperplasia occur in the lacrimal gland with an increase in
plasma cells (77). Heterophils and lymphocytes accumulate in the edematous head tissue
(77,80). Caseous exudates in the lacrimal gland, conjunctival sac and facial subcutis
contribute to facial swelling (77,84). Extensive facial swelling is facilitated by the
interconnection of perinasal, perisinusidal, periocular and facial subcutaneous connective
tissues (80).

Purulent inflammation has been seen in the air sacs of cranial spongy bones (80).
Marked exudation of heterophilis and fibrin was found in the tympanic cavity of the middle
ear (35,80). Nakamura reported histological changes in the spleen, liver, bursa of Fabricius
and thymus; however, involvement of these tissues is not supported by other reports or
clinical signs and are probably not due to disease.

Nucleic acid and viral proteins

The genome of APV is comprised of a single-stranded, non-segmented, negative
sense RNA virus approximately 13.3 kb in length (101). SDS-polyacrylamide gel
electrophoresis (PAGE) techniques have been used to study polypeptide composition and
have identified several virus-specific polypeptides. Three groups of workers have reported
Cavanagh et al. (20) identified 7 virus-specific polypeptides, some of which are glycosylated, with the estimated apparent molecular weights of: 82K, 68K, 53K, 15K, 43K, 40K and 35K. These polypeptides have been identified as the large glycopolypeptide or attachment protein (G), fusion protein precursor (FO), the fusion protein cleavage products F1 and F2, nucleocapsid (N), phosphorylated (P), and the matrix (M), proteins respectively. A 200K and 22K polypeptide have also been reported and considered to be virus specified. An additional 97K protein was detected, but the function and/or identity remains to be investigated further. Collins (23) also reported 7 viral polypeptides, however the Ms are somewhat different: 200K, 84K, 54K, 42K, 37K, 31K, and 14K with the 84K and 54K polypeptides being glycosylated. It was determined that the 200K polypeptide possibly represented host cell histones while the 42K, 37K, 31K, and 14K polypeptides were considered structural (23). The 42K and 31K appeared to be analogous to the two major non-glycosylated polypeptides VP40-44 and VP27-28 of established pneumoviruses representing the N and M polypeptides, respectively. Viruses in the family Paramyxoviridae have N and M polypeptides that are larger. The N polypeptide of NDV has a M, of 53-57K and a matrix M, of 40-44K.

SDS-PAGE studies conducted by Ling and Pringle (65) identified five viral polypeptides with a M, of 38K, 35K, 30K, 19K, and 15K (65). An additional 22K virus-specific polypeptide was revealed by in vitro translation which appeared to be either a nonstructural or a minor structural protein. The presence of a conserved 22K protein gene, a characteristic of pneumoviruses, strongly suggests that APV is closely related to the mammalian pneumoviruses (66). The 57K, 45K and 15K polypeptides were identified as
glycosylated with the 57K possibly being a disulfide-bonded dimer of the 45K and 15K
glycopolypeptides. In addition, a 83K M, was observed, but it is not known if the protein is
virus specific.

SDS-PAGE conditions differ from one researcher to another giving slight differences
in polypeptide mobility. The aforementioned polypeptide studies are summarized in table 1
below and the following conclusions have been drawn from the data. The approximate M, of
the G, FO, F1, F2, N, P, and M ploypeptides are 82K, 68K 53K, 15K, 42K, 40K, and 35K,
respectively. The 82K polypeptide is glycosylated and is the large glycopolypeptide or
attachment protein (G). The fusion protein precursor (FO) is a disulfide-bonded dimer with
the cleavage products of F1 and F2 which are glycosylated polypeptides. Inhibition of
glycosylation of p53 and p15 by tunicamycin shows that their glycans are of the N-linked
type, a feature common to the F proteins of three genera of the family Paramyxoviridae (20).

It cannot be determined, by SDS protein analysis, if the 200K or 22K polypeptide are virus
specific. According to Cavanagh, the 200K was virus specific and according to Collins the
protein represents host cell histones. The identity of this polypeptide has been confirmed by
genome sequence analysis. Sequence studies have identified 8 virus specific protein genes.
A 200K polypeptide was not found to be virus specific. The largest glycopolypeptide of the
Paramyxoviridae is the HN, H, and G protein of the Paramyxovirus, Morbillivirus and
Pneumovirus genera, respectively. On the basis of molecular weight alone, three
polypeptides differentiate pneumoviruses and metapneumoviruses from other genera of the
family Paramyxoviridae, p24/25 (not possessed by the morbilli and paramyxovirus genera)
and N and P which are smaller in size (20).
Table 1. Comparison of APV polypeptides and their molecular weights as
determined by three different workers, Collins, Cavanagh and Ling, with Pneumovirus,
Morbillivirus, and Paramyxovirus polypeptides.

<table>
<thead>
<tr>
<th>Poly-peptide</th>
<th>APV Collins</th>
<th>TRTV</th>
<th>APV Ling</th>
<th>Pneumovirus</th>
<th>Morbillivirus</th>
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<tr>
<td>L</td>
<td>200</td>
<td>200</td>
<td></td>
<td>160-200</td>
<td>160-200</td>
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<tr>
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<td>97</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>82</td>
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<tr>
<td>G, H, HN</td>
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<tr>
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</table>

Sequencing studies have provided information on gene order and identification. The
identity and order of the known genes in the APV genome (3'N-P-M-F-M2-SH-G-L 5') is
somewhat different from that of the mammalian pneumoviruses,
(3'NS1-NS2-N-P-M-SH-G-F-M2-L 5') (63). APV genome organization differs from
those of the Respirovirus and Morbillivirus genera in three ways: 1) Respirovirus and
Morbillivirus genera have two genes encoding, NS1 and NS2, small proteins of unknown
function upstream of the nucleocapsid protein gene, 2) the small hydrophobic (SH) protein
gene and the presumptive attachment (G) protein genes are located upstream of the F gene in
the Respirovirus and Morbillivirus genera, and downstream of the F in APV and 3) APV
codes for a 22K/M2 protein distinct from that of the M protein gene (64,103,122). APV does
conform to the pattern seen in all members of the family *Paramyxoviridae* with the N gene being the most promoter-proximal of the three major virus structural protein genes.

Sequence studies of the N protein gene of viruses from both subgroup A and B APV reveal a single open reading frame (ORF) potentially encoding a protein of 391 amino acid residues. The N genes of subgroup A and B HRS viruses are more conserved (14% difference) in nucleotide sequence than the N genes of subgroup A and B APV which differed by 24% (63). The nucleocapsid complex is formed by an association of the genomic RNA, N, P, and the L protein. The complex has a pivotal role in virus replication and is responsible for various RNA synthesis processes and transcription within the infected cell. The APV polymerase (L) gene encodes a single large ORF of 2004 amino acids, making the APV L protein the smallest to be described for any non-segmented, negative-strand RNA virus. Studies have demonstrated that L proteins of unsegmented negative-strand RNA viruses exhibited strongly invariant amino acids embedded in conserved blocks (11). An HRSV L protein N-terminal extension allows an overlap with the upstream 22K gene. APV and PMV L genes do not have this overlap. The APV L has the highest overall identity with the HRSV L protein when compared to 13 other L proteins. Unfortunately, only a single APV L gene has been sequenced so subgroup comparisons cannot be made at this time (122).

The APV small hydrophobic protein (SH) gene is 589 nucleotides in length, encoding a protein of 174 amino acids which is considerably larger than the RSV SH protein. Little is known about the probable function of the SH and 22K/M2 proteins, although in the case of RSV both are minor components of the virus envelope (64). The M2 protein gene has been identified only in the *Pneumovirus* and *Metapneumovirus* genera and is located downstream
of the G gene in HRSV and PVM while it is located downstream of the F gene in APV (64). Fluorescent antibody studies have indicated that M2 might be associated with the N and P proteins, similar to RSV. The hydropathy plot of the M2 of APV revealed that there were no extensive highly hydrophobic sequences present in proteins which are integrally associated with membranes. This observation is consistent with M2 which has an internal location (124).

The matrix protein of paramyxoviruses coordinates the assembly of virus components at the cellular membrane prior to budding of the mature particle and it regulates viral gene transcription (101). The many complex interactions of the M protein are believed to involve both hydrophobic and ionic processes. The hydropathy plots for APV and RSV indicate that the M protein is moderately hydrophobic with a highly hydrophobic sequence. This stretch may be membrane associated (122). APV M gene encodes a protein of 254 amino acids, very similar to that of RSV (256 residues) but very different from that of the morbilliviruses and paramyxoviruses (approximately 350 residues). This evidence indicates that APV is more closely related to the pneumoviruses than to the paramyxoviruses and morbilliviruses (122).

Among paramyxoviruses the matrix protein is the second most highly conserved protein following the polymerase which is frequently used for phylogenetic analysis. Comparison of subgroup A and B APV M gene sequences with US/CO APV M gene identified the US/CO as a separate avian pneumovirus (105). There is 75% homology in M gene nucleotide sequences between subgroup A and B APV, while the two subgroups share only 60% nucleotide sequence identity with the US/CO M protein gene (105). The 75%
homology seen between subgroup A and B M gene sequences is a lower degree of homology than is expected from an internal protein not exposed to immunological pressure (101). Subgroup A and B viruses have approximately 90% amino acid homology. There are 27 amino acid differences between the two matrix proteins, 19 of these being nonconservative alterations. Despite this divergence, the presence of a short hydrophobic domain, possibly a transmembrane region, from residue 194-209 is maintained in both strains, the conservation of this feature suggests it has a major role in the function of the protein. (101). The US/CO, which belongs to neither subgroup A or B, has only 78% amino acid identity with APV subgroup A and 77% identity with APV subgroup B M protein sequences. The majority of amino acid substitutions are, however, conserved (105). Phylogenetic analysis of M protein amino acid sequences demonstrates that avian pneumoviruses form a single clade relative to their mammalian counterparts. However, within the avian branch, APV US/CO is separate from the APV subgroup A and B cluster. Cross-neutralization and ELISA studies further support the unique antigenic nature of the US/CO APV (108). This data definitely suggests that the US/CO isolate is distinct from European viruses.

Pneumovirus F glycoproteins promote cell fusion. APV, subgroup A, FO gene was sequenced and found to consist of 538 amino acids, with the F2 and F1 subunits containing 102 and 436 residues, respectively. Each subunit has one potential N-linked glycosylation site. The APV F gene has virtually no non-coding regions at either end of the mRNA and encodes a F2 subunit which is shorter than that of most members of the family Paramyxoviridae. The FO gene has a high amino acid identity (40%) with the F gene of the RS virus, while identity profiles with five other paramyxoviruses range from 20-24%. When
conservative amino acid differences are taken into account the relatedness of the RS virus and APV F proteins increases to 85%. The overall amino acid identity and the number and distribution of cysteine residues support the inclusion of APV in the pneumovirus genus (123).

The two major antigenic determinants for pneumoviruses are the F and G glycoproteins, the G protein is considered to be the major antigenic determinant. The APV G gene is 1193 nucleotides in length, for subgroup A viruses and 1260 bp in subgroup B viruses (64). Five European APV G genes were sequenced and analyzed. Nucleotide sequence identity within each subgroup (A and B) is approximately 99%, while nucleotide sequence identity between the two subgroups ranged between 56.3 and 57%. A major feature of the G proteins is high proline, serine and threonine content, this feature is conserved between the two subgroups. Restriction enzyme digestion patterns, nucleotide sequence and predicted amino acid sequence clearly place the APV into two distinct groups (60). The UK and French isolates form one group recognized as subgroup A while other continental European isolates (Spain, Italy, and Hungary) form subgroup B (60).

A nested reverse transcription polymerase chain reaction (RT-PCR) assay with primers directed to the G gene of APV can differentiate subgroup A and B of APV (17). The initial PCR uses oligonucleotide primers that will bind to a conserved portion of the G protein gene of both subgroups. The second, nested PCR, uses primer sets where one of the primers is common to both subgroups, two other primers used are specific for either subgroup A or subgroup B. RT-PCR subgroup analysis agrees with the previous APV virus classification based on mAb (19).
Molecular subtyping of 21 APV was conducted with two different primer sets, one that amplified subgroup A viruses and one that amplified subgroup B viruses. Both primer sets were targeted to the G gene of APV. Ten of the viruses were typed as subgroup B viruses and 8 were typed as subgroup A viruses. One virus was determined to be a mixture of subgroup A and B viruses and two viruses were not typeable. The untypeable viruses were confirmed to be APV by a different set of N gene primers that detected all 21 APV (11). Molecular subtyping of APV utilizing the G protein gene has been shown by several researchers to be reliable. Subtyping results have been confirmed by monoclonal or polyclonal cross neutralization studies.

An ELISA test, as described by Chettle and Wyeth (21), using monoclonal and polyclonal sera has been used to evaluate antigenic relatedness. ELISA antigens were prepared by freezing/thawing and by solubilization with 0.5% (v/v) Nonidet P40. Crude ELISA antigens prepared by NP40 extraction were subgroup specific with little cross reaction whereas antigens prepared with freeze/thaw treatment were highly cross reactive (24). NP-40 antigens identified homologous sera, thus differentiating subgroups. Subgroup A and B ELISA antigens prepared with the NP-40 procedure were used in the initial serological testing for the US/CO APV. These subgroup A and B antigens failed to identify the US/CO antibody suggesting a unique subgroup identification (106). Neutralizing antibodies are likely to be directed against fewer epitopes than those for the ELISA with whole virus antigen and may therefore represent a far more sensitive test to assess antigenic variation (24).
Vaccination

Several live attenuated strains of APV have been licensed for commercial vaccine production. These vaccines have been used in commercial turkeys in the UK and France as well as several other countries (83). Vaccination programs implemented in 1990 in Spain were based on inactivated vaccines that were combined with IBV, ND, and IBD. Inactivated vaccines were initially selected because they were known to be safe and innocuous. A live attenuated and inactivated vaccine program has been used successfully in broiler breeders and layers for several years in France, to protect against a drop in egg production (38). Vaccination programs, similar to the French program, have reduced the loss of egg production in breeder turkeys, a major economic loss caused by APV (92).

Results differ between field and laboratory trials, even with turkeys of the same origin. Laboratory trials show seroconversion and good protection from the adverse effects of challenge, especially after two vaccinations. In the field, seroconversion is inconsistent and of short duration, while clinical signs are delayed and less severe (29,38,119). Several groups have reported testing live-attenuated strains of APV against virulent challenge. Single live-attenuated vaccination was compared to a live-attenuated/inactivated combination. Turkeys were primed with a live-attenuated vaccine at 1 week of age and boostered with an inactivated vaccine at 30 weeks of age. A pool of field isolates with log_{10} 7.5 CD_{50} doses of virus was used for the challenge. Vaccination with the live-attenuated vaccine alone failed to develop an antibody response, but provided partial protection against respiratory disease and good, but incomplete, protection against a drop in egg production (26). The combination of live-attenuated and inactivated vaccine provided complete
protection against respiratory disease, a reduction in egg production as well as a strong antibody response (26). In contrast to the previously described study, intraocular vaccination of 7-day-old turkey poults with a live-attenuated virus was shown to be protective against respiratory disease and to produce a significant antibody titer (36). The second study indicates that a single administration of live-attenuated vaccines can provide complete protection against respiratory disease and reduced egg production. Intraocular vaccination is costly as it requires manual vaccination. The virulence and titer of the challenge virus may be the reason for conflicting reports on the protective abilities of live-attenuated vaccines.

The normal method of attenuation of APV is serial passage in cell culture. Attenuation studies have shown that approximately 100 TOC passages does not effect virulence, but as few as 39 passages in CEF cells decreased both virulence and immunogenicity. Passage in Vero cell culture caused loss of virulence, but maintained immunogenicity (119). Virulent APV has also been attenuated by alternating serial passages from chicken embryos to TOC, approximately 30 passages are necessary. Multiple in vivo passaging of attenuated viruses have indicated stability of attenuation with no signs of reversion to virulence (30).

Experimental evidence suggests that vaccination with live subgroup A vaccines will protect against challenge with both subgroup A and B APVs (28). Similar experimental studies have shown that vaccination with live subgroup A and B viruses will protect against a US/CO challenge; however, vaccination with live US/CO APV will not protect against challenge with either subgroup A or B APV (52). Cook et al. demonstrated that turkey APV isolates are more pathogenic in turkeys than chickens and vice versa (32). Whether live turkey APV vaccine is equally protective in both chickens and turkeys remains to be
determined. At this time, no APV vaccine has been developed that will protect both turkeys and chickens against all subgroups of APV.

The role of neutralizing antibody in disease protection is unclear. Chemically bursectomized poults were vaccinated and challenged with virulent virus 3 weeks post vaccination. The bursectomized poults were completely protected against disease without any evidence of antibody response. Further evidence that circulating antibody is not essential for protection of the respiratory tract was reported by Jones et al. (56), when poults were B-cell immunosuppressed by cyclophosphamide and vaccinated at day-old. Despite their inability to produce circulating antibodies, they were immune to challenge with virulent APV at 21 days of age (56). Therefore cell-mediated immune responses may be more important in protection (52,59). Studies on T-cell and B-cell immunity to HRSV have revealed different roles for the various antigenic proteins. The F glycoprotein induced ELISA binding antibodies, virus-neutralizing antibodies, cytotoxic T-lymphocyte and T-helper cell responses while the G glycoprotein induced a narrow range of T-helper cell responses and no cytotoxic T-lymphocyte response (5). Natural infection to HRSV confers poor protection against reinfection to HRSV indicating that humoral immunity is inefficient in preventing upper respiratory tract infection (5). In poults the presence of high titers of maternal antibody does not prevent the development of clinical disease after virulent challenge, thus providing additional evidence that humoral antibody will not protect against clinical disease or reinfection with APV (82). Control of TRT will most likely require some novel approaches to vaccination and disease control.
A recombinant fowl pox virus carrying the fusion protein gene of APV (rFPVF+) successfully expressed the F protein and induced neutralizing antibodies in poults. Two weeks following a second vaccination poults were challenged superconjunctivally and intranasally with a virulent APV. Turkeys vaccinated with rFPVF+ experienced less severe clinical signs than the controls. The titer of virus recovered from challenged poults vaccinated with rFPVF+ was approximately 1000-fold greater than from birds in which the F protein was expressed (100). These studies indicate that the immune responses to the F protein plays a role in protection from disease.

Serology

The successful detection of antibody by ELISA and virus neutralization (VN) is dependent upon antigen selection. Significant antigenic differences exist between subgroup A and B viruses as demonstrated by monoclonal antibody cross-neutralization studies. Antigenic stability within subgroups has been demonstrated (33). Comparison of ELISA antigens and VN has shown that subgroup homology is essential for optimal detection of antibody (36,108). Incomplete antibody detection was found between UK and French antigens by both the VN and ELISA assays. Tests with homologous antigen/antibodies show significantly higher antibody titers, providing further evidence that the French and UK viruses are antigenically different and are in separate subgroups (36). The incorrect choice of ELISA antigen, the most economical test assay system, can hinder the early serological diagnosis of a TRT infection. Early detection of the US/CO APV was not possible because of antigenic differences between subgroup A and B ELISA antigens and US/CO antibody (108). ELISA assays can detect antibody to APV as early as 7-10 days post-exposure.
Antibody titers are indicative of exposure to the virus. Results obtained with commercial ELISA kits need to be viewed with caution as antigens are subgroup specific and will not reliably detect antibody to all APVs.

**PCR**

There is little doubt that it is, at times, essential for live pathogens to be isolated, for disease diagnosis or vaccine and diagnostic reagent production. Rapid identification of a pathogen, on the other hand, is often the goal for disease control. Polymerase chain reaction (PCR) is a procedure that uses nucleic acid technology to exponentially amplify the nucleic acid of a pathogen to a detectable level. DNA can be visualized with ethidium bromide staining or detected with fluorescent dyes by spectrographic readers. PCR is specific and sensitive but does not indicate presence of a live pathogen, it amplifies the viral nucleic acid. The specificity of the PCR relies upon the uniqueness of the nucleotide sequence of the oligonucleotide primers. The selected oligonucleotides should be unique to the pathogen and not anneal to nucleic acid of other avian pathogens or to the nucleic acid of host cells. Specificity is increased with a second nested PCR. The chances of the second oligonucleotide pair also binding to the “wrong” DNA to generate a product of the same size is very small (19).

The PCR assay amplifies a single copy of DNA to generate a second identical DNA copy. APV is a RNA virus and requires the initial step of generating a DNA complementary (cDNA) of the viral RNA. cDNA is created by a 30-minute reaction using the enzyme reverse transcriptase and complementary or random hexamer primers to produce a DNA copy. The cDNA is then amplified *in vitro* with polymerases to exponentially increase the
number of DNA copies. This procedure is referred to as reverse transcription-polymerase chain reaction (RT-PCR).

A RT-PCR with primers directed to the fusion gene of APV was used for viral RNA detection in subgroup A and B viruses. Selected primers amplified nucleic acid of subgroup A viruses identified by monoclonal antibody studies (25), but not subgroup B viruses (62). Tracheal/esophageal swabs from turkeys vaccinated with a live subgroup A vaccine were tested by the RT-PCR and found to yield viral nucleic acid from day 7 to 19 after infection (62). Virus identification was significantly extended from what is considered the optimum period for virus isolation (3-7 DPI). Separate VI studies reported that the maximum concentration of infectious virus is normally obtained 3-5 DPI. Titers drop precipitously after 5 DPI until day 7 at which time recovery is variable (31,81). Both tracheal and esophageal swabs when pooled were found to be good specimens for the detection of APV RNA. Southern blots utilizing a probe to the target nucleic acid increased the period of detection to day 19 p.i. for most samples and day 21 for one sample (62). Despite the success of APV detection with a complementary probe, there were groups of birds from which no virus could be detected after day 10 p.i. All bands detected by the probe from day 7 till the end of the study were “very faint.”

Li Jing et al. (62) found that tracheal/esophageal swabs could be dried for 2-3 days without any decrease in detection of viral RNA by RT-PCR. However, this procedure was tested only on two birds and on day 3 when a maximum concentration of viral RNA was present (62). Drying swabs reduces the possibility of bacteria and molds growing on the swabs and these microbes can damage nucleic acid. Cavanagh et al. (18) also found dry
swabs to be an excellent system for sampling the live bird and isolating viral RNA. Most of the turkey swabs were pooled in minimum essential medium (MEM) and placed directly on ice for maximum viral RNA isolation. This method proved to be successful.

RT-PCR has been helpful in the detection of APV in broiler chickens experiencing SHS in Japan. The fastidious nature of APV lead diagnosticians to investigate alternate means of detecting the virus. A RT-nested PCR was designed to detect the F protein gene of APV. The first PCR generated a 536 bp product using primers (F1 and F3) designed to TRT 3B, a subgroup A U.K. turkey virus. The nested PCR yielded a 347 bp product that included a restriction enzyme site, PstI. Primers were specific for APV and did not amplify nucleic acid of ND, avian influenza (AIV), IBV, avian reovirus, or BRSV. Sensitivity of detection by the 1st PCR corresponded to $10^{1.6}$ TCID$_{50}$ and of the nested PCR corresponded to 0.4 TCID$_{50}$. Both trachea and turbinates were collected from field samples, extracted and the nucleic acid was amplified using the RT-nested PCR. Turbinates proved to be a better source of viral RNA because 80% of the samples were positive while only 20% of the tracheal samples yielded RNA that could be detected. Duplicate samples were prepared for virus isolation. No virus was isolated from any of the samples by any of the several VI methods used (TOC, chicken kidney cell culture, and chorioallantoic cavity and yolk sac route of inoculating) (73). This finding illustrates the sensitivity, specificity and capability of a nested RT-PCR as a useful tool in the detection of APV.

An extensive investigation for a RT-PCR capable of detecting all subgroups of APV was recently conducted by Bayon-Auboyer et al. (11). Twenty seven primers corresponding to sequences either common to both subgroup A and B viruses, or subgroup specific, were
selected. These primers were designed to either the F, G, or N protein genes. Viral nucleic acid was isolated from tracheal swabs of experimentally inoculated turkeys. Only one of the RT-PCR's evaluated detected all 21 of the APV isolates tested. A primer set designed to the N gene successfully detected all APV isolates. Primers designed to the F and G gene detected only a portion of the viruses tested. The G-based genomic RT-PCR failed to detect two French APV isolates, making subgroup identification of these two viruses impossible by the use of molecular techniques. Subgroup identification was evaluated on all remaining viruses and results were in agreement with known serological subgroup classification.

Human respiratory syncytial virus, the prototype of Pneumoviruses, is a major cause of lower respiratory tract infections in infants. Traditionally, detection has included culturing the virus from nasopharyngeal secretions. Cell culture methods are slow and often the patient has left the hospital before the virus is identified. RT-PCR has been found to be more specific, sensitive and faster. Both the G and F proteins experience selective pressure from the host immune system leading to an expected high level of divergence. The G or attachment protein has been shown to have the greatest divergence among the 8 genes (51). The fusion gene appears to be the antigen mainly responsible for induction of neutralizing antibody. Despite this antigenic characteristic, Plows and Pringle (98) have concluded that the F₂ sequences are more variable than the F₁ subunit sequences, but overall, the F protein gene is among the most conserved genes of RS virus. Primers directed to the F₁ subunit for the fusion glycoprotein have been successfully used for the detection of HRSV in hospital samples (94). The portion of the F₁ subunit used for detection of HRSV is conserved in subgroup A and B RS viruses. Portions of the F gene are quite variable in A and B viruses
and have been used for epidemiological studies (51,76,90). A RT-nested PCR assay amplifying a conserved portion of the BRSV F gene was found to amplify all strains of BRSV and a useful tool in detection of the virus in clinical specimens (90,115). All 8 of the APV genes have been sequenced, and the sequences follow the same pattern as Pneumoviruses with the G protein having the greatest diversity, and the F and G proteins receiving the most immunological pressure. The nucleocapsid, matrix, and polymerase genes are highly conserved. However, the majority of the genome analysis has been conducted only in one of the two subgroups of viruses preventing subgroup comparisons and identification of conserved sequences. RT-PCR directed to the F protein gene for both BRSV and HRSV was found to be specific and was capable of detecting all strains of the virus in clinical samples.

TaqMan® PCR is a PCR assay that applies standard amplification and extension techniques while incorporating a fluorogenic probe for detection of the amplified product. During PCR, a fluorogenic probe consisting of an oligonucleotide with both a reporter and a quencher dye attached, anneals specifically between the forward and reverse primers. The fluorescent oligonucleotide probe has a 5’ reporter dye, FAM, and a downstream 3’ quencher dye, TAMRA. The emission of the 6-carboxyfluorescein (FAM) moiety is normally quenched by virtue of its proximity to the dye 6-carboxy-tetramethylrhodamine (TAMRA); when the FAM moiety is cleaved from the oligonucleotide by the activity of the polymerase its fluorescence is detected by a charge-coupled device camera of the ABI Model 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA, USA). With each cycle, additional reporter dye molecules are cleaved from their respective probes, and the
increased fluorescence intensity is monitored during the PCR (47). Because of the specificity of the probe, the possibility of false-positive reactions due to a fluorescence signal associated with the generation of non-target amplification are minimized with this system (50). ABI 7700 Sequence Detection software allows for either real-time quantitative or endpoint detection to be used for product analysis. The real-time detection monitors the fluorescence intensity at each cycle. Relative normalized fluorescence (ΔRn) vs. time (PCR-cycle number) is plotted to allow real-time assessment. The ABI 7700 employs a computer algorithm to calculate a value termed ΔRn as follows: ΔRn = Rn+ - Rn-, where Rn+ is the emission intensity of the reporter divided by the emission intensity of the quencher at any given time in a reaction tube and Rn- is the emission intensity of the reporter divided by the emission intensity of the quencher measured before PCR amplification in that same reaction tube (47). The average background fluorescence emission is calculated from cycles 1-15 in all reactions, and a standard deviation is derived. A threshold fluorescent intensity is established at 10-fold above this standard deviation. Any sample that exceeds the fluorescence threshold is considered positive (47,68). Endpoint detection reads the fluorescence intensity at the last cycle of the PCR reaction and using the same threshold calculation determines positive and negative samples. Endpoint optical density (OD) readings are reported for each sample, these OD readings are the only quantitative data provided with the endpoint detection system. In this way the endpoint detection data resembles ELISA endpoint data. To induce fluorescence during PCR, a laser light is distributed to the 96 sample wells via a multiplexed array of optical fibers. The resulting fluorescent emission returns via the fibers and is directed to a spectrograph with a
charge-coupled device camera (47). Both amplification and detection processes are contained within a closed system, thus minimizing the potential for product carry-over contamination. TaqMan® utilizes universal conditions for buffer (reagent concentration) and thermal cycling. PCR primers are designed to have a Tm range of 58-60 °C and the amplified target needs to be between 50 and 150 bp. An exogenous internal positive control (IPC), which is included in the reaction, distinguishes between samples identified as negative because they lack target sequence and those negative due to the presence of a PCR inhibitor. The IPC has a different reporter fluorescence signal than the sequence specific target probe. The universal thermal cycling includes a enzymatic degradation of carry-over DNA by UNG (50C for 2 min.), a 95 °C for 10 min. hot start, and 40 two step amplification cycles (denaturation at 95C for 15 sec and annealing/extension at 60 °C for 1 min.). Samples tested in triplicate had minimal variation based on standard deviation and coefficient of variance. Each triplicate amplification was highly reproducible, showing that real time PCR using the ABI 7700 Sequence Detector instrumentation introduces minimal variation into the quantitative PCR analysis (47).

The specificity and sensitivity of the TaqMan® has been evaluated by a number of workers for a variety of applications. The TaqMan® system was reported to be species specific with a detection threshold of 2.1 x 10^5 copies of the pla target or 1.6 pg of total cell DNA (50). The detection of experimentally infected tissue samples was successful and the system was found to be rapid for the detection of field samples. Other diagnostic applications include the detection of Marek's disease in leukocyte DNA (49), the detection of APV in turkeys in Minnesota (78, 109) and the quantitation of chronic myeloid leukemia
Mensink et al. (75) reported that the TaqMan® system could detect as few as 10 copies of Bcr-Abl DNA and is a sensitive and accurate method for quantitation of CML. The TaqMan® sequence detection system has many applications and has been utilized more extensively in human medicine than veterinary medicine. It is reported to be a sensitive assay system capable of detecting low copy numbers of DNA, an important factor when developing detection systems to diagnose APV infections after 7 DPI.

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DETECTION OF AVIAN PNEUMOVIRUS IN TISSUES AND SWAB SPECIMENS FROM INFECTED TURKEYS

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ABSTRACT

Avian pneumovirus (APV), a member of the family Paramyxoviridae and genus Metapneumovirus, was recently isolated for the first time in the U.S. The APV/turkey/Colorado/97 virus was isolated in chicken embryos and cell culture. This technique proved to be slow and labor intensive. Detection techniques that are rapid and sensitive are needed for detection of APV. Two separate nested reverse transcription-polymerase chain reaction (RT-PCR) assays for the detection of APV were evaluated and compared to virus isolation (VI) for sensitivity and specificity. Primers for both the conventional nested RT-PCR and the TaqMan® RT-PCR were directed to the APV fusion gene. The outside PCR primers detected the subgroup A APV and the US/CO APV, while the nested primers were specific for US/CO APV. Respiratory tissues and tracheal swabs were collected from experimentally inoculated turkeys between 1 and 21 DPI and

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tested by all three detection methods. Tracheal swabs were shown to be good specimens for the detection of APV between 3 and 8 DPI, while turbinate and sinus specimens were the most productive samples over the entire collection period. Two separate tracheal swab samples were collected, dry swabs and swab pools, both proved to be good specimens. VI detected APV between 3 and 7 DPI and was the most labor intensive method. APV was detected by both RT-PCR methods as early as 1 DPI and as late as 17 DPI. All APV inoculated turkeys were positive or suspect positive for US/CO APV antibody at 10 DPI by enzyme-linked immunosorbent assay (ELISA). The TaqMan® RT-PCR was found to be the more sensitive assay for the detection of APV in tissue and swab specimens from infected turkeys.

**INTRODUCTION**

Avian pneumovirus (APV) is the etiological agent of turkey rhinotracheitis (TRT) in turkeys (21,33) and swollen head syndrome (SHS) in chickens (4,24,27). TRT, an upper respiratory disease, is characterized by rapid onset with morbidity and mortality rates reported to be as high as 100% and 30%, respectively (3). Clinical signs include snicking, rales, nasal and ocular discharge, depression, and swollen infraorbital sinuses (23,18). Clinical signs in breeder hens also include reduction in egg production and quality (23). Turkeys of all ages are susceptible; (3,23,32) however, poults younger than 6 weeks have been reported to experience a severe clinical disease (2). Secondary bacterial agents and poor husbandry increase the severity of disease and extend recovery time (1,3). With the exception of Australia and Canada, most countries with a commercial turkey industry have experienced the disease (16). The first isolation of APV in the United States (U.S.) was in
1997 following a respiratory disease outbreak in turkeys in Colorado (30,31). Since the initial isolation, APV has been identified serologically in Minnesota, South Dakota, North Dakota and isolated from turkeys in Minnesota (30).

APV, a non-segmented single-stranded negative sense RNA virus, is a member of the family Paramyxoviridae and genus Metapneumovirus (8,25). Cross-neutralization studies, using polyclonal and monoclonal antibodies, and sequencing studies of the glycopolypeptide (G) attachment protein gene have identified two subgroups of APV. Subgroup A consists of the United Kingdom (UK) (prior to 1993) and South African viruses, while subgroup B includes the European viruses (9,11,15). Due to limited cross-neutralization with subgroup A and B APV, initial detection and identification of the APV/turkey/Colorado/97 (US/CO) strain of APV was challenging. Enzyme-linked immunosorbent assay (ELISA) tests with subgroup A and B antigens failed to detect antibodies in turkeys infected with US/CO APV (30). Cross-neutralization studies and recent analysis on the US/CO APV matrix (M) protein genome have determined that the US/CO APV is antigenically unique (28,30,31). Subgroup classification for the US/CO strain of APV is not known at this time.

Several other common avian pathogens including, *Bordetella avium*, *Mycoplasma gallisepticum* (MG), and avian influenza produce respiratory signs similar to those produced by APV (14). A diagnostic tool capable of quickly identifying APV would be helpful in identification of the causative agent. Isolation of APV is labor intensive, slow, difficult, due to the fastidious nature of the virus and rarely successful in birds showing severe signs of the disease (1). The reverse transcription-polymerase chain reaction (RT-PCR) procedure has
been shown to be a specific diagnostic assay for avian pathogens and would provide the speedy, specific diagnostic tool needed for detection of APV (6).

The objectives of this study were to 1) determine which specimens are the most suitable for ease of collection and successful detection of the virus over time and 2) to compare the conventional nested RT-PCR, TaqMan® RT-PCR and virus isolation (VI) techniques for sensitivity and specificity.

**MATERIAL AND METHODS**

**Experimental design and sample collection.** Turkeys used in the study were 4 weeks of age and obtained from a local commercial source. The turkeys were negative for circulating antibodies to subgroup A, subgroup B and the US/CO APV, as determined by ELISA. Two separate experiments were performed; experiment 1 with 30 turkeys and 10 different collection points (1,3,5,7,10,12,14,17,19 and 21 DPI) and experiment 2 with 42 turkeys and 14 different collection points (1,3,4,5,6,7,8,9,10,12,14,17,19 and 21 DPI). Upon arrival birds were randomly divided into two groups, control and inoculated. All turkeys were housed in a biosafety level 3 (BL-3) animal facility. Control and APV inoculated birds were housed in separate negative-pressure animal rooms. Control birds were sampled and cared for prior to APV inoculated turkeys at all times. All inoculated turkeys were inoculated intraocularly and intranasally with $10^4$ TCID$_{50}$ of the US/CO APV. At each collection point, 2 inoculated and 1 control bird were sacrificed for tissue collection and all remaining turkeys were swabbed and observed for respiratory signs.

Turbinate, lung, sinus and trachea samples were collected from all sacrificed birds. Two separate tracheal swabs, using standard cotton swabs, were collected from all turkeys at
each collection point. The first swab specimen, referred to as tracheal swab pool, was immersed in tris buffered tryptose broth (TBTB) with antibiotics, swirled, squeezed against the side of the tube and discarded; while the second, referred to as the dry swab, was wiped on a 2mm circular piece of drape laboratory paper. Two tracheal swab pools were collected at 1, 3 and 5 DPI in both experiments and 2 dry swab pools were collected at 1 and 3 DPI in experiment 1. All inoculated birds were included in 1 tracheal swab pool and 1 dry swab pool at all other collection points. Nasal discharge collected on a separate dry cotton swab will be referred to as sinus exudate swab. Blood samples were collected from all control and APV inoculated turkeys at 0 DPI for both experiments. During experiment 2, blood was collected from all APV inoculated turkeys at 7, 10, 14 and 21 DPI and from sacrificed birds at each collection point. Sinus exudate, the preferred sample, was used when available. When this sample was not available the choanal/infraorbital sinus area was swabbed. All tissue samples and tracheal swab pools were processed for both virus isolation and nucleic acid isolation the same day they were harvested, while dry swabs and sinus exudate swabs were only processed for nucleic acid detection.

Statistical analysis of the three detection methods was conducted using the Fisher exact test to evaluate sensitivity. Tissues specimens, lung, trachea, sinus, turbinate and tracheal swabs were compared using the same technique to determine which specimens were the most reliable source of the virus.

Viruses. The US/CO APV, at passage 4, was purified by 4 limit dilution passages in chicken embryo fibroblast (CEF) cell culture. The virus used in this study represented the 12th passage of the virus. A dilution of the virus representing $10^4$ TCID$_{50}$ per 0.3 ml was
prepared in sterile 0.01 M PBS. A total of 0.3 ml of the diluted virus was inoculated by the intraocular and intranasal routes into each APV inoculated turkey.

Viruses used to evaluate specificity of the RT-PCR were avian nephritis virus (ANV), avian influenza virus (AIV), avian paramyxovirus types I, II, and III (APMV-1, APMV-2, APMV-3), infectious bronchitis virus (IBV), infectious bursal disease virus (IBD), avian encephalomyelitis virus (AEV), reovirus (Reo), APV/UK/14/1/1986 (UK 14/1 APV), and APV/Hung/657/4/1990 (Hung APV). AIV, APMV-I, APMV-II, APMV-III, and IBV were derived from infected amniotic/allantoic fluid (AAF) while the IBD, Reo, ANV, UK 14/1 APV, and Hung APV were propagated cell culture material. AEV was a homogenized embryo suspension. Viral RNA nucleic acid material for all RNA viruses included in the specificity study were extracted using the QIAamp viral RNA (Qiagen Inc., Valencia, CA) isolation kit and the manufacturer's protocol. Viral DNA nucleic acid material for all DNA viruses was isolated using the PureGene DNA isolation kit (Gentra Systems, Minneapolis, MN) and manufacturer's protocol.

Conventional nested RT-PCR. Immediately following tissue collection, samples were placed on wet ice. RNA nucleic acid was extracted using the Qiagen Rneasy mini tissue isolation kit (Qiagen Inc., Valencia, CA) and manufacturer’s protocol, with several modifications. Sterile tenbrook tissue grinders were used to homogenize 30 mg of tissue in 600 µl of RLT lysis buffer. Immediately following homogenization the lysed tissue homogenate was passed through a Qiagen QIAshredder (Qiagen Inc., Valencia, CA) for complete sample disruption. Tracheal swab pools and sinus fluid (140 ul) were lysed in 600 µl RLT buffer by repetitive pipetting. Dry swab drape paper was vortexed in lysis buffer and
allowed to incubate for 10 min. Total RNA was isolated by passing the clarified lysed tissue homogenate through Qiagen collection columns. Any homogenized sample which failed to pass through the column with the initial 15 second centrifugation was centrifuged for an additional 30 seconds at 10,000 x g. Ethanol RPE buffer was removed by centrifuging for 5 min. at maximum speed. Isolated RNA was eluted from the collection column using a single collection procedure and 30 µl of RNase-free water.

Two separate sets of oligonucleotide primers designed to a region of the F protein gene were used for the nested PCR. The first stage primers, DIF-1 and DIR-2, were designed from F protein gene sequence TRT 3B and have been previously described (20). The second stage primers, 2F and 2R, were designed (13) from US/CO APV F protein gene sequence. The first stage reaction produces a 536 bp product with subgroup A APV and a 380 bp product with the US/CO APV while the second stage reaction is specific for the US/CO APV and produces a 280 bp product.

For cDNA synthesis by reverse transcription (RT) 5 µl of RNA solution was mixed with 2.5 µl of 10X (100 mM Tris-HCl, pH 8.3, 500 mM KCl) PCR buffer, 4 µl of 25 mM MgCl₂, 1 µl 5 nM random hexamers, 1 µl of 10 mM each of deoxyribonucleotide triphosphates (dATP, dGTP, dCTP, dTTP), 20 units of RNase inhibitor and 50 units of murine reverse transcriptase. A 25 µl total reaction volume was obtained by adding diethylpyrocarbonate (DEPC) treated water. The reaction solution was incubated for 10 min. at 30 °C followed by 30 min. at 42 °C for the production of cDNA by RT. The RT reaction was stopped by heating to 92 °C for 10 min. For the first PCR, 5 µl of cDNA solution, described above, was mixed with 2.0 µl of 10 X PCR buffer (see above), 2 µl of 25 mM
MgCl₂, 0.25 µl of 20 µM of each primer, and 2.5 units of Perkin Elmer (PE) Amplitack Gold DNA polymerase. A 25 µl total reaction volume was obtained by adding DEPC treated water. For the nested PCR, 5 µl of 1st stage PCR products were mixed with 2 µl of 10 X PCR buffer, 1 µl of 25 mM MgCl₂, 0.5 µl (10mM) each DNTP, 2.5 units PE Amplitack Gold DNA polymerase and 0.25 µl of 20 µM of each primer. A 25 µl total reaction volume was obtained by adding DEP treated water. The first PCR was performed with 30 cycles of 94 °C for 1 min., 62 °C for 1 min. and 72 °C for 1 min. following an initial 10 min. 95 °C denaturation incubation while the nested PCR was conducted under identical conditions for 35 cycles. Both the first and nested PCR reaction programs were concluded with a final 72 °C 7 min. extension period. Second stage amplified products (20 µl) were subjected to electrophoresis in a 3 % agarose gel, stained with ethidium bromide (Et Br) and visualized with a transilluminator ultraviolet light.

A separate nested second stage TaqMan® PCR reaction was performed for product confirmation. All reagents and equipment mentioned in the following paragraph were obtained from PE Applied Biosystems (PE ABI, Foster City, CA). In addition to the target DNA an exogenous internal positive control (IPC) was included in the PCR reaction to distinguish true target negatives from samples testing negative due to inhibition of the PCR. For the PCR 3 µl of the previously described first stage product was mixed with 12.5 µl of the universal master mix, 2.5 µl of 10 X IPC probe mix (JOE-labeled), 0.5 µl of 50 X IPC exogenous DNA, 0.375 µl of 20 µM of each oligonucleotide primer, and 0.125 µl of 10 µM APV F protein gene FAM labeled probe. A 25 µl total reaction volume was obtained by adding DEPC treated water. The PCR reaction was conducted in a microamp optical 96-well
reaction plate with six no template control (NTC) wells and six no amplification control (NAC) wells. Blocker was added to each NAC well in lieu of 3 µl of first stage product and 3 µl of DEPC treated water was added to each NTC well. A two stage (95 °C for 15 sec. and 60 °C for 1 min.) 40 cycle PCR procedure was conducted following two initial incubation steps (50 °C for 2 min. followed by 95 °C for 10 min.) using a 7700 PRISM sequence detector. The forward primer F10 (GCTGTGAATGATCTCAAGGACTTTAT), reverse primer R129 (GAACCTCCGATTGTATTGTCCAA) and FAM APV-US 280 probe (AACAAGTGTGACATCTCAGACCTTAAGATGGCA) were designed to amplify and detect a (119 bp) segment of the US/CO F protein gene. This 119 bp segment is located within the 280 bp second stage RT-PCR product. Results were interpreted using the endpoint post-PCR plate read analysis and real-time PCR analysis.

**Sensitivity.** Sensitivity of the nested RT-PCR and TaqMan® RT-PCR procedures was determined by making five-fold serial dilutions of $10^{3.75}$ TCID$_{50}$ of the US/CO APV in sterile DEPC treated water. RNA was extracted with the QIAamp viral RNA isolation kit and manufacturer’s protocol. RT-PCR procedures were performed as described in conventional RT-PCR and TaqMan® RT-PCR materials and methods. Five-fold dilutions were used to accurately determine the detection limit of the assay. Two independent sensitivity trials were performed.

**ELISA.** Antibody to APV was detected using an ELISA procedure as described by Chettle and Wyeth (7). Three different ELISA antigens were used; APV/UK/14/1/1986 a subgroup A APV, APV/Hung/657/4/1990 a subgroup B APV, and the APV/US/CO/1997 representing the U.S. subgroup of APV. All 0 DPI sera were assayed against all three APV
antigens to detect previous exposure to APV. Subsequent bleedings were tested against only the US/CO APV ELISA antigen. Antigen and conjugate titrations were conducted with each antigen to optimize assay sensitivity. A 1:40 dilution of the sera was used on all three ELISAs. An optimal working dilution of a goat origin anti-chicken horseradish peroxidase-labeled conjugate was applied to the plate followed by an ortho-phenylenediamine substrate. The enzymatic reaction was stopped with 2.5 M H₂SO₄. Results were read using a single 490 nm wavelength (OD₄₉₀) and interpreted according to the method described by Chettle and Wyeth. Confirmation testing was conducted on all suspect sera with the indirect fluorescent antibody assay (IFA).

**Virus isolation.** A 20% tissue homogenate was prepared in tris buffered tryptose broth (TBTB) with antibiotics (10,000 IU/ml Penicillin G, 2,000 µg/ml Streptomycin sulfate, 650 µg/ml Kanamycin sulfate, 1,000 µg/ml Gentamicin sulfate and 20 µg/ml Amphotericin B) and centrifuged at 1,500 x g for clarification. The supernatant was recovered, filtered with a 0.45 µM filter and 0.3 ml was inoculated into each of five 8 day-old specific pathogen-free (SPF) embryos by the yolk sac (YS) route (29). Eggs were candled daily for embryo death and incubated with humidity at 37 °C for 9 nine days. All dead embryos (between day 2 and 9) as well as live embryos were harvested. AAF and yolk sac material were collected and the embryo was observed for hemorrhagic lesions and stunting. First passage yolk sac material and AAF were homogenized in TBTB and processed for an additional YS embryo passage. Four 8-day SPF embryos were inoculated by the YS method with 0.2 ml of clarified homogenized first passage material. Following the second embryo passage, YS and AAF material were processed in TBTB, centrifuged at 1,500 x g for 30 min. and 1.0 ml of the
supernatant material was inoculated onto a 25 cm² flask of chicken embryo fibroblast (CEF) cell culture. Two blind passages were made in CEF cells followed by IFA staining of inoculated CEF cells for detection of viral antigen.

RESULTS

Clinical signs. No clinical signs were observed at 1 DPI in either experiment. Clinical signs commenced at 3 DPI with sneezing and mouth breathing; nasal exudate could be produced with gentle squeezing of the nares. At 4 DPI all APV inoculated birds were depressed and exhibited sneezing and snicking. Two turkeys at 4 DPI died, tracheal plugs were observed in the air passages. At 5 DPI the nares were crusted over with nasal discharge and feed material, mouth breathing was common. Swollen infraorbital sinuses were observed at 7 DPI and by 8 DPI most clinical signs had subsided. All control turkeys remained healthy throughout both experiments.

Sensitivity and specificity. A virus dilution of 1:15,625 was detected by the conventional nested RT-PCR in both trials, this represents a calculated value of $10^{-0.44}$ TCID₅₀ of US/CO APV. The same first stage amplified products were also tested by the TaqMan® RT-PCR where a virus dilution of 1:15,625 was detected in two separate RT-PCR assays. A virus dilution of 1:78,125 was detected in only one of the two sensitivity titrations. The 1:78,125 virus dilution represents a calculated value of $10^{-1.69}$ TCID₅₀.

Samples from all control turkeys produced negative results by all three detection methods. As demonstrated in figure 1, the second stage RT-PCR fusion gene primers did not amplify any avian viral pathogen, other than the US/CO APV. This included subgroup A, UK 14/1 APV, and subgroup B, Hung APV. The first stage fusion gene primers produced
the expected 536 bp product with the UK 14/1 APV and a 380 bp product with the US/CO
APV. No PCR product was observed with subgroup B, Hung APV. The 536 UK 14/1 APV
bp product was sequenced and found to have >99% sequence homology with published TRT
3B (20) fusion protein gene sequence.

**Conventional nested RT-PCR.** Conventional nested RT-PCR positive results are
shown in table 1 for experiment 1 and in table 2 for experiment 2. Collection points 1 & 3
DPI represent the first stage of infection, 4-7 DPI the middle stage and 8-21 DPI the final
stage (figure 6-8). APV nucleic acid was detected as early as 1 DPI in the turbinate and sinus
from one turkey in experiment 2 and as late as 17 DPI from the sinus material of two
different turkeys in experiment 1 (figure 9). Viral nucleic acid was consistently detected in
both the turbinate and sinus between 3 and 7 DPI with a detection rate of 79%. Detection
became less consistent at 8 DPI; however, when viral nucleic acid was detected after 7 DPI it
originated from the sinus or the turbinate. All dry swab samples between 3 and 7 DPI
contained APV viral RNA, with the exception of 1 swab at 3 DPI (figure 6). The results for
tracheal swab pools were similar; with the exception of 1 swab at 4 DPI, all samples were
positive for viral nucleic acid. Only 4 lung or tracheal samples produced the expected 280 bp
band product by the nested RT-PCR.

Agarose gels illustrating results for 7 and 10 DPI are shown in figures 2 and 3. As
shown in figure 2, the conventional nested RT-PCR produced specific, easily interpretable,
single 280 bp bands. Weaker bands were seen for some trachea and lung samples and 17 DPI
sinus samples (figure 4). At 7 DPI the presence of strong bands indicated a large quantity of
RNA was detected; however, at 10 DPI no specific 280 bp bands were observed. The
dramatic decrease in detection between 7 and 10 DPI promoted the inclusion of an 8 and 9 DPI collection point in experiment 2. Results for the 17 DPI collection point are shown in figure 4; multiple bands inconsistent with the 280 bp product were observed in the Et Br stained agarose gel. Nonspecific bands were detected from both control (lane 3 & 5) and APV inoculated turkeys (lanes 6-18). Interpretation of a specific 280 bp band (lane 9 and 13) vs nonspecific products was difficult. Nonspecific bands were observed on 3 other collection points, but single 280 bp bands were observed in the majority of all Et Br stained gels.

**TaqMan® RT-PCR.** TaqMan® RT-PCR positive results are shown in table 1 for experiment 1 and in table 2 for experiment 2. Viral nucleic acid was first detected at 1 DPI from the sinus and turbinate of one turkey, while at 17 DPI, the last collection point yielding positive results, 2 sinus samples were positive (figure 9). Turbine and sinus samples were a consistent source of the virus between 1 and 9 DPI with 71% of the turbinate and 88% of the sinus samples positive for APV nucleic acid in experiment 2. Results for experiment 1 are similar, with a 63% detection rate in both the turbinate and sinus. The combined detection rate for lung and tracheal samples was 50% between 3 and 7 DPI in experiment 1 and 37% between 3 and 9 DPI in experiment 2. No APV nucleic acid was detected at 1 DPI or after 8 DPI in tracheal swab pools or dry swabs in either study. However, between 3 and 8 DPI 100% of all tracheal swab pool and dry swab samples were detected by the TaqMan® RT-PCR procedure.

Both real-time PCR and endpoint analysis were used to evaluate the FAM probe fluorescent emission reading. A sequence specific signal was generated from the F protein
probe reporter dye (FAM) and the IPC JOE probe. Fluorescence emission was detected by optical fibers connected to a spectrograph. Endpoint analysis reports the results for all samples as either positive or negative based on a calculated threshold value of the fluorescence intensity at the last cycle of the PCR reaction. Real time detection monitors the fluorescence intensity at each cycle plotting relative normalized fluorescence (ΔRn) vs. time. Threshold values are calculated in the manner as they are for endpoint analysis.

Determination of positive or negative status in real-time PCR was decided by evaluation of the amplification plot for each sample. Results from the two analytical methods were in agreement. All samples were tested in duplicate. The results from the duplicate wells were in agreement for 99% of the samples. Repeat testing of the remaining 1% produced results that were in agreement. IPC DNA was amplified in 100% of all test samples.

Statistical analysis. No overall difference was observed between the 2 experiments indicating the results are repeatable. The sinus (p<0.01) and turbinate (p<0.01) tissues when compared to lung and trachea tissues for both experiment 1 and 2 were found to be significantly better sources of APV virus and viral RNA by all three detection methods. The conventional nested RT-PCR was found to be less sensitive than the TaqMan® RT-PCR in detecting viral RNA from lung specimens (p<0.05). Tracheal swab pools were shown to be more productive sources of viral RNA than trachea tissue by both the conventional nested RT-PCR (p<0.01) and TaqMan® RT-PCR (p<0.01) as were dry swab specimens, conventional nested RT-PCR (p<0.01) and TaqMan® (p<0.05). The TaqMan® RT-PCR was shown to be a more sensitive assay than the conventional nested RT-PCR (p<0.03) and VI (p<0.01) for the detection of APV in tissue and swab specimens.
Serology. All sera collected between 1 and 7 DPI were negative for antibodies to the US/CO APV by ELISA. Between 8 and 12 DPI all sera were positive for APV antibody by IFA (figure 5). Several of the sera during this time frame were positive by ELISA, but most sera tested in the ELISA suspect positive category. All APV infected birds were positive by ELISA between 14 and 21 DPI.

Virus isolation. Tables 1 and 2 include virus isolation results for experiment 1 and 2, respectively. Virus was isolated between 3 and 7 DPI from a combination of turbinate, lung, sinus, trachea, and tracheal swab pool samples. In experiment 1 the sinus of one turkey was positive at 7 DPI, while in experiment 2 virus was recovered from the sinus and trachea tissue of a single turkey at 6 DPI. Virus was recovered from tracheal swab pools at 5 and 6 DPI in experiment 2 and from the 5 DPI pool in experiment 1. Isolation was most successful at 3 and 5 DPI with the highest overall recovery rate in the turbinate, and sinus samples. Virus was isolated from only 23 of the total 98 samples tested in experiment 1 and 2 between 1 and 7 DPI. All samples were virus negative after 7 DPI.

DISCUSSION

Turkeys from both experiment 1 and 2 exhibited clinical signs typical of APV infection; sneezing, swollen infraorbital sinuses, tracheitis and nasal exudate (18,23). However, some differences were noted between experiment 1 and 2; turkeys in experiment 1 exhibited more nasal discharge while in experiment 2 two birds died at 4 DPI. Turkeys in both experiments were from the same commercial source, but received at different times. Neither group was cultured to determine what, if any, secondary bacterial infections were circulating in the turkeys upon arrival. The different clinical responses noted in the two
experiments could possibly be attributed to different secondary bacterial infections. Research has shown that secondary bacterial infections increase the severity of infection (10).

Antibody to APV was detected in 50% of the sampled APV inoculated birds at 8 and 9 DPI by ELISA as shown in figure 5. The proportion of birds positive for APV antibody increased to 75% by 10 DPI. Many of these OD490 values fell in the suspect positive category, but were confirmed with IFA techniques. Early sero conversion (8-10 DPI) allows antibody detection to be used as a early screening tool for APV.

APV was detected by the RT-PCR procedures in turbinate, sinus, trachea, lung, tracheal swab and dry swab samples. Throughout the study, sinus (p<0.01) and turbinate (p<0.01) tissue were significantly more productive sources of virus and viral RNA than were the lung and turbinate specimens for all detection methods. In vivo studies, conducted by Cook et al., reported large quantities of virus recovered from the turbinate at 3 ($10^{5.9}$ CD50/gm) and 5 ($10^{3.6}$ CD50/gm) DPI (12). Turbinate tissue has been demonstrated by Mase et al. (20) to be an excellent source of viral RNA for RT-PCR. Neither study reported on the detection of APV from sinus swabs or exudate. Trachea and lung samples did yield viral RNA, but in fewer samples (see table 1 & 2). In vivo studies have demonstrated the presence of APV, occasionally, in the lung, but in very low concentrations (12,18), explaining the low detection rate for lung samples when tested by the RT-PCR method. Isolation of APV has been demonstrated in 3 to 7 DPI trachea samples (12). In the current study, virus was recovered from four (3-6 DPI) trachea samples. Of these four samples, 2 were negative for APV by the conventional RT-PCR and 1 was negative by both RT-PCR’s. This can possibly be contributed to sample processing. Pieces (30 mg) of the trachea were processed for RNA
extraction. An alternative processing procedure that would yield a higher mass of infected cilia would most likely increase the rate of detection in trachea samples. Amplification of IPC DNA, by the TaqMan® procedure, in all tracheal samples shows that no PCR inhibitory substances were present.

Tracheal swabs are good specimens for isolation of APV nucleic acid as demonstrated in these and other studies (6,17). They are easily collected and eliminate the need to sacrifice live birds. As shown in figure 6 and 7, tracheal swab pools produced positive results by all three detection methods. Several swabs are needed to create a pool; however, TRT morbidity rates are as high as 90-100% within a flock of infected birds (3). For this reason, a flock test is appropriate. The number of swabs needed to create a pool is influenced by the stage of infection at which the swabs are collected. Tracheal swab pools, dry swabs and nasal/sinus exudate swabs were a very consistent source of the viral RNA between 3 and 8 DPI; however, after 8 DPI APV was only detected in tissue specimens. Dry swabs and nasal/sinus exudate swabs are easy to collect. Ambient shipping temperatures and endogenous RNases may degrade viral RNA before samples can be received for processing. A temporal study testing the liability of RNA in these types of samples needs to be conducted. The ease and consistency of collection and detection favor swab specimens when samples are collected during the active clinical phase of the disease.

As shown in figure 9, APV was detected by both RT-PCR detection methods between 1 and 17 DPI, while virus was isolated only between 3 and 7 DPI. RT-PCR extends the period of detection from 4 to 17 days. There was a dramatic decrease in detection by the RT-PCR procedures at 8 DPI; however, viral RNA was detected in sinus tissue at 8, 9, 10
and 17 DPI. Li Jing et al. (17) also reported detection of APV at 17 to 19 DPI using RT-PCR procedures. Studies evaluating the concentration of virus recovered from a variety of respiratory tissues reports large quantities of recovered virus between 3 and 7 DPI with a dramatic decrease in virus detection at 7 DPI (12). APV was detected in sinus and turbinate specimens by RT-PCR, in 37 of 44 samples between 5 and 7 DPI. As illustrated in figure 9, detection of APV was most successful, between 3 and 7 DPI, by any of the three detection methods.

Figures 6, 7, and 8 compare the conventional nested RT-PCR, TaqMan® PCR and virus isolation detection methods at 3, 5 and 9 DPI, representing the first, middle and end stages of infection, respectively. Both the conventional nested RT-PCR and the TaqMan® RT-PCR assay produced more positive results than virus isolation as shown in tables 1 & 2 and figures 6-8. Detection capabilities were extended to 17 DPI with the TaqMan® RT-PCR and conventional RT-PCR, as demonstrated in figure 9. Virus isolation was the least sensitive method for the detection of APV. Virus was recovered from only 23 of the 98 3-7 DPI samples, defining this procedure as labor intensive, and unsatisfactory.

Both the conventional nested RT-PCR and TaqMan® RT-PCR were found to be specific and sensitive methods for the detection of the US subgroup of APV. Overall, the TaqMan® RT-PCR, detecting a calculated value between $10^{-0.44}$ to $10^{-1.69}$ TCID$_{50}$ of the US/CO APV, was shown to be a more sensitive assay than both the conventional nested RT-PCR ($p<0.03$) and virus isolation ($p<0.01$) for the detection of APV in swab and tissue specimens. Tissues with concentrations of viral RNA below the detection limit of the nested RT-PCR, but within the detection limit of the TaqMan® probe method were identified. The
most significant improvements in detection were with lung and trachea specimens. The conventional nested RT-PCR was less sensitive than the TaqMan® RT-PCR in the detection of APV form lung specimens (p<0.05). The combined detection rate for lung and tracheal samples, between 3 and 9 DPI, increased from <1% with the conventional nested RT-PCR to 40% with the TaqMan® RT-PCR. PCR product confirmation is traditionally performed with an oligonucleotide probe complementary to the amplified product or restriction enzyme analysis. The TaqMan® RT-PCR procedure combines the second stage reaction and product confirmation using a fluorogenic probe complementary to the target sequence. Not only does this method provide a faster detection system, it is sensitive and increases the rate of detection over conventional RT-PCR. Originally the TaqMan® RT-PCR was developed for the purpose of product confirmation. When working with diagnostic field samples there will be occasions when nonspecific bands may confuse interpretation of conventional RT-PCR results. Improved sensitivity and specific product confirmation justify routine implementation of the TaqMan® RT-PCR. Detection of the US APV is necessary, as both subgroup A and B APV are exotic to the U.S. The first stage fusion gene primers amplify both subgroup A and the US subgroup of APV. This allows the detection of two of the three representative subgroups of APV. The second stage PCR is specific for the US/CO APV only. The TaqMan® RT-PCR was shown to be the most sensitive procedure for detecting APV RNA in tissue and swab contents from APV infected turkeys. Although, negative results with this assay cannot be interpreted as negative findings for all APVs, it will differentiate the US/CO APV from subgroup A and B APV.
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28. Seal, B. Matrix protein gene nucleotide and predicted amino acid sequence demonstrate that the first US avian pneumovirus isolate is distinct from European strains. Virus Res. 58:45-52. 1998.


ACKNOWLEDGMENTS

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Table 1. Conventional nested RT-PCR, TaqMan® RT-PCR, and virus isolation results for experiment 1. Two APV inoculated turkeys were sampled at each collection point. Results are listed as the number of positive specimens/ the number of total APV inoculated specimens tested. All control specimens were negative.

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TaqMan® RT-PCR results from APV inoculated turkeys (experiment 1)

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Virus isolation results from APV inoculated turkeys (experiment 1)

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^A Sinus exudate swab samples were collected only on days that the turkeys had nasal discharge.

^B Not done
Table 2. Conventional nested RT-PCR, TaqMan® RT-PCR, and virus isolation results for experiment 2. Two APV inoculated turkeys were sampled at each collection point with the exception of 4 DPI, 3 APV inoculated turkeys were sampled. Results are listed as the number of positive specimens/ the number of total APV inoculated specimens tested. All control specimens were negative.

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Conventional nested RT-PCR results from APV inoculated turkeys (experiment 2)

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TaqMan® RT-PCR results from APV inoculated turkeys (experiment 2)

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Virus isolation results from APV inoculated turkeys (experiment 2)

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\(^A \text{Not done}\)
Figure 1.
Figure 3.
Figure 4.
Fig. 1. Electrophoresis of amplified PCR products included in the specificity study of the nested RT-PCR. Lane 1, avian nephritis; lane 2, avian encephalomyelitis; lane 3, infectious bronchitis virus; lane 4, infectious bursal disease; lane 5, paramyxovirus type I; lane 6, paramyxovirus type II; lane 7, paramyxovirus type III; lane 8, avian influenza virus; lane 9, reovirus; lane 10, APV/US/CO/97; lane 11, APV/Hung/657/4/90; lane 12, APV/UK/41/1/86; lane 13, APV/US/CO/97; lane 14, negative control.

Fig. 2. Second stage F gene 7 DPI PCR products. Lane 1, control (C) tracheal swab pool (Tr Sw P); lane 2, C turbinate; lane 3, C lung; lane 4, C sinus; lane 5, C Tr; lane 6, principal (P) Tr Sw P; lane 7 & 11, P turbinate; lane 8 & 12, P lung; lane 9 & 13, P sinus; lane 10 & 14, P Tr; lane 15, C dry swab; lane 16, P dry swab; lane 17, sinus exudate swab; lane 18 positive C; lane 19, negative C.

Fig. 3. Second stage F gene 10 DPI PCR products. 100 molecular weight markers; lane 1, negative control (C); lane 2, positive C; lane 3, C tracheal swab pool (Tr Sw P); lane 4, C turbinate; lane 5, C lung; lane 6, C sinus; lane 7, C Tr; lane 8, principal (P) Tr Sw P; lane 9 & 13, P turbinate; lane 10 & 14, P lung; lane 11 & 15, P sinus; lane 12 & 16, P Tr; lane 17, C dry swab; lane 18, P dry swab; lane 19, P sinus exudate swab; lane 20 & 21, P sinus swab; lane 22, C sinus swab.
Fig. 4. Second stage F gene 17 DPI PCR products. 100 bp molecular weight markers; lane 1, control (C) tracheal swab pool (Tr Sw P); lane 2, C turbinate; lane 3 C lung; lane 4, C sinus; lane 5, C Tr; lane 6, principal (P) Tr Sw P; lane 7 & 11, P turbinate; lane 8 & 12, P lung; lane 9 & 13, P sinus; lane 10 & 14, P Tr; lane 15, C dry swab; lane 16, P dry swab; lane 17, sinus exudate positive control; lane 18, P Tr; lane 19, P turbinate; lane 20, P Tr; lane 21, positive C; lane 22, negative C.
Fig. 5. The percentage of sampled APV inoculated birds from experiment 2 that tested as either suspect or positive for APV antibody by the enzyme-linked immunosorbent assay (ELISA). All APV inoculated birds prior to 8 days post inoculation (DPI) were negative by ELISA.
Fig. 6. Comparison of conventional nested RT-PCR, TaqMan® RT-PCR and virus isolation detection methods at 3 days post inoculation (DPI). Chart bars represent the percent of APV inoculated samples that tested positive for APV by each detection method.
Fig. 7. Comparison of conventional nested RT-PCR, TaqMan® RT-PCR and virus isolation detection methods at 5 days post inoculation (DPI). Chart bars represent the percent of APV inoculated samples that tested positive for APV by each detection method.
Fig. 8. Comparison of conventional nested RT-PCR, TaqMan® RT-PCR and virus isolation detection methods at 9 days post inoculation (DPI). Chart bars represent the percent of APV inoculated samples that tested positive for APV by each detection method.
Fig 9. Comparison of the nested RT-PCR, TaqMan® RT-PCR and virus isolation detection methods between 1 and 21 days post inoculation. Graph lines indicate the number of samples that tested positive for US/CO APV at each collection point.
THE SENSITIVITY AND SPECIFICITY OF A RT-PCR ASSAY FOR THE AVIAN PNEUMOVIRUS (COLORADO STRAIN)

A paper submitted to Avian Diseases

Janice C. Pedersen¹, Donald L. Reynolds²³, A. Ali

ABSTRACT

Avian pneumovirus (APV), the etiological agent of turkey rhinotracheitis in turkeys, was first isolated in the U.S. in 1997. Cross neutralization studies and matrix gene sequence analysis has shown that the US APV is antigenically distinct from previously described avian pneumoviruses. A reverse transcription-polymerase chain reaction (RT-PCR) assay for the detection of APV, Colorado strain (US/CO), was evaluated for sensitivity and specificity. The single-tube RT-PCR assay utilized primers developed from the matrix (M) gene sequence of the US/CO APV. Representatives of subgroup A and subgroup B APV and the US APV along with Newcastle disease virus (NDV), a member of the family Paramyxoviridae, and bovine respiratory syncytial virus (BRSV), a member of the genus Pneumovirus, were tested. It was found that the RT-PCR amplified the US/CO APV, but did not detect subgroup A or B APV, NDV or BRSV. These findings further confirm the unique

¹National Veterinary Services Laboratories, Veterinary Services, Animal and Plant Health Inspection Service, U.S. Department of Agriculture, and ²Veterinary Medical Research Institute, College of Veterinary Medicine Iowa State University
characteristic of the US/CO APV. The RT-PCR was capable of detecting between $10^{0.25}$ TCID$_{50}$ and $10^{-0.44}$ TCID$_{50}$ of the US/CO APV. These results have demonstrated that the single-tube RT-PCR assay is a specific and sensitive assay for the detection of US/CO APV.

INTRODUCTION

Avian pneumovirus (APV) is the etiologic agent of turkey rhinotracheitis (TRT) in turkeys (19,33) and swollen head syndrome (SHS) in chickens (7,23,26). Turkey rhinotracheitis is an acute, rapidly spreading, upper respiratory disease causing nasal and ocular discharge, rhinitis, swollen infraorbital sinuses, and impairment of tracheal cilia (16,18,20,21). A short incubation period of 1-3 days facilitates quick transmission of the disease (14,17). Turkeys of all ages are susceptible; (4,21,31) however, poult younger than 6 weeks have been reported to experience a more severe clinical disease (2). Mortality and morbidity rates as high as 30% and 90% respectively, have been reported (1,4,8). Several contributing factors including secondary bacterial infections, poor husbandry, ventilation, and higher stocking densities can influence mortality and morbidity (4). Clinical signs in turkey breeder hens include reduction in egg production and quality (21). APV was first isolated in the U.S.A. in 1997 following a respiratory disease outbreak in turkeys from Colorado (29,30).

APV, a non-segmented single-stranded negative sense RNA virus, is a member of the family Paramyxoviridae and genus Pneumovirus (10,25). Only one serotype of APV has been described (9), but on the basis of differences in the sequence of the glycopolypeptide (G) gene and cross-neutralization studies, two subgroups (A and B) within the one serotype have been identified (5,9,11,15,22). Subgroup classification for the U.S. /Colorado (US/CO)
strain of APV has not been determined. Detection of APV viral antigen and antibodies are traditionally accomplished with immunofluorescence and enzyme-linked immunosorbent assay (ELISA) techniques and is subgroup dependent. Cross-neutralization studies have determined that US/CO is antigenically unique and has only limited cross-neutralization with subgroup A and B viruses (29,30). Consequently, ELISA tests with subgroup A and B antigens failed to detect antibodies in turkeys infected with US/CO APV. A recent report on the US/CO APV M protein genome analysis indicates the US/CO forms a single clade separating it as a unique virus (27).

A RT-PCR diagnostic procedure has been developed for the detection of US/CO APV utilizing primers designed from the matrix gene sequence (3). The objective of this study was to determine if the RT-PCR procedure could detect pneumoviruses other than US/CO and to determine the sensitivity of this RT-PCR assay.

**MATERIALS AND METHODS**

**Viruses.** Three APVs, the bovine respiratory syncytial virus (BRSV), and Newcastle disease virus (NDV), LaSota strain, were used in this study. The three NVSL reference APV strains, are as follows: US/CO, a 1997 U.S., Colorado APV turkey isolate; UK 14/1, a turkey subgroup A APV (15) isolated in the UK in 1986; and subgroup B Hungarian 657/4 (15) isolated from turkeys in Hungary. All APVs were propagated in chicken embryo fibroblast (CEF) cells. NDV was propagated by the allantoic route (28) in specific pathogen free (SPF) chicken embryos, and BRSV was grown in bovine turbinate (BT) cell culture (32). Taxonomic classification and titers of the respective viruses are shown in Table 1.
RT-PCR procedure. Previously described RNA extraction and RT-PCR procedures (3) were used with minor modifications. Briefly, RNA was extracted from 140 ul of CEF or BT cell culture supernatant and amniotic-allantoic fluid (AAF) using the QIAamp viral RNA (Qiagen Inc., Valencia, CA) isolation kit and manufacturer's protocol. Viral RNA was eluted from the Quiagen column with 50 ul of diethylpyrocarbonate (DEPC) treated water. Matrix gene primers (0.2 uM) used in this study were selected using the US/CO APV matrix gene sequence that has been previously described (3). Single tube cDNA synthesis and DNA amplification were conducted using the Promega Access™ RT-PCR (Promega Inc., Madison, WI) system in a 50 ul reaction with 10 ul of isolated RNA.

Each virus, along with uninfected cell culture supernatant and AAF, was extracted and tested by the RT-PCR procedure. Amplified products (20 ul) were subjected to electrophoresis in a 2% agarose gel, stained with ethidium bromide and visualized with a ultraviolet transilluminator. RT-PCR controls included a negative control, substituting sterile DEPC treated water for extracted material, and uninfected cell culture supernatant and AAF. The entire RT-PCR procedure was performed twice for each virus and their corresponding controls.

Sensitivity of the RT-PCR procedure was determined by making five-fold serial dilutions of the US/CO virus in sterile DEPC treated water. RNA extraction and the RT-PCR procedure were conducted as described above. Five-fold dilutions were used to accurately determine the detection limit of the assay. Two independent sensitivity procedures were performed.
RESULTS

Figure 1 displays the RT-PCR assay results for the described viruses. Both lanes 1 and 9 contain US/CO and produced the expected 631 base pair (bp) product. Lane 3 (UK 14/1) and lane 4 (Hungarian 657/4) had no visible bands. No visible bands were observed in lanes 2, 6, 8, 5, 7 and 10 that contained uninfected cell culture supernatants from CEF cells and BT cells, AAF from uninfected embryos, BRSV, NDV and the negative control, respectively.

Figure 2 demonstrates the results of the RT-PCR assay performed on various dilutions of the US/CO virus stock solution. In both trials, the RT-PCR was positive at 1:3125 dilution of the virus solution representing a calculated value of $10^{0.25}$ TCID$_{50}$ of US/CO APV. In one trial a distinct, but faint band, was present at $10^{-0.44}$ TCID$_{50}$, however, this band was not apparent in the second trial. Table 2 shows the assay sensitivity at each virus dilution.

DISCUSSION

Most countries with a commercial turkey industry, with the exception of Canada and Australia, have been affected by TRT (6,13). TRT has been problematic for U.S. turkey producers in Colorado and more recently in Minnesota (12,29). Several other avian respiratory pathogens including mycoplasma, avian influenza virus, infectious bronchitis virus, and Newcastle disease virus can cause a similar clinical disease (21). A RT-PCR capable of detecting US/CO APV may prove useful in the identification and differentiation of respiratory pathogens.
Representatives of APV subgroups A, UK 14/1, and B, Hungarian APV 657/4, were used to determine specificity of the RT-PCR for APV. Other viruses that were evaluated in this study to determine the specificity of the RT-PCR assay included BRSV, a member of the family Paramyxoviridae and genus Pneumovirus, and NDV, a member of the family Paramyxoviridae and genus Rubulavirus. The results of this study clearly indicated that the RT-PCR assay was specific for only US/CO APV and not subgroups A and B of APV or the other viruses that were evaluated. A RT-PCR capable of detecting all subgroups of APV may be a valuable screening tool; however, it may be limited in its capacity to differentiate the subgroups.

In both sensitivity trials, a clearly identified 631 bp band was observed at the dilution corresponding to 1:3125. Figure 2 demonstrates the sensitivity of the RT-PCR as observed in the second trial where a faint band was observed at the 1:15,625 dilution. This band was not apparent in the alternate trial. This work was performed only twice, but we are satisfied that the RT-PCR can detect between $10^{-0.25}$ and $10^{-0.44}$ TCID$_{50}$ of US/CO APV. This RT-PCR provides a sensitive and specific assay for the detection of the US/CO APV.

Pneumoviruses generally encode eight genes with the matrix and polymerase genes being considered two of the more conserved (27). Therefore, a RT-PCR using primers from the matrix gene may be able to detect all APVs including the subgroup A and B viruses as well as the US/CO. However, recent reports on sequencing of the matrix gene phylogenetically separates US/CO from both subgroup A and B (27). It was reported that subgroup A and B APV matrix (M) genes share 75% identity in their coding sequences; however, they have only 60% identity with the US/CO APV M protein gene. Predicted M
proteins of European APV subgroup A and B isolates share 89% identity in their amino acid sequence. The APV/CO has only 78% identity with APV subgroup A and 77% identity with subgroup B protein sequences (27). Seal concluded that the US/CO is a unique virus forming its own clade (27). This phylogenetic uniqueness is supported by serological studies (30). In this study the inability of the RT-PCR using matrix gene primers to detect either the subgroup A or B APV further corroborates the unique character of the US/CO.

The RT-PCR procedure has been shown to be a specific diagnostic assay for avian pathogens. Cross-contamination of samples is not uncommon with PCR procedures. Single tube techniques reduce this potential and are less resource intensive than multistep RT-PCR procedures. Other approaches such as a multiplex RT-PCR or RT-PCR using primers targeted to other genes may be useful for APV screening assays. Although this assay was conducted on a purified virus sample, it has good potential for clinical applications. Modifications of the procedure may be necessary for use with clinical specimens, however we would not expect the sensitivity and/or specificity to be greatly reduced. Further development of this assay for clinical specimens warrants future studies.

REFERENCES


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


ACKNOWLEDGEMENTS

The National Veterinary Services Laboratories is gratefully acknowledged for their financial support.
Table 1. Virus identification, taxonomic classification and virus titer for viruses used in this study.

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<td><em>Paramyxoviridae</em> /</td>
<td>$10^{3.75}$ TCID₅₀/ml ³</td>
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<td>U. K./avian pneumovirus 14/1 (UK 14/1)</td>
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<td>Hungarian/avian pneumovirus 657/4</td>
<td><em>Paramyxoviridae</em> /</td>
<td>$10^{4.83}$ TCID₅₀/ml</td>
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<td><em>Rubulaviruses</em></td>
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³ The titer of all APVs and BRSV were determined by titration in cell culture and calculation of the mean tissue culture infective dose (TCID₅₀).

² The titer of NDV was determined by titration in embryos and calculation of the mean embryo infectious dose (EID₅₀).
Table 2. The sensitivity of the RT-PCR as determined by amplification and ethidium bromide staining of 5-fold serial dilutions of the US/CO APV.

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<td>1: 125</td>
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<td>+</td>
</tr>
<tr>
<td>1: 625</td>
<td>$10^{0.95}$ TCID$_{50}$</td>
<td>+</td>
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<tr>
<td>1: 3125</td>
<td>$10^{0.25}$ TCID$_{50}$</td>
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<tr>
<td>1: 15.625</td>
<td>$10^{-0.44}$ TCID$_{50}$</td>
<td>+/-</td>
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<tr>
<td>1: 78,125</td>
<td>$10^{-1.69}$ TCID$_{50}$</td>
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The presence of a 631 bp is reported as positive (+) while the absence of a 631 bp band is reported as negative (-). A faint band is reported as positive/negative (+/-).
Figure 2
Fig. 1. Electrophoresis of RT-PCR amplified products in a 2% agarose gel stained with ethidium bromide. Lane 1, US/CO APV; lane 2, uninfected CEF supernatant; lane 3, UK 14/1 APV; lane 4, Hungarian APV; lane 5, BRSV; lane 6, uninfected BT supernatant; lane 7, NDV; lane 8, uninfected AAF; lane 9, US/CO APV; lane 10, negative RT-PCR control; lane 11, 100 bp molecular weight markers.

Fig. 2. Electrophoresis of RT-PCR amplified dilutions of US/CO APV in a 2% agarose gel stained with ethidium bromide. Lane 1, undiluted; lane 2, 10^{3.05} TCID_{50}; lane 3, 10^{2.35} TCID_{50}; lane 4, 10^{1.65} TCID_{50}; lane 5, 10^{0.95} TCID_{50}; lane 6, 10^{0.25} TCID_{50}; lane 7, 10^{-0.44} TCID_{50}; lane 8, 10^{-0.69} TCID_{50}; lane 9, negative RT-PCR control; lane 10, 100 bp molecular weight markers.
CONCLUSION

APV was first detected in the U.S. in 1997 and continues to be a disease problem in the state of Minnesota. Sequencing and cross-neutralization studies have shown the US/APV to be antigenically unique, thus prompting the formation of a third subgroup or a second serogroup within the *Metapnumovirus* genus. Subgroup A and B APVs are still considered exotic to the U.S. and need to be differentiated from the US/APV. A rapid and sensitive assay that is capable of detecting the US/APV and distinguishing it from European subgroup A and B APVs is needed.

RT-PCR is a very rapid, sensitive and specific assay for the detection of APV. The single-tube M gene and the F gene nested RT-PCR assays are sensitive, detecting between $10^{0.25}$ to $10^{-1.69}$ TCID$_{50}$ of the US/CO APV. This study demonstrated that RT-PCR can be conducted on a variety of specimens including dry swabs, tracheal swab pools and respiratory tissues. Results can be produced in as little as 48 hours. RT-PCR was shown to increase the detection rate and extend the detection period from 4 days with VI to approximately 16 days with RT-PCR. Sinus and turbinate specimens are the most productive tissues for the detection of the virus between 1 and 17 DPI, however birds need to be sacrificed for sample collection. Tracheal swab pools and dry swabs were shown to be good samples for detection of the virus between 3 and 8 DPI. Swabbing live birds is an efficient and rapid method for the detection of APV viral RNA. Swabs from several birds can be pooled. Virus isolation was demonstrated to be the least sensitive method for the detection of APV, producing significantly less positive results over a shorter time period than either RT-PCR assay.
A screening RT-PCR capable of detecting all subgroups of APV would be a helpful diagnostic tool, however one was not identified. The first stage reaction for the F gene RT-PCR will detect both subgroup A and the US/APV, while the second stage reaction is specific for the US/APV. The M gene and F gene RT-PCR assay were shown to be specific for the US/APV. This differentiation is necessary in view of the fact that both subgroup A and B viruses are exotic diseases in the U.S. A sensitive RT-PCR assay that will rapidly detect the U.S. subgroup of avian pneumovirus from tracheal swabs, dry swabs or respiratory tissues was identified. This diagnostic tool will be helpful in the detection of the U.S. subgroup of APV.