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PATHOGENICITY AND ANTIGENICITY OF AN AMERICAN AVIAN PNEUMOVIRUS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

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2002

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ABSTRACT

The pathogenicity, antigenicity, and transmissibility of an American avian pneumovirus (APV) were studied using turkey poults. In addition, a comparison between three different diagnostic methods for APV was performed. Three studies were conducted to investigate these objectives. In the first study, the pathogenicity, persistence, transmissibility, and tissue distribution of the APV in turkey poults were investigated. In the second study, a comparison between two different enzyme linked immunosorbent assays (ELISA) and virus neutralization (VN) test was performed. In the third study, the antigenic relationships between the APV and four avian paramyxoviruses were studied.

In trial one of the first study, we investigated APV infection alone or in combination with *Bordetella avium*, which is a common cause of respiratory disease in turkey poults. Two-week-old turkey poults were allotted to four groups. Three groups were inoculated oculonasally with APV or, *B. avium* alone or a combination of APV and *B. avium*. The fourth group was used as a control group. The viral RNA was detected by reverse transcriptase-polymerase chain reaction (RT-PCR) up to 10 days post inoculation (PI) in birds inoculated with both the virus and bacteria and up to 5 days in birds inoculated with the virus alone. The virus was isolated up to 7 days PI from the dually infected birds and up to 3 days PI from birds inoculated with the virus alone. *B avium*
was recovered from the sinuses and tracheas of all inoculated birds throughout the experiment. In addition, *B. avium* was recovered from the lungs of the birds infected with the virus and bacteria from day 8 PI up to the end of the experiment. The antibody response to the virus and bacteria in the dually infected birds was higher than that of birds inoculated with either agent alone using the VN test, ELISA and microagglutination test (MAT).

In the second trial, the tissue distribution of APV in experimentally inoculated two-week-old turkey poults was studied. Birds were allotted to two groups. Birds in group one serve as non-inoculated controls and birds in group two were inoculated oculonasally. Birds from both groups were euthanized at 2, 4, 7, 14, and 21 days and samples from sinuses, tracheas, lungs, thymuses, burses, livers, spleens, ileums, jejunums, and feces were collected and examined by RT-PCR and used for virus isolation attempts. Only samples from sinuses, tracheas, and lungs were positive for APV by both RT-PCR and virus isolation. Antibodies to APV were detected as early as 7 days PI.

In the third trial, the ability of APV to spread among birds was studied. One-week-old commercial turkey poults were allotted into five groups. One half of the birds in groups 1a and 2b were inoculated oculonasally and placed in different rooms, the other half were placed in the same cage to serve as direct contact birds. Birds in groups 1b and 2a were not inoculated and placed in the same room but in different cages with groups 1a and 2b to serve as indirect contact birds. Birds in groups 1a and 1b were placed in a different cage in the same room where the airflow was directed from the infected group (group 1a) towards the uninfected indirect contact group (group 1b). Birds in groups 2a and 2b were placed in a different cage in the same room where the airflow was directed from the
uninfected indirect contact group (group 2a) towards the infected group (group 1a). The fifth group was a non-inoculated control and was placed in a separate room. The virus was isolated and the viral RNA was detected in the inoculated and direct contact birds. The virus was not isolated, viral RNA was not detected, and no antibodies were detected in the indirect contact birds.

In the second study, a comparison was conducted between two different whole virus ELISAs, developed in Ohio (OH) using APV/Minnesota/turkey/2a/97 and Minnesota (MN) using APV/Colorado/turkey/97, and a Virus neutralization (VN) test. Serum samples from 270 turkeys from 27 Minnesota turkey flocks were tested using two different ELISAs and a VN test to detect the APV antibodies. In addition, 77 turkey serum samples and 128 ostrich serum samples from OH were tested. None of the turkey samples from OH had antibodies to APV using the VN test and OH ELISA. The ostrich samples were tested only with the VN test and were all negative for antibodies to APV. For the MN serum samples, 107, 115 and 120 were positive using the VN test, the OH ELISA and the MN ELISA respectively. The kappa value of 0.938 and 0.825 showed excellent agreement between the VN test and the OH ELISA and the MN ELISA, respectively, for detection of antibodies to the APV. The OH ELISA and MN ELISA had a sensitivity of 1.0 and .953, specificity of .950 and .889 and accuracy of .970 and .914, respectively. Our results indicate that the three methods are sensitive and specific for diagnosis of the APV infection.

In the third study, the antigenic relationships between the APV and four avian paramyxoviruses (PMV-1, PMV-2, PMV-3, and PMV-7) were studied using a
hemagglutination inhibition test, ELISA, and VN test in vero cells and chicken embryos. All the viruses were isolated in the USA or Canada. No antigenic relationships between APV and the PMVs were detected using the three tests.
Dedicated to My Parents, My wife and My children

Mohammed, Norh, Majd, and Nasser
ACKNOWLEDGMENT

I would like to express my appreciation to my advisor Professor Y. M. Saif for his guidance, advice, encouragement and unending patience during my Ph.D program. Thanks are also due to my graduate committee members Dr. Teresa Morishita, Dr. Daral Jackwood, and Dr. John Gordon for their valuable suggestions, advices and counsel during the performance of my research and write-up of this dissertation.

I would like to acknowledge Dr. Lucy. Ward, Dr. Kenneth Theil, Dr. Srinand Sreevatsan, Dr. Qijing Zhang, and Dr. Jeff Lejeune for their suggestions and criticisms of the manuscripts.

To Robert Dearth, many thanks and appreciations for helping during posting birds and supplying chicken embryos and also for his help with providing bacterial media for my experiment. I wish to thanks Arden Agnes, Christine Nielsen, and Christine McCloskey for their help with preparation of the histopathology slides. To my fellow graduate students, thanks for your support during my research, there have been great discussions from which I learned great information, scientifically and socially. It is a friendship that I will not forget.

Throughout this study I have had moral support from faculty, staff, and graduate students of the Department of Food Animal Health Research Program in Wooster and Veterinary Preventive Medicine in Columbus of the Ohio State University; I appreciate their supports.
Finally, I am grateful to my parents and my parents-in-law for their love and concerns. Many thanks are due to my wife for her love, understanding and support during these past years.
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Studies in avian Microbiology, Virology, Infectious Diseases, and Molecular Biology.
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I. Avian Pneumovirus

The Avian pneumovirus (APV) is the etiologic agent of turkey rhinotracheitis (TRT) and swollen head syndrome (SHS) in turkeys and chickens, respectively (15, 37, 38, 40, 52, 64, 76, 99, 103). The APV causes respiratory diseases in young birds and a drop in egg production in breeder flocks (3, 32, 55). Although, all ages of turkeys are susceptible to APV, the severity of the disease varies. Alexander et al. reported that the disease was more severe in day old poults than 6-week-old turkeys (1). The clinical disease is characterized by nasal and ocular discharge, swollen infraorbital sinuses and sneezing (14, 52, 64, 103). These clinical signs have been observed in different countries and found to be similar but different names were given to the disease, which reflects the complexity of the etiology when associated with secondary infections.

Lister and Alexander listed many pathogens that might cause rhinotracheitis in birds, which include bacterial agents such as \textit{Bordetella avium} and Bordetella-like bacteria, \textit{Escherichia coli}, mycoplasma, chlamydia, and viral agents such as Newcastle
Disease, avian paramyxovirus type-1 (PMV-1), Yucaipa virus (PMV-2), PMV-3, Infectious barsal disease virus (IBDV), Avian influenza virus, and avian Adenovirus (32,58).

In the late 1970s a disease causing rhinotracheitis, but with much higher infectivity, morbidity and mortality, became apparent in South Africa (14) and later in several other countries. The causal agent was isolated and later shown to be a pneumovirus, which is the cause of the infection in turkeys and other birds such as chickens and ducks (13, 35, 36, 38, 50, 62, 74, 97, 101).

Pneumoviruses are members of the subfamily Pneumovirinae in the family Paramyxoviridae. APV is classified in the genus Metapneumovirus (76, 81). APV has a single-stranded, nonsegmented RNA (83) and has close genetic homology with human, ovine, caprine, and bovine respiratory syncytial virus (83,104). In 1994, a new subgroup designated subtype B appeared and placed within the same serotype (56, 70).

Respiratory illness caused by APV infection in turkeys was first reported in the United States in 1996 in Colorado. The virus was subsequently isolated from a respiratory disease outbreak in Minnesota in 1997, where the disease incidence has continued to increase over the past years (41, 43). In 1999, 37% of the turkey flocks in Minnesota experienced APV outbreaks, resulting in economic losses of $15 million (43).

**HISTORY**

The disease was first observed in 1978 in South Africa (16). Buys and Preez 1979 reported the use of turkey tracheal ring organ culture (TOC) for the isolation of a
ciliostatic agent from young turkeys in South Africa (16). In the winter of 1978-79 a wave of outbreaks of a respiratory disease occurred and spread through a number of turkey farms in different parts in Israel. The disease was characterized by symptoms, which ranged from mild to severe respiratory signs. Low egg production was observed in breeder farms. Morbidity was high, reaching 100%, while mortality fluctuated between 5-90%. Yucaipa virus was isolated and blamed for these outbreaks. The APV, however, was not isolated (59).

In 1981 an epizootic of rhinotracheitis appeared in France. Andral et al. (6, 7, 8) suggested that the initial causative agent for this respiratory disease was Hemorrhagic Enteritis virus. They also suggested that chlamydia psittaci was the main agent responsible for this epizootic. In addition, they isolated many bacteria from sick turkeys including mycoplasma, and Alcaligenes Faecalis. However, the APV was not isolated from these cases.

The first appearance of the disease in the UK was in 1985 as a highly infectious and contagious respiratory disease of turkeys, designated TRT (64). A new hypothesis concerning the etiological agent was introduced. In this hypothesis TRT appeared to be a viral disease because none of the other agents seemed likely to cause such a rapid onset of clinical symptoms with very high morbidity and subsequent drop of egg production (9).

The variable mortality suggested that bacteria might be involved as secondary pathogens. This was confirmed by the observation that experimental infection of turkeys...
with one pathogen or more would increase the severity of the disease. Studies were reported earlier on inoculating APV and *B. avium*, *Pasteurella maltocida* (30), *Mycoplasma gallisepticum* (66), and *E. coli* (62).

Finally, McDougall and Cook in 1986 recovered a virus, which was ciliostatic in both turkey and chicken tracheal ring organ culture. This agent was isolated from cases of TRT (64). Wilding *et al.* pointed out that these ciliostatic agents could be isolated only from those outbreaks, where clinical signs were either about to occur or had been present for less than three days (99). This may explain previous difficulties in isolation of turkey rhinotracheitis virus (TRTV). Based on the size, morphology, and lack of detectable hemagglutination, the authors suggested the virus might be a pneumovirus (99). More work was carried out to confirm this hypothesis by nucleic acid analysis. Subsequently, several research groups confirmed the identity of the virus as a pneumovirus (15, 37, 38, 40, 52, 64, 76, 99, 103).

In 1992 an outbreak of TRT in turkey breeders was reported from North Carolina, USA (85). However, no avian pneumovirus was recovered from this outbreak. In late 1996 an outbreak of rhinotracheitis was observed in turkeys in the State of Colorado, USA and an avian pneumovirus was successfully isolated (86). Soon after, an APV was isolated from Colorado and it was successfully eradicated. In 1997, Goyal *et al.* detected antibodies to APV in Minnesota turkeys. In addition, five virus isolates were obtained and confirmed by reverse transcriptase polymerase chain reaction (RT-PCR). The eradication of the APV from Minnesota was not successful and the APV is still causing respiratory disease outbreaks in turkey flocks in Minnesota (41).
GEOGRAPHICAL DISTRIBUTION

After the first recovery of APV in South Africa from a turkey flock (16), many countries reported the disease in turkeys. In the early 1980s, the disease was reported from France (37), the UK (64), Spain (9), Israel (98) and others European countries. At the same time the disease was reported from Asia, South America, and Central America (24). In the USA, the APV was reported in 1997 in turkeys in Colorado and after eradication from Colorado it appeared in Minnesota and continues to cause respiratory problems in the turkey farms (41, 86). While there are no reports from many countries, the only two countries reported to be free from APV infection are Australia and Canada (24).

ECONOMIC SIGNIFICANCE

Avian pneumovirus infection causes considerable economic losses through poor weight gains, higher food consumption, mortality due to secondary infections, and increased medication and production costs. Morbidity rates in poults are very high, usually approaching 100%, whilst mortality rates, although low in uncomplicated cases, could reach significant levels where secondary infections intervene (60). In breeder turkey flocks, the APV infection causes losses in egg production and sometimes paleness of the shell eggs (50). In chickens, many workers reported a sharp drop of egg production and high incidence of soft and thin-shelled eggs (27, 50, 55).
ETIOLOGY

Historically, the *Paramyxoviridae* family consisted of three genera: paramyxovirus, morbillivirus and pneumovirus. Within the pneumovirus genus there were: human, bovine, ovine, and caprine respiratory Syncytial virus, Mouse pneumovirus, and avian pneumovirus (2). The family *Paramyxoviridae* has been reclassified in 2000 by International Committee on the Taxonomy of Viruses into two subfamilies, the *Paramyxovirinae* and the *Pneumovirinae*. *Paramyxovirinae* contains three genera, Respirovirus, Rubulavirus, and Morbillivirus. *Pneumovirinae* contains the genera pneumovirus and Metapneumovirus. However, the APV differs from mammalian pneumoviruses at the molecular level and has been reclassified in the genus *metapneumovirus* (77, 81).

In 1993, there were several reports about the differences between APV strains that were isolated in different countries or continents. One year later, Juhasz and Easton reported the appearance of two distinct subgroups (56). In this study, the sequences of the attachment (G) protein of five different continental and European isolates and an UK isolate were compared to each other. The results indicated that the UK and French isolates formed one subgroup, which was designated subgroup A, and isolates from the other countries formed the second group, which was designated subgroup B (56). Consequently, Naylor et al. 1997 reported the appearance of subgroup B in Great Britain, which was detected by RT-PCR. They used RT-PCR primers designed to discriminate
between APV subgroups A and B. This study confirmed that outbreaks of respiratory
disease in turkeys in Britain during 1994-1995 were caused by APV infections and that
the virus was subtype B (70).

Researchers showed that the APV isolated from the USA differs from the
European A and B subgroups (29, 82). Cook et al. found that monospecific antiserum to
A or B strains did not neutralize Colorado strains (APV/CO) and vice versa; nor did
monoclonal antibodies, which neutralize subgroup A or B strains, neutralize (APV/CO)
strain. However, it was partially neutralized by a subgroup A hyperimmune serum. A
homologous enzyme-linked immunosorbent assay (ELISA) antigen was essential for the
detection of APV/CO antibodies, since the ELISA in which subgroup A or B strains were
used to detect antibody to APV/CO gave poor results (29).

The predicted sequence of the matrix (M) proteins of European APV type A and
B isolates share 89% identity in their amino acid sequence. However, the predicted
sequence of the M protein of APV/CO is only 78% similar to the APV type A and 77%
similar to the APV type B protein sequence (83). The predicted amino acid sequence of
the APV/CO fusion (F) protein has 72% sequence identity with the F protein of APV type
A and 71% sequence identity with the F protein of the APV subtype B. This compares
with 83% sequence identity between the predicted amino acid sequence of the F protein
of APV subtypes A and B (84). Phylogenetic analysis of the M and F proteins, together
with the serological uniqueness of the USA isolates supports their classification as a new
APV subgroup, designated subtype C (82). The lack of heterogeneity among the USA isolates that were obtained during the past few years suggests that these viruses are a relatively stable population (82).

Furthermore, the new French APV isolates differed from not only the European A and B subtypes (12), but also from the APV/CO subtype C (94). Using RT-PCR, which is based on attachment (G) protein primers, Toquin et al. suggested that at least three antigenically different viruses were present in France in 1985-86 and that APV/CO is different from all European APVs (94).

CHARACTERISTICS OF THE AVIAN PNEUMOVIRUS

The APV are enveloped RNA-viruses, with a non-segmented single stranded genome of negative sense that is approximately 13.3 kilobases in length (84). The genus of metapneumovirus exhibits three main differences when compared to the other viruses of the paramyxoviridae. These differences are in enveloped structure, morphology, and structural and nonstructural polypeptides.

ENVELOPE. The APV was found to be an RNA virus with a lipid-containing envelope with spikes that are derived from the plasma membrane of the host cell in which the virus is grown. Analysis of the TRTV fusion protein suggested that the TRTV is more similar to the viruses which belong to subfamily pneumovirinae than the viruses which belong to subfamily paramyxovirinae (104). The fusion glycoprotein (F) of the APV,
consisting of two subunits protein (F1 and F2), has 38 % amino acid identity with the F-protein of respiratory syncytial virus but only about, 20 % with members of the paramyxoviruses and morbilliviruses (104).

Neither neuraminidase nor hemagglutination activity can be observed in the APV genome, a property characteristic of the pneumovirus. The viral genome apparently does not code for any protein with hemagglutination activity or neuraminidase but it codes for the attachment protein (G), which plays an important role in the adsorption to cellular receptors (84). Therefore, attempts to demonstrate hemagglutination of chicken, human and guinea pig erythrocytes fail thus confirming the identity of a virus as pneumovirus. The APV also fails to agglutinate turkey, duck, goose, rabbit, pig, horse, cow, sheep, and mouse erythrocytes at +4 C, room temperature, or +37 C (103). In addition, the APV envelope exhibits sensitivity to ether and chloroform (22, 23).

**MORPHOLOGY.** The APV has pleomorphic-fringed particles, roughly spherical with sometimes bizarre shapes possessing long filaments (68). A naked strand of the nucleocapsids with helical shape, derived from disrupted particles might be observed. Mean virus particle size is between 100 nm and 200 nm but can range between 70 nm and 600 nm. Filamentous projections are usually present which can be 1000 nm long. In TOC fluid, pleomorphic virions could be observed whilst the virions seen in cell culture fluid were mainly regular spherical particles. Occasionally, round particles with a diameter of 500 nm or more were observed (68).

The helical nucleocapsid diameter of APV is present with a 14 nm diameter and 7 nm pitches. These are smaller than those of the subfamily paramyxovirinae, which have a
diameter of about 18 nm together with smaller pitches (23, 37, 103). The fringe spikes consist of surface projections of approximately 13-15 nm at the base and appeared broader at the distal end. Their width measured 2-3 nm at the base and 3-5 nm at the extremity, while the interspike space is about 2-3 nm (68).

**STRUCTURAL AND NON-STRUCTURAL POLYPEPTIDES.** The APV polypeptides appear to be different from other members of the paramyxoviridae family. Three groups (19, 23, 58) of researchers have reported the polypeptides composition of APV Table 1. Eight structural polypeptides of which two were glycolysated (G = 82-84 KD and F1 = 45-54 KD), and three nonstructural virus-specified proteins, can be detected in sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) techniques. Although, there was not a total agreement between the results, the molecular weights for these polypeptides were between 14 and 200 KD. These polypeptides have been identified as nucleoprotein (N), phosphorprotein (P), matrix protein (M), second matrix protein (M2), surface glycoprotein, attachment (G), fusion protein (F), a small hydrophobic protein (SH), and a viral RNA-dependent RNA polymerase (L).

Three polypeptides distinguish APV from other viruses in the subfamily paramyxovirinae and group it with other subfamily pneumovirinae, such as respiratory syncytial virus (RSV). The first is the nucleocapsid protein designated N (38-43 KD), is much smaller (38-43 KD) than those of the other two genera (56-61 KD). The second is the P protein is much smaller (35-40 KD) than those of the others (70-84 KD). The third is the small polypeptide of 22-19 KD, designated M2, is not found in the other subfamily.
paramyxovirinae (19, 23, 58). The summary of these three studies and a comparison between APV and paramyxovirus, pneumovirus, and morbillivirus polypeptides are listed in Table 1.1.

**Viral nucleic acid**

Pneumoviruses are members of the family paramyxoviridae that contains single stranded and non-segmented negative sense RNA genomes of approximately 15 KD in length (82). Viruses related to APV include human, bovine, ovine, and caprine respiratory syncytial viruses and pneumonia virus of mice (82). Subfamily pneumovirinae generally encodes ten genes, versus six or seven in subfamily paramyxovirinae. The putative genes order of APV (3' N-P-M-F-M2-SH-G-L 5') are unlike its mammalian counterparts (3' NS1-NS2-N-P-M-SH-G-F-M2-L 5'), wherein the SH and G genes are located at the 5' to the M2 gene (82). The extreme 3' and 5' ends of one European APV isolate's genome were determined and established that the NS1 and NS2 genes are absent in these avian viruses (79). This is also distinct from the mammalian pneumovirus and along with a smaller L gene result in APV having a genome of only 13.3kb (80). Consequently, since APV lacks NS1 and NS2 genes, but has an M2 gene with structural characteristics like other pneumoviruses, it has been suggested to designate it as the type virus of a new genus within the metapneumovirus (77, 81).
Physical and chemical properties

Townsend et al. reported the susceptibility of APV isolated from Minnesota turkeys to physical and chemical agents, which could be summarized as follows (97):

**The effect of different solutions.** There was no difference in the virus titer among 4% fetal bovine serum-minimum essential medium (FBS-MEM), 4% FBS-MEM-SPG (sucrose 0.218 M, KH$_2$PO$_4$ 0.0038 M K$_2$HPO$_4$ 0.0072M, potassium glutamate 0.0049 M), or 4% FBS-MEM SPG- dimethyl sulfoxide (DMSO) for a specific temperature and storage for up to 26 weeks.

**The effect of various temperatures.** After 4 weeks at 20°C the virus was no longer viable. After 26 weeks at 4°C the virus was not viable. The APV stored at -20°C and -70°C remained viable for 58 weeks. However, the virus stored at -20°C lost some titer, but no loss occurred at -70°C. The virus was completely inactivated in 6 hours at 50°C. When APV was exposed to 37°C for 6 hours the TCID$_{50}$/ml decreased 1.4 log. In 24 hours at 37°C, the APV had a titer of less than one TCID$_{50}$/ml, and it was completely inactivated after 72 hours.

**The effect of freeze/thaw cycles.** There were no differences noticed in the APV viability after 12 freeze and thaw cycles.

**The effect of pH.** Between pH level of 4.5 and 9.0, there was no significant loss of titer. At pH of 4.1 and 10.0 for one hour, losses of virus activity were 1.0 and 3.5 log TCID$_{50}$/ml, respectively.

**The effect of Chloroform.** The APV in PBS titer was $10^{5.6}$ TCID$_{50}$/ml and the chloroform-treated virus titer was zero.
The effect of drying. One hundred microliters of APV/MN/2a in 1% and 5% FBS-MEM was spread in a spot with a diameter of 1.5 cm on a sterile Petri dish and allowed to dry for one hour, 24 hours, 72 hours, or 7 days in a vertical laminar flow hood. After that, one hundred microliters of sterile water was added to the dry spot, and washed around with a pipette, placed in a tube, and then titrated. The virus in 1% FBS-MEM was titrated at $10^{4.4}$ TCID$_{50}$/ml at the beginning of the experiment. After one hour, 24 hours, 72 hours, and 7 days, the titrations were $10^{3.5}$, $10^{3.0}$, $10^{2.5}$, and $10^{2.5}$ TCID$_{50}$/ml, respectively. The virus in 5% FBS-MEM was titrated at $10^{4.6}$ TCID$_{50}$/ml at the beginning of the experiment. After 24 hours, 72 hours, and 7 days, the titrations were $10^{2.7}$, $10^{3.0}$, and $<1.0$ log TCID$_{50}$/ml, respectively.

The effect of disinfectants. Bleach, Ethyl alcohol, Quaternary ammonia, Iodophor, phenol derivative, and Biguanide (Chlorhexedine diacetate solution) inactivated the APV in various concentrations after ten minutes exposed at room temperature.

Laboratory host systems

The APV grows in extremely low infectivity titers in primary isolation systems such as embryonated chicken and turkey eggs or tracheal organ cultures (2). In addition, the APV can grow in a variety of primary cell culture systems such as tracheal organ culture (TOC) (15, 51, 64, 03), chicken embryo fibroblasts (CEF) (42) and chicken kidney cell (CK) (100) and continuous cell culture system such as Vero cells (15,100).
BSC-1 (57), and QT (21, 41). The infected cell cultures result in cytopathic effect (CPE) characterized by cell rounding and syncytium formation that starts around day three post inoculation (PI) (15, 60).

**Natural and Experimental Hosts.**

Turkeys and chickens, of any age, are known to be natural hosts of APV. Additionally, Picault *et al.* found APV antibodies in a flock of guinea fowl and were able to produce a rhinotracheitis-like disease and reisolate the virus from guinea fowl, which were inoculated with a virus isolated from TRT-affected turkeys (76). In experimental infection with APV, Gough *et al.* studied susceptibility, clinical signs and virus isolation from turkeys, chickens, guinea fowl, Pigeons, pheasants, geese, and ducks (39). The clinical signs of APV were observed in turkeys, chickens, and pheasants only. The virus was isolated from turkeys and chickens but not from pheasants. At the same time, antibodies to APV were detected in all the bird species using ELISA and virus neutralization (VN) test (39). Nonetheless, APV of turkey origin was isolated from the nasal turbinates of ducks using Vero cells and viral RNA was detected using RT-PCR up to 21 days PI (88). These ducks had a serologic response to APV but no clinical signs of the disease were noticed in these ducks (88). In breeder ducks, the APV was isolated in Vero cells from 42-week-old Muscovy ducks, which were naturally infected. An antibody response to APV was detected and respiratory signs and a drop in egg
production were noticed in these ducks (95). Antibodies to APV have been reported in ostriches in Zimbabwe (17) and in gulls in the Baltic Sea (48), but the APV has not been isolated from these species.

Pathogenicity

Experimental infection with APV often results in milder signs of the disease than those seen in the field (91, 92). The severity of the clinical disease in turkeys and chickens are affected by management such as ventilation, hygiene, and stocking density (92). In the field, other pathogens may play a great role and lead to more severe disease. The two main affected species by the virus are the turkey and chicken. The APV causes turkey rhinotracheitis (TRT) and swollen head syndrome (SHS) in chicken (15, 37, 38, 40, 52, 64, 76, 99, 103).

Turkey rhinotracheitis (TRT). The TRT is an acute, highly contagious disease of upper respiratory tracts of turkeys (24). Onset of the signs of the disease is usually rapid and the infection may spread through a house within 24 hours with 100 % morbidity and variable mortality (92). The disease is characterized initially by clear nasal discharge, which may become thicker and mucopurulent as a result of secondary bacterial infections. Ocular discharge increases from day five after infection, sometimes becoming frothy but then clear. Sneezing, tracheal rales, depression, and swollen infraorbital sinuses are noticed. Coughing, head shaking, and depression are commonly observed (15, 50, 64, 103). In addition, a drop in feed consumption during the acute stage is noticed.
(15). In uncomplicated infection, recovery is rapid and birds may appear normal and recover completely within 10 - 14 days post infection (15). In some cases, mortality is the major problem and can be as high as 30% (9) or may be over 50% (92).

In breeding flocks, clinical signs are not as severe as in the young pouls. Nevertheless, the APV infection results in poor egg production and eggshell quality. The drop in egg production could be as low as 40% of expected level (64). Moreover, Poor feed conversion is noticed in the breeder flock. Recovery of infected laying flocks can take up to three weeks and increased incidence of thin-shelled eggs, and misshapen eggs are seen during this period (9, 92). Egg peritonitis was also reported (55).

**Swollen head syndrome (SHS) in chickens.** The SHS was reported in chickens at the same time that APV infection was first seen in turkeys. The role of APV infection in chickens is less clear than that in turkeys. The APV can infect chickens without necessarily being responsible for the clinical disease and it induces an antibody response (23, 100). Moreover, the APV has been isolated from naturally infected chickens of all ages (14, 39, 46, 54, 76, 92) and chickens have been experimentally infected with APV (18, 33, 51, 61).

In addition, the APV is not the only agent associated with SHS. Many pathogens were isolated from SHS cases. These pathogens include infectious bronchitis virus (IBV) (65) in South Africa and IBV and Escherichia coli in California, USA (35). The SHS was reproduced in experimentally infected SPF chickens with E. coli alone after inoculation into submucosal tissue of the nasal membrane or subcutaneous tissue of eyelids with four different strains of E. coli (66).
The disease associated with SHS usually lasts for 2-3 weeks. It is characterized by apathy, lack of locomotion, sneezing, coughing, head shaking, head scratching, periorbital edema around the eyes which is often unilateral but then may extend over the whole skull. In addition to the above signs, cerebral disorientation, torticollis and opisthotonus are observed. Other reported signs include birds resting their head on their backs, ear and eye discharge, and foul smelling diarrhea (71, 73). The mortality rarely exceeds 2%, while morbidity could reach 10 % (27). In laying broilers, reduction in egg production of about 2-3% of the normal level was observed (68).

Clinical sings following experimental infection of chickens with APV have been described as a very mild respiratory disease (14, 51).

**Virus replication**

*In vivo replication*. The APV naturally infects the upper respiratory tracts of turkeys and chickens. The APV replication is very much limited to the upper respiratory tract (24). In addition, the APV was detected in the following parts of the reproductive tracts, middle magnum, vagina, and uterus in experimentally inoculated turkeys and chickens (27, 32, 33, 55). In chickens, the virus was isolated from respiratory tracts and blood (89).
Immunity

**Humoral immune response.** Serological response to APV was studied and detected using ELISA (20, 42), virus neutralization (VN) test (25), and indirect immunofluorescence (9, 11), however, the class of the immunoglobulin was not determined in these studies.

The antibody to APV was reported to be detected as early as five days after the clinical sings appeared using VN test in CEF cells, then the antibody titer stared declining by days 13 after the appearance of the clinical sings (11). It has been found that after experimental infection of mature turkey hens with APV, the antibody titer rose to over 12 log₂ and were still at the same levels at day 89 PI using ELISA and VN test (55).

**Cellular immunity.** No antibodies were detected in vaccinated turkey poults that were chemically bursectomised using cyclophosphamide at a dose which had been shown to cause only B cell immunosuppression in turkeys (53). Yet, there was no increase in the clinical sings of the disease when poults were challenged with virulent strain of APV, implying that the cellular protection provides the main resistance to the APV infection (53).

Diagnosis

**Clinically.** The diagnosis of APV infection in turkeys based on clinical sings is believed to be possible (91, 92). However, in chickens diagnosis based on clinical sings is
not recommended. Because of nonspecific clinical signs in the APV infection, the necessity of using the laboratory methods to isolate or detect the virus or antibody is needed to confirm the diagnosis (50).

**Virus isolation.** It is very rare to attempt to isolate the APV from field cases in order to diagnose the APV infection because of the fastidious nature of the APV and the very short period of virus persistence after infection (3). The APV isolation should be attempted at the very first signs of clinical disease. If the obvious clinical signs are seen, the best advice is to select birds from the same house which are not yet showing clinical signs (24, 99). The nasal secretions or tissue scraped from the sinuses of affected birds are the best samples for isolation of the APV (50). Nonetheless, the APV has been successfully isolated from naturally infected birds such as turkeys, chickens, and ducks by many workers (88, 95).

The most sensitive and reliable system to isolate the APV from field cases is using the chicken or turkey embryo tracheal organ cultures (TOC) (64, 103) and chicken embryos inoculated via the yolk sac at 6 days of incubation (15, 32).

Once the virus has been isolated, it can be readily adapted to grow in cell culture. Different kinds of cell culture have been used to cultivate the APV. These cells are chick embryo fibroblasts (CEF) (42), and chick embryo kidney (CEK) (101), and a continuous cell line such as Vero (15, 101) cells and QT-35 (21, 41).

**Virus detection.** Reverse transcriptase-polymerase chain reaction (RT-PCR) has been used to diagnose APV infection (4, 5, 57, 63, 74, 75, 87). This technique is much
faster than virus isolation. However, the RT-PCR detects the viral RNA, therefore, positive RT-PCR results are less significant in terms of detecting an active infection in the field (24).

Direct or indirect immunofluorescence have been used on sections of trachea or turbinates in experimental infection, but not from field materials (51, 100). The use of monoclonal antibodies specific for the subtype of APV would further enhance immunofluorescence value (50). A good correlation was found between virus isolation and immunofluorescence, which imply that immunofluorescence is a reliable test to detect APV (100).

**Serology.** Serological tests such as ELISA and VN test are the most commonly used methods to diagnose the APV infection. The VN test is performed in a variety of systems, including TOC, CEF, or Vero cells. However, ELISA and VN test show similar sensitivity (11) but the ELISA is the most commonly used assay (20, 36, 42, 72, 96).

Currently, there are at least three different types of ELISA kits available for APV infection diagnosis. In the first type, plates coated with crude or purified virus were used to detect antibodies to APV in serum samples (20, 36, 42, 72, 96).

The second type is a blocking ELISA, where the test serum competes with a mouse monoclonal antibody against an epitope on the virus coating the plates. In this case, the test serum is applied then after rinsing, the mouse monoclonal antibody is applied then the anti-mouse conjugate is added. If the color develops, this mean the serum samples are negative. The advantage of this method is that it can be used with sera of any species of birds (50).
In the third type, APV antibodies are detected using ELISA, which is based on recombinant viral proteins. These proteins are generated in a vector such as *E. coli*. The APV matrix (M) protein is used to coat the ELISA plates to detect antibodies to APV (44). A Nucleocapsid (N) protein-based sandwich ELISA was developed to detect antibodies to APV (45).

**Differential diagnosis**

Various bacterial and viral etiologies cause clinical symptoms similar to those of the TRT and SHS in turkeys and chicken, respectively. In turkeys, the symptoms associated with APV infection have a close similarity with infection caused by *B. avium* (avian Bordetellosis). Whilst, the APV causes disease in all ages of turkeys, the *B. avium* causes disease mostly in young turkeys. In young turkeys, culturing the *B. avium* is important to determine the causative agent of the disease (90).

Diseases caused by *E. coli* can be differentiated during post mortem such as airsacculitis, perihepatitis and pericarditis. In addition, other bacterial pathogens such as *Mycoplasma gallisepticum, Mycoplasma meleagridis,* and *Chlamydia Psittaci* can produce similar disease but less contagious and culturing the affected birds would show the causative bacteria (60).

Moreover, there are several viral pathogens, which have similar clinical sings to APV infection. The most common viral pathogens are Orthomyxoviruses (avian
influenza), paramyxoviruses type-1 and type-2 (PMV-1, PMV-2) and avian Adenoviruses (60). Rotaviruses, reoviruses, enteroviruses-like particles, and paramyxovirus type-3 (PMV-3) are less common (6, 7, 8, 60).

In chicken, SHS can be confused with bacterial infections such as infectious Coryza caused by *Haemophilus paragallinarum* which could be distinguished by bacteriological examination (13). Some of the features of SHS encountered in broiler breeder chickens are similar to Fowl Cholera caused by *pasteurella multocida* (73).

Viral infections in chickens can cause problems in diagnosis of APV due to the similarity of clinical signs to APV infection. These diseases include IBV (35) and infectious laryngotracheitis (ILT) (71).

In addition, the pathogens that affect the immune system have to be considered. These are: Adenovirus in turkeys (55), infectious barsal disease (IBDV) (60), and Aflatoxicosis in chickens (49, 60).

**Prevention and control**

There is no effective drug to treat the APV infection. However, antibiotics that were used to control secondary bacterial infections associated with APV infection show little or no success (47, 91). Meanwhile, the main aim of controlling APV infection in turkeys and chickens is vaccination.

**Vaccination.** Because maternally derived antibodies do not protect against infection, nor do they prevent the successful application of vaccines (26, 31, 69), several
types of vaccines have been developed to protect the susceptible birds from APV infection. Live attenuated vaccines are widely used in turkey and chicken flocks. Cook et al. 1995 implied that the vaccine would give excellent protection in turkeys and chickens flocks if the vaccine is administrated carefully and correctly applied to ensure that all birds receive an adequate dose of the vaccine at the same time (28).

A number of attenuated vaccines have been developed and have met with variable degree of success in the field. All attenuated vaccines have been produced by repeated passage of virulent virus in a laboratory culture system to achieve different degrees of attenuation (50). Live attenuated vaccines are used in growing turkeys and broiler chickens. While the inactivated vaccines are used in the layers and breeders before the onset of lay (24). Lifelong protection of broilers should be possible with one vaccination of attenuated vaccine. For the mature birds, the administration of inactivated vaccine should come after at least one vaccination of live attenuated vaccine before the onset of lay to give complete protection (24).

Gulati et al. used a vaccine attenuated in CEF (seven passages) and Vero cells (34 passages) to vaccinate 10 flocks of 2-4-week-old turkey poults, each 20,000-50,000 bird. For the ten flocks, two birds per 1000 birds were vaccinated via nasal and ocular routes. In another flock all the birds were vaccinated in drinking water. For the ten flocks, very little symptoms were appeared and within three weeks, all birds were seropositive for APV antibodies using ELISA. The flock that was vaccinated using the drinking water became seropositive within two weeks. All 11 flocks remained seropositive until 10
weeks post vaccination. The medication coast, total condemnation, and mortality rates were lower in the vaccinated flocks than in the unvaccinated flocks on the same farm from the previous year (43).

Moreover, a recombinant fowl pox virus vaccine was produced which expressed the fusion protein (F) of APV (78). Turkey poult's were vaccinated twice at an interval of two weeks, intramuscularly and by wing web on each occasion. Two weeks after the second vaccination the poult's were challenged superconjunctivally and intranasally with virulent APV. A partially protective immune response was achieved. Antibodies to APV were detected using ELISA and virus neutralization test in turkeys after the second vaccination. The vaccine was able to reduce the clinical sings and the amount of virus that been recovered from the nostril and tracheas (78).

In general, there is excellent cross protection between subgroups A and B (28, 36, 96). Subgroups A and B vaccines protect turkeys against challenge with Colorado strain. However, while Colorado strain vaccine protected turkeys, and to some extent chickens, against subgroup A strains, protection against a subgroup B challenge was less good in both species (29). At the same time, subgroup A and B vaccines protect against the non-A and non-B strains reported from France (24).
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virus has greater identity with that of human respiratory syncytial virus, a
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A= Data from Cavanagh and Barrett, 1988.  
B= Data from Collins and Gough, 1988.  
C= Data from Ling and Pringle, 1988.  
¹ Data from Alexander, D. J. 1990.

Table 1.1: Comparison of the virion proteins of the APV and three genera of the paramyxoviridae.


II. Bordetella Avium

*Bordetella avium* is the causative agent of turkey Coryza (TC) or Bordetellosis, which is a highly contagious upper respiratory disease that affects young turkey poultts. *B. avium* is a gram-negative, and non-glucose-fermenting bacillus. The bacteria can colonize the ciliated epithelium, which results in distortion of the mucus membranes of the upper respiratory tract. The clinical signs of *B. avium* are characterized by onset of sneezing, rhinitis, conjunctivitis, and loss of the ciliated epithelial cells (22).

The disease is widely known as turkey Coryza. Other synonyms that have been used are alcaligenes rhinotracheitis (ART), adenovirus-associated respiratory disease, acute respiratory disease syndrome, *Bordetella avium* rhinotracheitis (BART), and turkey rhinotracheitis (22).

**HISTORY**

The disease was first reported by Filion et al from Canada in 1967 (4). Nearly a decade later, a similar syndrome was recognized in Germany and the United State, where the causative agent was identified as *Bordetella bronchiseptica*-like (6) and *Alcaligenes faecalis* (21) respectively. The name *Bordetella avium* was eventually proposed and generally accepted (12).
In the United States, investigators initially focused on viruses such as adenoviruses, which were frequently associated with the disease. Several attempts to reproduce the disease experimentally with either adenoviruses or infectious bursal disease virus (IBDV) failed. At the same time, concurrent inoculation of *B. avium* and IBDV failed to exacerbate experimental Bordetellosis. Other infectious agents, including mycoplasma, paramyxoviruses, and chlamydia have been considered in the etiology of turkey rhinotracheitis (22).

**MORPHOLOGY AND GROWTH**

The *B. avium* is gram-negative, non-glucose-fermenting, motile, capsulated, fimbriated and strictly aerobic bacillus. The generation time for *B. avium* is about 35-40 minutes at 35 C (1, 22). The *B. avium* grows on MacConkey, Bordet-gengou, veal infusion, trypticase soy blood agar, and brain heart infusion (BHI) broth but not on minimal essential medium. Filamentous forms have been observed following growth of *B. avium* in broth media high in nutrients (22).

Three different types of colonies are usually observed. Type one colony is produced by most of the *B. avium* strains, which produce small, compact, translucent, pearl-like colonies with entire edges and glistening surface (12). They are 0.2 to 1.0 mm in diameter after 24 hours of incubation and 1.0 to 2.0 mm in diameter after 48 hours of incubation (12). Type two colonies are generally larger than type one colonies. Type three colonies are characterized by a serrated irregular edge, smooth surface, and larger size than type two colonies (22).
VIRULENCE FACTORS

Major virulence factors of the *B. avium* can be divided into those involved in adhesion, local mucosal injury, or systemic effects. The surface structures or molecules of *B. avium* responsible for adhesion are fimbriae (pili) and the hemagglutinin (9, 13). As with other *Bordetella* species, there are more than one surface molecule is likely to be responsible for adhesion to cilia (22).

The mucosal injury is attributed to different toxins that have cytotoxic and ciliostatic effect on mucosal membranes. *B. avium* produces a dermonecrotic toxin which is capable of killing young turkeys and mice. This toxin appears not to be responsible for ciliostasis or local epithelial damage (15, 16). Another toxin is the tracheal cytotoxin (TCT), which was shown to cause damage to ciliated epithelial cells leading to loss of epithelium and poor clearance of mucus (5). A heat-stable toxin was identified and was shown to be capable of causing diarrhea and death in mice inoculated intraperitoneally. However, there is no evidence that the toxin produces adverse effects in poultry (3). A toxin called osteotoxin was identified and shown to have lethal effect on different cell lines. This toxin might be responsible for the cartilage lesions that lead to tracheal softening and collapse (22).

Several systemic pathophysiologic effects have been attributed to *B. avium* infection. These effects include elevation of serum corticosterone, enhanced leukocyte migration, altered electrocardiograms, reduced body temperature, reduced levels of monoamines in brain and lymphoid tissues, reduced levels of liver tryptophan2, 3-
dioxygenase, and reduced thyroid hormones in conjunction with fasting (22). In addition, reduction in bursa size and defective immune functions were observed in B. avium affected poults (19, 20).

**PATHOGENESIS**

Bordetellosis is a highly contagious disease, which is transmitted to susceptible poults through close contact with infected poults or through exposure to contaminated litter or water (18). Naturally occurring infection with B. avium is recognized in turkeys 2 to 6-weeks-old (6) although, older birds and breeder flocks may develop clinical disease (11). Turkeys are the natural host of B. avium. Moreover, B. avium was isolated from chickens and other species. Strains of B. avium, which were isolated from avian species, are still pathogenic for day-old turkeys (12, 22).

The symptoms associated with B. avium include sneezing, submaxillary edema, mouth breathing, and nasal discharge, which may become brownish (22). Other changes in birds behavior include activity reduction, huddling, decreased feed and water consumption, and poor weight gain, which leads to growth stunt, are also observed (1). In turkeys 2 to 6-weeks of age, morbidity could reach 80 to 100%, whereas the mortality rate is less than 10% but could reach more than 40% if Escherichia coli are present (17). Infection of a breeder flock resulted in 20% morbidity and no mortality (11).
TREATMENT AND CONTROL

Treatment of an infected flock with antibiotics such as tetracycline-HCl or oxytetracycline-HCl administrated by drinking water or aerosol caused a reduction in mortality and improved the clinical signs (11, 23). Vaccines that are available commercially for prevention of Bordetellosis in turkeys include a live temperature-sensitive (ts) mutant of \textit{B. avium} and a whole cell bacterin (22). These vaccines are administrated by spray, drinking water, or eye drop have equal effectiveness in reducing severity of the disease but do not prevent the infection (8, 22). Maternal immunity may be useful for prevention of Bordetellosis in progeny poult (2, 7, 14). Vaccination of breeder hens with either heat-killed (7) or formalin-killed (2) adjuvanted bacterins have delayed the onset and severity of clinical disease in experimentally challenged poult.

Jackwood and Saif (10) studied whether poult infected with nonpathogenic \textit{B. avium}-like bacteria would develop immunity to \textit{B. avium}. The \textit{B. avium}-like bacteria failed to persist for a significant period in the respiratory tracts and failed to induce either a serologic response or protection to \textit{B. avium} challenge.
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III. Avian Paramyxoviruses

Avian viruses that belong to the family paramyxoviridae are the cause of respiratory and reproductive diseases in several avian species. Nine serotypes of avian paramyxoviruses PMV-1 to PMV-9 have been recognized (3). Of these, Newcastle disease virus (NDV) (PMV-1) remains one of the most important pathogen of poultry. In addition, PMV-2, PMV-3, and PMV-7 can be responsible for respiratory and reproductive diseases in turkeys (2, 21).

The family paramyxoviridae has been reclassified into two subfamilies, the paramyxovirinae and pneumovirinae. The subfamily pneumovirinae contains two genera, pneumovirus and the metapneumovirus. The subfamily paramyxovirinae contains three genera, Respirovirus, Rubulavirus, and Morbillivirus. The avian paramyxoviruses belong to the genus Rubulavirus (20). The new classification is based on morphologic criteria, the organization of the genome, the biologic activities of the proteins, and the sequence relationship of the encoded proteins (20).

It has been reported that there are some cross relationships between avian paramyxoviruses serotypes (2). Lipkind et al (18) found minor cross relationships between PMV-1, 3, 4, 7, 8, and 9 and between PMV-2 and 6. However, the relationship between PMV-1 and PMV-3 appears to be closer and more important than the others (2, 4, 23). In addition, chickens infected with PMV-3 viruses may be protected against
challenge with virulent NDV strain (6). Hemagglutination inhibition (HI) antibodies to PMV-3 viruses may be detected in turkeys and chickens showing high vaccine-induced titers to NDV, and ND-vaccinated birds infected with PMV-3 viruses show a rise in HI titer to both viruses (5, 10). More recently, a monoclonal antibody against the pigeon variant PMV-1 inhibited PMV-3 viruses isolated from exotic birds in HI tests (12).

HISTORY

NEWCASTLE DISEASE VIRUS. The first outbreak of the Newcastle disease virus (NDV) occurred in 1926, in Java, Indonesia and in Newcastle, England (13, 15). In addition, there were reports of outbreaks in central Europe with symptoms similar to those caused by NDV (2). In the United States, in the 1930s there was an outbreak of respiratory and nervous signs which was described and named pneumoencephalitis (9). It was shown that the cause of this outbreak is a virus indistinguishable from NDV in serologic tests (2, 9). A few years later, the NDV was isolated from different parts of the world (2).

AVIAN PARAMYXOVIRUS TYPE 2. In 1956, in Yucaipa, California, the PMV-2 was isolated from chickens (8). Serological surveys of poultry in the United States showed that the PMV-2 was more frequently infecting turkeys than chickens (11). The PMV-2 was isolated mostly from imported caged birds, primarily from passerines and paittacines (22). Several reports suggested that viruses belong to the same PMV-2 serotype are present around the world (3).
AVIAN PARAMYXOVIRUS TYPE 3. The isolation of PMV-3 was made in turkeys in Ontario in 1967 and Wisconsin in 1968 (24). Antibodies to PMV-3 were consequently detected in several European countries (2).

AVIAN PARAMYXOVIRUS TYPE 7. The PMV-7 was first isolated from a hunterkiller dove in Tennessee in 1975 (7), but it has not been associated with disease in avian species. Saif et al (21) isolated PMV-7 in 1997 from commercial breeder turkey flock that had respiratory and reproductive problems.

ETIOLOGY

CLASSIFICATION OF AVIAN PARAMYXOVIRUSES. Members of paramyxoviridae family are RNA viruses showing helical capsid symmetry. They contain a nonsegmented, single stranded genome of negative polarity. Their envelope formed from modified cell membrane as the virus is budded from the cell surface after capsid assembly in the cytoplasm (19).

MORPHOLOGY. Negative contrast electron microscopy reveals pleomorphic virus particles, but usually spherical in shape, although filamentous and other forms are common. They are about 150-500 nm in diameter, the filamentous forms of about 100 nm across and variable length are often seen. Virions consist of lipid envelope, derived from host cell membrane, surrounding a nucleocapsid. The surface of the virus particle is covered with spike-like projections about 8-12 nm in length and spaced 7-10 nm apart (2, 20).
HEMAGGLUTINATION and NEURAMINIDASE ACTIVITIES

The hemagglutinin-neuraminidase (HN) protein in the virus envelope has the ability to bind to receptors on the surface of red blood cells (RBCs). Chicken RBCs are usually used in the hemagglutination (HA) test or HI test for these viruses. In addition, the NDV agglutinates all amphibian, reptilian and avian RBCs (16). Moreover, the other avian paramyxoviruses have the ability to agglutinate a wide range of RBCs. Cells other than RBCs could be agglutinated by Paramyxoviruses if they have the correct receptors (2).

All members of the subfamily paramyxovirinae possess the enzyme neuraminidase (mucopolysaccharide N-acetyleneuraminyl hydrolase EC 3.2.1.18) which is also part of the HN molecule. The exact function of the neuraminidase in virus replication is unknown, but it seems likely that it removes virus receptors from the host cell and this prevents the attachment of newly budded virus particles (2). In the HA and HI tests, possessing the enzyme neuraminidase may be important for the gradual elution of agglutinated RBCs (1).

PATHOGENICITY

NEWCASTLE DISEASE. Natural and experimental infection with NDV has been demonstrated in at least 236 species from 27 of the 50 orders of birds, although there were variations in severity of the clinical signs even with different species of the same genus (14). Chickens of any age are the natural host of NDV and the most frequently used laboratory animal (2). The virus is transmitted from bird to bird by small
droplets containing virus. In addition, the virus is excreted in the feces and ingestion of the infectious feces by susceptible birds usually results in infection. Vertical transmission of NDV from hen to chicks is controversial. However, embryos could be infected by contaminating the outside of the egg then the virus penetrates the shell after laying. Cracked or broken infected eggs may serve as a source of virus for newly hatched chicks. Chicks may hatch from eggs that have been infected vertically from hens, which were vaccinated or infected with lentogenic viruses (2).

The pathogenicity of NDV in chickens is determined by the strain of virus causing the infection. For example, the clinical signs of the VVNDV or the velogenic strains include listlessness, and then death. The neurotropic velogenic form of the disease start with sudden respiratory disease then neurologic signs and sharp decrease of egg production. Mortality frequently reaches 100% in flocks of fully susceptible chickens (2).

The mesogenic strains of NDV cause respiratory disease and drop in egg production. Nervous symptoms may occur but are not common. Mortality is affected by exacerbating conditions and usually low. The Lentogenic viruses cause disease in only susceptible young birds and do not usually cause disease in adult birds (2).

**AVIAN PARAMYXOVIRUS TYPE 2.** These viruses cause mild or unapparent symptoms in chickens and turkeys, although, it is more severe in turkeys than chickens (17). Nonetheless, severe respiratory symptoms, elevated mortality, and low egg production was reported (2)

**AVIAN PARAMYXOVIRUS TYPE 3.** Turkeys are the host of these viruses that mainly cause drop in egg production with mild respiratory disease (2).
AVIAN PARAMYXOVIRUS TYPE 7. The clinical signs were primarily respiratory with elevated mortality. When laying hens are infected, the egg production may drop significantly (21).

DIAGNOSIS

There are several serological tests that could be used to diagnose the paramyxoviruses infection in birds. These tests include, agar gel precipitin, single radial immunodiffusion, single radial hemolysis, virus neutralization (VN) test in chick embryos, HA and HI tests, and enzyme linked immunosorbent assays (ELISA) (2). Good correlation has been reported between ELISA and the HI test, which are the most commonly used serologic tests to diagnose these infections. Virus isolation is important to diagnose the infection as well as characterization of the infecting strain (2).

PREVENTION AND CONTROL

Controlling the paramyxoviruses need to be applied at different levels such as international, national, regional and farm level. For the international level, poultry and poultry products are usually organized and managed by multinational companies. For better control of these diseases, all multinational companies and countries must report any outbreaks within their borders to international agencies to prevent the disease from spreading to other countries. At the national level, this will be directed to prevent the introduction of the virus and preventing it from spreading within the country. On the farm, the level of biosecurity and management that are practiced at the farm are important
to prevent the introduction of the virus into the farm, and preventing these viruses from spreading to other farms if any of these viruses are present in the farm (2).

**VACCINATION.** Vaccination is the most important tool used to prevent diseases caused by paramyxoviruses. Paramyxoviruses other than NDV cause diseases with low mortality but egg production problems especially that associated with PMV-3 infection in turkeys are significant. For PMV-3, oil-emulsion vaccines are available and prevent the serious egg production losses for turkey breeder farms (2).

There are several kinds of vaccines used to induce immunity against NDV to prevent infection and replication of the virus in susceptible birds. Live vaccines could be grouped into two groups; mesogenic and lentogenic viruses derived vaccines. The mesogenic vaccines must be used as secondary vaccines due to their greater virulence. The live vaccines could be used to vaccinate bird individually or by mass application of the vaccine by spray or drinking water. Inactivated vaccines are produced by treating the infective allantoic fluid with β-propiolactone or formalin to kill the virus then mixed with a carrier adjuvant. These vaccines are administrated by injection either intramuscularly or subcutaneously (2).
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PATHOGENICITY, TRANSMISSIBILITY AND TISSUE DISTRIBUTION OF
AVIAN PNEUMOVIRUS IN TURKEY POULTS

SUMMARY

The pathogenicity, transmissibility, tissue distribution, and persistence of avian pneumovirus (APV) in turkey poults were investigated in three experiments. In the first experiment, two-week-old commercial turkey poults were inoculated oculonasally with APV alone or in combination with *Bordetella avium*. In the dually infected group, there were more severe clinical signs, the virus persisted longer, the bacteria invaded more respiratory tissues, and the birds had higher antibody titer than the group exposed to APV or *B. avium* alone.

In the second experiment, the distribution of APV in different tissues in experimentally inoculated two-week-old commercial turkey poults was studied. Only
samples from sinuses, tracheas, and lungs were positive for APV by both reverse transcriptase-polymerase chain reaction (RT-PCR) and virus isolation (VI).

In the third experiment, the ability of APV to spread among birds was studied in one-week-old commercial turkey poults inoculated oculonasally. The virus was isolated and the viral RNA was detected in the inoculated and direct contact birds. The virus was not isolated, viral RNA was not detected, and no antibodies were detected in the indirect contact birds. These birds were placed in different cages in the same room where the airflow was directed from the infected towards the uninfected indirect contact group.

INTRODUCTION

Turkey rhinotracheitis (TRT) is an acute infectious respiratory disease of turkeys of all ages (4), which is more severe in young poults than in older birds (2). In early 1997, Senne et al reported the first isolation of avian pneumovirus (APV) in Colorado from a turkey flock with respiratory symptoms (27). The etiologic agent APV (19, 27), belongs to the genus metapneumovirus, subfamily pneumovirinae, within the paramyxoviridae family (25). Infected young turkeys develop respiratory signs consisting of nasal exudates, ocular discharge, and swelling of the infraorbital sinuses (21). In addition, there is a sharp reduction in egg production and misshaped eggs are produced by breeder flocks (21).

APV causes more severe disease in the field than under experimental conditions (31). In addition to the effect of management practices, secondary pathogens play an important role in the disease process. The interactions of a variety of pathogens with
APV have been reported. These included; *Bordetella avium* (8), *pasteurella*-like organism (8), *Mycoplasma gallisepticum* (20), *Ornithobacterium rhinotracheale* (10), *Escherichia coli* (18), and *Mycoplasma imitans* (11). It was shown that co-infection with each of these pathogens could exacerbate and prolong the clinical signs of the disease caused by APV.

*B. avium* is a pathogen of turkeys that infects the upper respiratory tract and causes a respiratory disease designated Bordetellosis or turkey Coryza (30). The clinical signs of TRT can be confused with other respiratory diseases such as that caused by *B. avium* in turkeys (1).

The APV was isolated from respiratory tracts of commercial turkey poult s (8) and detected in oviducts of 30-weeks-old turkey hens (17). In addition, the virus was detected from the respiratory tract when chickens were inoculated oculonasally and from the respiratory tract and the intestines when inoculated orally (28).

In the United States, APV has been endemic in Minnesota since 1997 but did not spread to many States, leading to the question about the transmissibility of the virus. Cook *et al* 1991 reported that the APV did not transmit from infected to uninfected poult s placed in separate cages but in the same room (8) but Giraud *et al* 1986 indicated that the APV could spread to SPF poult s placed in different cages (12).

In this study, an experimental APV infection model was used to study the pathogenicity, persistence, tissue distribution, and transmissibility of the virus. These studies were designed to extend our knowledge of the pathogenesis and epidemiology of the infection.
Materials and Methods:

**Virus.** The avian pneumovirus (APV/ Minnesota / turkey /2a/97) was obtained from D. Senne at the National Veterinary Service Laboratories (NVSL), Ames, Iowa. The virus was passaged in Vero cells twelve times at the NVSL and three times in our laboratory. **Virus Isolation.** Sections from different organs were collected and mixed 1:10 (v/v) in serum-free minimum essential medium (MEM). The samples were homogenized and centrifuged at 3000g for 15 minutes. The supernatants were filtered through 0.45 μm syringe filter (Nalgene) and stored at −70 C until used. Virus isolation was attempted in nine to eleven day old chicken embryos inoculated via the allantoic route using 0.2 ml of the homogenized liquid for each egg. The inoculated eggs were incubated at 37 C for four days and the allantoic fluids were collected and stored at −70 C until used.

**Bacteria.** The media and procedures used to isolate and grow *B. avium* are described earlier (26). Isolate B that was isolated in our laboratory was used for challenge, antigen production, and serology, enzyme linked immunosorbent assays (ELISA) and microagglutination test (MAT), as described earlier (5, 14, 15).

**Animals.** Commercial turkey poults from the same genetic background were used in these studies. They were housed in cages inside high-security isolation rooms provided with HEPA-filtered intake and exhaust air. Upon arrival to our premises five poults were randomly chosen and tested for antibodies to APV using ELISA and virus neutralization (VN) test and for antibodies to *B. avium* using ELISA and MAT.
Histopathology. Sections from sinuses were collected at necropsy, placed in Prefer fixative® (Nalge Co, Rochester, NY), processed through graded alcohols, and embedded in paraffin. Four-micron thick sections were cut and stained by Hematoxylin and Eosin (H&E).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). The viral RNA was extracted with Trizol ®LS Reagent (GibcoBRL) using the manufacturer's protocol. Two hundred fifty µl of the allantoic fluid or the homogenized tissue samples were used for viral RNA extraction. The RNA pellet was suspended in 20 µl of diethylpyrocarbonate (DEPC) treated water and stored at -20 C until used.

The primers were designed from the M protein sequence and synthesized at Integrated DNA Technologies Inc, Coralville, Iowa, using published sequence (3). The RT-PCR was performed with the Titan™ One Tube RT-PCR System (Boehringer Mannheim) following the manufacturer’s recommendations. Ten µl of the viral RNA was used for reverse transcription (cDNA synthesis). The RNA and primers mixture were incubated at 70 C for 10 minutes then chilled on ice. The reverse transcriptase (RT) reaction was started at 45 C for one hour followed by a denaturation step at 94 C for three minutes. Forty cycles of denaturation, annealing, and extension at 94 C for 30 second, 50 C for one minute, and 72 C for one minute, respectively, were performed. The final amplification was performed at 72 C for ten minutes. The resulting products were subjected to electrophoresis in 1.2% agarose gel (SeaKem LE Agarose Rockland, Maine). The gel was stained with ethidium bromide and product bands were visualized with an ultraviolet transilluminator.
ANTIGEN PREPARATION FOR ELISA. The preparation of APV antigen for ELISA was similar to that described (7) with some modifications. Briefly, the supernatant of infected Vero cells was collected and treated by centrifugation at 100,000x g for three hours at 4 C (Beckman L7-55 ultracentrifuge, rotor SW 41. Palo Alto, CA). The pellets were resuspended in PBS (pH 7.2) and placed on a sucrose gradient (35% and 55%) and centrifuged at 100,000x g for three hours at 4 C. The virus was harvested from the sucrose interphase and diluted with PBS (pH 7.2), then centrifuged at 100,000x g for three hours at 4 C. The concentration of the virus protein was determined by electrophotometry at wavelengths of 280 and 260 nm (13). A negative control of Vero cells was treated the same as the APV-infected Vero cells. The ELISA procedures were performed as described earlier (22). Positive control sera were prepared in specific pathogen free (SPF) turkeys that were inoculated with inactivated virus.

Virus neutralization test. The procedure for conducting the VN test was described earlier (22) with some modification as follows: the serum samples were serially diluted two fold in serum free tissue culture medium (MEM) starting from 1:10 up to 1: 1280. A volume of 50 µl containing 100 tissue culture infective dose_{50} (TCID_{50}) of APV (Minnesota/turkey/2a/97) was added to an equal volume of each serum dilution contained in sterile 96-well flat-bottom plates (Corning Incorporated, Corning, NY). A volume of 50 µl of the virus/serum mixture of each dilution was transferred to a duplicate of monolayer of Vero cells contained in 96-well flat-bottom plates. The cells were incubated at 37 C for 5-6 days and checked every day for cytopathic effect (CPE) consisting of large syncytial formation and rounded cells.
EXPERIMENTAL DESIGN

Expt. one. Pathogenicity and persistence of APV and B. avium were studied. One hundred twenty, 2-week old poults were allotted equally into four groups. Each bird in group one was inoculated with 0.2 ml tissue culture fluid containing $10^{5.25}$ TCID$_{50}$/ml of the APV via the oculonasal route. Birds in group two were each inoculated with 0.2 ml tissue culture fluid containing $10^{5.25}$ TCID$_{50}$/ml of the APV via the right nostril and right eye. Two days later group two poults were inoculated with 0.2 ml broth containing B. avium at a titer of $3.5 \times 10^6$ colony-forming units/ml (cfu/ml) via the left nostril and left eye. Birds in group three were inoculated each with 0.2 ml broth containing B. avium at a titer of $3.5 \times 10^6$ cfu via the oculonasal route. Group four was kept as uninfected controls. Three poults from each group were sacrificed at 1, 2, 3, 4, 5, 7, 10, 14, 21, and 28 days post inoculation (PI). Swabs and sections from sinuses, tracheas, and lungs were collected at necropsy and placed in tubes containing serum free medium (MEM) for bacterial and viral isolation, respectively. Blood was collected from sacrificed poults at 14, 21, and 28 days PI for serological tests. A score of 0-3 was used to describe the clinical signs of the three sacrificed poults as follows: 0 = absence of clinical signs, 1 = clear nasal exudates, 2 = turbid nasal exudates, 3 = nasal exudates, frothy eyes and/or swollen infraorbital sinuses.

Expt. Two. Tissues distribution of APV in turkey poults was studied. Thirty, 2-week-old poults were allotted equally into two groups. Birds in group one were inoculated each with 0.2 ml tissue culture fluid containing $10^{5.25}$ TCID$_{50}$/ml of the APV via the oculonasal route. Birds in group two were kept as an uninfected control. Three
poults were sacrificed at 2, 4, 7, 14, and 21 days PI. Sections from sinuses, tracheae, lungs, thymuses, bursae, livers, spleens, ilea, jejuna, and feces were collected and placed in separate tubes containing serum free-MEM for virus isolation and viral RNA detection. Extreme care was taken during necropsy (i.e. frequent changes of sterilized equipment and gloves) to avoid cross contamination of tissue samples. Blood was collected from sacrificed poults at 2, 4, 7, 14, and 21 days PI for serological tests.

Expt three. The transmissibility of APV in turkey poults was studied. One hundred seventy, one-week-old turkey poults were allotted to five groups, 34 each. Poults in 1a and 1b groups were placed in different cages in one room. Poults in 2a and 2b groups were placed in different cages in a separate room. A control group was housed in a different room. Each room had two cages (3'x3'x6') that were placed two feet apart. Seventeen poults from 1a and 2b groups were inoculated each with 0.2 ml tissue culture fluid containing $10^{5.5}$ TCID$_{50}$/ml of the APV via oculonasal route. The remaining 17 poults in 1a and 2b groups were not inoculated and kept as direct contact poults in the same cage for each group. Poults in 1b and 2a groups were kept as indirect contact birds. The direction of the airflow was from group 1a to group 1b (infected to indirect contact) and from group 2a to group 2b (indirect contact to infected). Four poults, two from inoculated and two from direct contact, from each group were sacrificed at 2, 4, 7, 10, 14, 21, and 28 day PI. The extra six poults from each group were sacrificed at day 28 PI to be examined by serological tests. Sections from sinuses, tracheas, and lungs from each poult
were collected and placed in separate tubes containing serum free medium (MEM) for virus isolation and RNA detection. Blood was collected from sacrificed poults at 14, 21, and 28 days PI for serology tests.

**RESULTS**

**Experiment one.** The pathogenicity and persistence of APV and *B. avium* were studied in this experiment.

**Clinical signs.** Birds in the uninfected control group had no clinical signs during the course of the experimental period. Poults challenged with the virus, *B. avium*, or both had nasal discharge of varied severity at different times. The mean scores for clinical signs in the infected groups are shown (Fig 2.1). The greatest mean score [X =1.26] for the APV inoculated group was seen at days 3 and 4 PI and declined thereafter until day seven when the poults had no clinical signs. The greatest mean clinical score for the APV and *B. avium* inoculated group [X = 2] was seen at days 7 and 10 PI. For the *B. avium* infected group, the greatest mean clinical score [X = 1.59] was seen at day 7 PI. The dually inoculated group and the *B. avium* inoculated group had a mean score of [X = 1] starting from day 21 until day 28 PI when the experiment ended.

**Virus persistence.** Viral RNA was detected in the APV infected group up to day 5 PI by RT-PCR and the virus was isolated up to 3 days PI Table 1. The viral RNA was detected in the dually inoculated group up to 10 days PI and the virus was isolated up to 7 days PI Table 2.1. The APV was not detected in the control group or the *B. avium* inoculated group.
**Bacterial persistence.** *Bordetella avium* was isolated throughout the experimental period from the sinuses and the tracheas of poults inoculated with *B. avium* alone and those inoculated with *B. avium* and APV. In the group inoculated with *B. avium* only the bacteria was not recovered from the lungs, whereas the group inoculated with both agents had *B. avium* in the lungs beginning at day 8 PI of *B. avium* and consistently thereafter.

**Serology.** Antibodies to APV were detected by ELISA and VN test as shown (fig 2.2). Antibodies to *B. avium* were detected by ELISA and MAT as shown (fig 2.3). The antibody response against APV and *B. avium* was higher in the dually infected group than in groups infected with either pathogen alone in all the tests performed.

**Histopathology.** Sections from infraorbital sinuses were collected at day 14 and 21 PI from sacrificed poults in each group and were histopathologically examined. A preliminary study indicated that lesions were most consistent at day 14 and 21 PI. The respiratory epithelium of the infraorbital sinuses from the APV inoculated group was intermittently and mildly hyperplastic with scattered submucosal lymphoid infiltrates forming aggregates within the surrounding connective tissues. The ciliated epithelium was intact. Epithelium of infraorbital sinuses from the dually infected group was intermittently hyperplastic with variable disruption of the overlying cilia and hyperplastic mucous glands. Variable amounts of underlying lymphoid infiltrates were noted in the submucosal and connective tissue. Some exudates characterized by mucus, cellular debris and scattered bacteria were noticed within sinus lumens. The respiratory epithelium of infraorbital sinuses from the *B. avium* inoculated group was intermittently hyperplastic with variable disruption of the overlying cilia and variable amounts of underlying
lymphoid infiltrates. Some exudates characterized by mucus admixed with cellular debris and scattered bacteria were observed overlying the epithelial lining as shown (fig 2.4). No significant microscopic changes were seen in sections from tracheas and lungs of inoculated groups. Turkey poult in the control group did not have any microscopic changes.

Experiment two. Tissues distribution of APV in turkey poult that were inoculated oculonasally was studied. Mild respiratory signs were observed in the inoculated poult. These signs included nasal exudates and depression, which continued until day 7 PI. No virus or viral RNA was detected from thymuses, bursae, livers, spleens, ilea, jejuna, and feces. The viral RNA was detected from sinuses and tracheas starting from day 2 until day 7 PI, while the virus was isolated until day 4 PI. Viral RNA was detected from the lungs on day 4 and 7 PI and the virus was isolated only on day 4 PI as shown in Table 2.2. Poult inoculated with APV had antibodies detectable by ELISA and VN test as early as 7 days PI. The non-inoculated group had no clinical sings or antibodies to APV for the whole experimental period.

Experiment three. The transmissibility of APV in one-week-old turkey poult that were placed in different cages but in the same room was studied. Inoculated and direct contact poult in 1a and 2b groups had mild respiratory signs consisting of nasal discharge and depression. The APV was isolated and the viral RNA was detected only in inoculated and direct contact birds as shown in Table 2.3. The APV was isolated up to day four PI and the viral RNA was detected up to 10 days PI as shown in Table 3. The indirect contact poult in 1b and 2a groups had no signs of respiratory disease and the APV was not
detected at any time. Inoculated and direct contact poults in la and 2b groups had similar antibody responses as shown in (fig 2.5 and 2.6). No antibodies to APV were detected in the indirect contact poults in 1b and 2a groups during the whole experimental period.

**Discussion**

Most of the early studies on APV were conducted using non-American strains of the virus. Nonetheless, recent publications dealt with pathogenicity and tissue distribution of American strain of APV (16, 23, 24, 28, 29). This study was designed to extend this information and to investigate the transmissibility of the virus.

In this study, the clinical disease following inoculation of two-week-old poults with the APV and *B. avium* was more severe than in birds inoculated with either pathogen alone. This is in agreement with previous work (8, 9) where the combination of APV and *B. avium* infections increased the severity of the disease. Turkey poults that were inoculated with APV and *B. avium* had respiratory signs for longer time than poults inoculated with either agent alone.

APV was isolated up to 3 days PI in poults infected with APV only and was isolated up to 7 days PI in poults inoculated with both APV and *B. avium*. However, by using the RT-PCR, we were able to detect the viral RNA up to 5 days PI in the single infection and up to 10 day PI in the mixed infection. These results indicate that isolation attempts could yield negative results if not done early in the course of the infection. In addition, these results suggested that the virus persists longer in the complicated infections than in the APV infected poults.
Similar to the results reported by Saif et al (26), *B. avium* was isolated only from sinuses and tracheas in the group inoculated with *B. avium* only. In this study, *B. avium* was isolated from the lungs of poult's in the group inoculated with the two pathogens, which contributed to the severity of the disease in this group. On the other hand, Cook et al found that *B. avium* could be recovered from sinuses, tracheas, and lungs in poult's inoculated with APV, *B. avium* and *pasteurella*-like organism and in poult's inoculated with *B. avium* alone (8). In the group inoculated with both agents, the antibody responses against APV and *B. avium* were higher than in the groups inoculated with either agent alone. This could be a result of the longer persistence and the wider tissue distribution of the pathogens.

In experiment 2, the APV was detected only from respiratory tracts of inoculated poult's but not from thymuses, bursas, livers, spleens, ilea, jejuna, and feces. Cook et al 1991(8), found APV only in respiratory tracts when poult's were inoculated with APV alone. However, they also isolated APV from hearts, livers, spleens, kidneys, and caecal tonsil of birds inoculated with APV, *B. avium* and *pasteurella*-like organism. These results indicated that APV in vivo replication is restricted to the respiratory tracts in uncomplicated infections. However, when other pathogens are present that may cause damage to the upper respiratory tracts tissues, resulting in spread of APV to other organs (8).

Antibodies to APV were detected as early as 7 days PI using the VN test and ELISA. Baxter-Jones et al (6) reported the detection of antibodies to APV five days after the clinical signs appeared using VN test in chicken embryo fibroblast (CEF) then the
antibody titer declined by days 13 after the appearance of clinical signs. These results indicate that the ELISA and VN tests are good diagnostic tools during the early stage of APV infection.

In experiment 3, the APV did not spread to the indirect contact poults, which were placed in different cages. Although, the direction of the airflow in one room was from the inoculated poults to the indirect contact poults and the airflow in the second room was from the indirect contact poults to the inoculated poults. In addition, there was no antibody response to APV in the indirect contact poults. At the same time, the direct contact poults contracted the infection within two days. These results are in agreement with those of Cook et al 1991 (8) who could not detect antibody to APV in sentinel birds that were placed in the same room in different cages that were solid-sided and one meter high and one meter apart, with recently infected poults for 21 days. The airflow direction was from the infected poults towards the uninfected poults. On the other hand, Giraud et al 1986 (12) reported that the APV could spread from infected poults to SPF poults placed in separate cages but in the same room. However, information about the experimental procedures such as number of birds, size of cages, distance between cages, and direction of the airflow were not provided. Our results indicated that the USA isolate of APV used in this study is not highly transmissible.

In summary, compared to single infection, the combined infection resulted in a more severe disease that persisted longer, and spread to the lower part of the respiratory tract. The dually infected group had higher antibody response in all serologic tests performed. The APV was isolated only from the respiratory tracts when poults were
inoculated oculonasally. In addition, antibodies to APV were detected as early as 7 days PI. The virus was shown not to be indirectly transmissible. Moreover, there were no major differences in the pathogenicity or persistence of APV in one-week-old or two-week-old turkey poults.
REFERENCES


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Clinical signs of three sacrificed poult were scored from 0 to 3 as follows: 0 = absence of clinical signs. 1 = clear nasal exudates. 2 = turbid nasal exudates. 3 = nasal exudates. frothy eyes and/or swollen infraorbital sinuses.

Figure 2.1: Scores for clinical signs following APV and/or B. avium inoculation groups in experiment one.
<table>
<thead>
<tr>
<th>Day PI</th>
<th>APV Inoculated</th>
<th>APV and B. avium Inoculated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RT-PCR(^A)</td>
<td>VI(^B)</td>
</tr>
<tr>
<td>1</td>
<td>+(^c)</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
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<tr>
<td>28</td>
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</tbody>
</table>

\(^A\) RT-PCR reverse transcriptase-polymerase chain reaction.

\(^B\) VI virus isolation.

\(^c\) Tissues material from three poults, from sinuses, tracheas, and lungs were all pooled in one tube at each necropsy day.

Table 2.1: APV isolation and Viral RNA detection from APV inoculated groups in experiment one.
Negative controls and *B. avium* inoculated group had no detectable antibody to APV using ELISA and the VN test.

Figure 2.2: Antibody response to APV in experimentally inoculated poults in experiment one using ELISA and the virus neutralization (VN) test.
Negative controls and APV inoculated group had no detectable antibody to *B. avium* using ELISA and MAT.

Figure 2.3: Antibody response to *B. avium* in experimentally inoculated poulti in experiment one using ELISA and microagglutination test (MAT).
Figure 2.4: Photomicrograph of sinuses sections. A = negative control. B = APV inoculated group. C = *B. avium* inoculated group, and D = APV and *B. avium* inoculated group in experiment one.
<table>
<thead>
<tr>
<th>Days PI</th>
<th>Sinuses</th>
<th>Tracheas</th>
<th>Lungs</th>
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<tr>
<td></td>
<td>RT-PCR&lt;sup&gt;A&lt;/sup&gt;</td>
<td>VI&lt;sup&gt;B&lt;/sup&gt;</td>
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<tr>
<td>2</td>
<td>+&lt;sup&gt;C&lt;/sup&gt;</td>
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<td>21</td>
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</table>

<sup>A</sup> RT-PCR reverse transcriptase-polymerase chain reaction.

<sup>B</sup> VI virus isolation.

<sup>C</sup> +/- Tissue from sinuses, tracheas, or lungs were pooled separately from three birds at each sampling period.

Table 2.2: APV isolation and viral RNA detection from poults in experiment two.
<table>
<thead>
<tr>
<th>Days</th>
<th>PI</th>
<th>Group 1a</th>
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<th>Group 1b</th>
<th></th>
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<th>Group 2b</th>
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<td></td>
<td></td>
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<td>Contact</td>
<td>Indirect contact</td>
<td>Indirect contact</td>
<td>Inoculated</td>
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A VI virus isolation.

B RT-PCR reverse transcriptase-polymerase chain reaction.

C No. Positive/No. Tested.

D Each sample tested represent pooled tissues from sinus, trachea, and lung of one bird.

Table 2.3: APV isolation and viral RNA detection from poults in experiment three.
Geometric mean titers using VN test

Negative controls and indirect contact groups had no antibody response to APV using the VN test.

Figure 2.5: Antibody response to APV using VN test for inoculated and direct contact poultis in experiment three.
Geometric mean titers using ELISA

Negative controls and indirect contact groups had no antibody response to APV using the ELISA.

Figure 2.6: Antibody response to APV using ELISA for inoculated and direct contact poults in experiment three.
CHAPTER 3

COMPARISON OF ENZYME LINKED IMMUNOSORBENT ASSAY AND
VIRUS NEUTRALIZATION TEST FOR DETECTION OF ANTIBODIES TO
AVIAN PNEUMOVIRUS

SUMMARY

Two different whole virus enzyme linked immunosorbent assays (ELISA), developed in Ohio (OH) using APV/Minnesota/turkey/2a/97 and Minnesota (MN) using APV/Colorado/turkey/97, and Virus neutralization (VN) were used to test 270 turkey serum samples from 27 Minnesota turkey flocks for avian pneumovirus (APV) antibodies. In addition, 77 turkey serum samples and 128 ostrich serum samples from OH were tested. None of the turkey samples from OH had antibodies to APV using the VN test and OH ELISA. The ostrich samples were tested only with the VN test and were all negative for antibodies to APV. For the MN serum samples, 107, 115 and 120 were positive using the VN test, the OH ELISA and the MN ELISA.
respectively. The kappa value of 0.938 and 0.825 showed excellent agreement between the VN test and the OH ELISA and the MN ELISA, respectively, for detection of antibodies to the APV. The OH ELISA and MN ELISA had a sensitivity of 1.0 and .953, specificity of .950 and .889 and accuracy of .970 and .914, respectively. Our results indicate that the three methods are sensitive and specific for diagnosis of the APV infection.

INTRODUCTION

In 1997, avian pneumovirus (APV) emerged in the United States as a cause of respiratory disease in turkeys (13). The virus was first isolated by Buys and Preez (2) in 1980 in South Africa and later in Europe and other countries (1) where it causes the respiratory disease designated turkey rhinotracheitis (TRT) characterized by coughing, nasal discharge and swelling of the sinuses. Morbidity could reach as high as 100% with mortality reaching up to 50% (14).

Because TRT clinical signs are not specific to APV and could be confused with other respiratory diseases it is necessary to use laboratory procedure for diagnosis. Confirmation of APV infection depends on detection of the virus or its antibodies. Clinical specimens usually contain low virus titer and the virus is shed over a short period of time (10). Hence, virus isolation is not the preferable method for diagnosis of the APV infection. The most commonly used serologic method and virus detection procedure are enzyme linked immunosorbent assay (ELISA) and reverse
transcriptase-polymerase chain reaction (RT-PCR) respectively. There are different types of ELISA kits available commercially for diagnosis of APV in different species of birds (10). Monoclonal antibody based ELISA is used to differentiate between different APV isolates or subtypes in countries other than the USA (6). A crude and purified virus based ELISA (4, 12) are widely used. Recombinant viral protein based ELISA such as the matrix (M) protein (7) and nucleocapsid (N) protein (8) expressed in Escherichia Coli were developed in different laboratories.

In our laboratory we developed an ELISA using a purified whole virus as an antigen and a virus neutralization (VN) test using Vero cells to detect antibodies in serum samples but these tests were not compared as to their sensitivity, specificity, or accuracy. In this study, a comparison was made between the VN test and two ELISAs using purified whole virus, (APV/Minnesota/turkey/2a/97) and (APV/Colorado/turkey/97), respectively, for detection of APV-specific antibodies. Serum samples tested were from commercial turkey flocks in Minnesota (MN), commercial turkeys in Ohio (OH) and commercial ostriches in OH. Ostriches were tested since there was a report of detection of pneumovirus antibodies in that species (3).

**MATERIALS AND METHODS**

**Virus.** The avian pneumovirus (APV/Minnesota/turkey/2a/97) was obtained from Dr. D. Senne at the National Veterinary Service Laboratory (NVSL), Ames,
Iowa. The virus was passaged in Vero cells twelve times at the NVSL and three times in Vero cells in our laboratory. The (APV/Colorado/turkey/97) virus was passaged between 10 and 13 times in Vero cells.

Samples. Serum samples (n = 270) from commercial turkeys flocks in MN and 77 from OH were used for this comparative study. In addition, 128 ostrich serum samples collected at slaughter time from slaughter plants in Ohio and Indiana were tested using VN test only.

Virus neutralization test. The procedure for conducting the VN test was performed as described earlier (12) with some changes as follow. The serum samples were serially diluted two fold in serum free tissue culture medium starting from 1:10 up to 1:1280. A volume of 50 µl containing 100 TCID50 of (APV/Minnesota/turkey/2a/97) was added to an equal volume of each serum dilution contained in sterile 96-well flat-bottom plates (Corning Incorporated, Corning, NY). A volume of 50 µl of the virus/serum mixture was transferred to a monolayer of Vero cells contained in 96-well flat-bottom plates. The cells were incubated at 37 C for 5-6 days and checked every day for cytopathic effect (CPE) consisting of large syncytial formation and rounded cells.

OH ELISA Antigen preparation. Preparation of (APV/Minnesota/turkey/2a/97) antigen for ELISA was performed as described (4) with some modifications described below. The clarified supernatant of infected Vero cells was centrifuged at 100,000x g for three hours at 4 C. The pellets were resuspended in PBS (pH 7.2) and placed on top of two layers of 35% and 55% sucrose concentration and centrifuged at 100,000x g.
g for three hours at 4 C. The bands between the two layers of sucrose were collected and diluted with PBS, then centrifuged at 100,000x g for three hours at 4C without sucrose. The concentration of the virus protein was determined by electrophotometry at a wavelength of 280 nm and 260 nm (9). A negative control of Vero cells was treated the same as the APV-infected Vero cells. The ELISA procedure was performed as described (12). Positive control sera were prepared in specific pathogens free (SPF) turkeys using inactivated virus.

**MN ELISA Antigen preparation.** The (APV/Colorado/turkey/97) antigen preparation and ELISA procedure were performed as described (5).

**Data analysis.** A Kappa value was calculated to determine the agreement beyond chance between the VN test and ELISA conducted in OH and ELISA conducted in MN. The VN test was used as the reference test. The Kappa test (K or Kappa) was calculated using the following formula:

\[
K = (Po-Pc)/(100-Pc)
\]

where Po = observed agreement and Pc = chance agreement. While \( Po = (A+D)/n \times 100 \) and \( Pc = \{[(a+c) \times (a+b)/n^2] + [(b+d) \times (c+d)/n^2]\} \times 100 \). When the \( K = 0 \), there is no agreement beyond chance levels; if \( K < 0.3 \) the agreement is poor; a value of \( K \) between 0.3 and 0.5 is acceptable; a \( K \) value between 0.5 and 0.7 is good; and a \( K \) value of >0.7 is excellent (11).

The sensitivity, specificity and accuracy of the OH ELISA and MN ELISA in detecting antibodies to APV in serum samples were compared with the standard VN test and calculated (11) using the following formulas:
Sensitivity = \( \frac{a}{a+c} \)

Specificity = \( \frac{d}{b+d} \)

Accuracy = \( \frac{a+d}{a+b+c+d} \)

Where (a) is a positive result from an infected birds, (b) is a positive result from an uninfected birds, (c) is a negative result from an infected birds and (d) is a negative result from an uninfected birds.

Results

The results of the VN test and the two ELISA methods for detection of antibodies to APV in the turkey serum samples are presented in table 3.1. All serum samples from OH were negative using the OH ELISA and the VN test. The ostrich sera were also negative when tested using the VN test. Of the 270 sera from MN, 107, 115 and 120 were positive using VN test, the OH ELISA and the MN ELISA, respectively. The percentage of the positive sera was higher using the MN ELISA (44.44%) and the OH ELISA (42.59%) than that obtained with the VN test (39.62%).

The correlation between the VN test and the OH and the MN ELISA is shown in table 3.2. The VN test was used as the reference standard for calculation of a Kappa value that had been used to determine the strength of agreement between the VN test and the OH ELISA and MN ELISA, using the 270 samples from Minnesota turkeys. The Kappa value showed excellent agreement between the VN test and the OH ELISA (K = 0.938) and MN ELISA (K = 0.825). Using the data in Table 2, and considering the VN test as the reference standard, the OH
ELISA sensitivity was 1.0 and the specificity was 0.950, and the accuracy was 0.970. The MN ELISA sensitivity was 0.953, and the specificity was 0.889 and the accuracy was 0.914.

Discussion

The turkey and ostrich sera from OH had no antibodies to APV and the infection has not been diagnosed in OH. The percentage of the positive serum samples that have been tested using the VN test (39.62%), were lower than that detected by the OH ELISA (42.59%) or the MN ELISA (44.44) respectively.

Nonetheless, the kappa values indicated that the correlations between the VN test and the OH and MN ELISA (0.938 and 0.825) respectively were excellent for the naturally infected birds. Although, the kappa value was excellent for the OH and MN ELISA, the OH ELISA had a higher kappa value than the MN ELISA. The OH ELISA sensitivity, specificity and accuracy were slightly higher than the MN ELISA. In addition, OH ELISA results were more similar to the VN test results than those of the MN ELISA. The virus used in the VN test and the OH ELISA (APV/Minnesota/turkey/2a/97) is different from that used in the MN ELISA (APV/Colorado/turkey/97) which might explain the above results. Nonetheless, the Kappa values obtained for the tests with the two virus strains indicate similarities between the viruses.

The VN test had high sensitivity in detection of antibodies to APV. All the samples that were negative in the VN test were also negative in the OH ELISA.
and only five samples that were negative in the VN test were positive in the MN ELISA. At the same time the OH ELISA detect eight positive samples that were negative in the VN test while the MN ELISA identified 18 positive samples that were negative in the VN test. Similar to our results, Chiang et al found that the ELISA based on whole (APV/Minnesota/turkey/2a/97) or (APV/Colorado/turkey/97) yielded similar results when used for detecting antibody to APV in turkey sera (5). Our results indicate that the ELISA and VN test are useful diagnostic tools for detecting antibodies to APV.
REFERENCES


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<td>(39.62%)&lt;sup&gt;E&lt;/sup&gt;</td>
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<sup>A</sup>ND. Not determined.

<sup>B</sup>OH. Ohio

<sup>C</sup>MN. Minnesota

<sup>D</sup>No. Positive/No. tested.

<sup>E</sup>Positive percentage.

Table 3.1: Antibody detection using different procedures.
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<sup>A</sup> OH. Ohio  
<sup>B</sup> MN. Minnesota

Table 3.2: Agreement between the VN test and ELISA's for detection of APV antibodies.
CHAPTER 4

LACK OF ANTIGENIC RELATIONSHIPS BETWEEN AVIAN PNEUMOVIRUS
AND FOUR AVIAN PARAMYXOVIRUSES

SUMMARY.

Avian paramyxoviruses (PMV) and pneumovirus (APV) belong to the family paramyxoviridae. Antigenic relationships between PMV were shown previously, hence, this study was designed to investigate possible antigenic relationships between APV and four avian PMVs (PMV-1, PMV-2, PMV-3, and PMV-7). Enzyme linked immunosorbent assay (ELISA), Hemagglutination inhibition (HI) test, and virus neutralization (VN) test in chicken embryos and in vero cells were used. The HI test was performed using the PMVs as antigens against the APV and PMVs antisera. The ELISA and VN test in chicken embryos were performed using PMVs and APV antigens and antisera. The VN test in vero cells was performed using the APV as an antigen against
the PMVs antisera. All the viruses were isolated in the USA or Canada. No antigenic relationships between APV and the PMVs were detected using the described tests.

INTRODUCTION

Avian pneumovirus (APV) is the causative agent of turkey rhinotracheitis (TRT) and swollen head syndrome (SHS) in turkeys and chickens, respectively. The APV and avian paramyxoviruses (PMVs) belong to the family paramyxoviridae which has two subfamilies, the paramyxovirinae and pneumovirinae (17). The subfamily pneumovirinae contains two genera metapneumovirus and pneumovirus. The subfamily paramyxovirinae contains three genera, Respirovirus, Rubulavirus, and Morbillivirus. The APV belongs to the genus metapneumovirus, subfamily pneumovirinae, within the paramyxoviridae family. The Rubulavirus genus has several viruses including Newcastle disease virus (NDV), which is designated avian paramyxovirus type 1 (PMV-1) and eight other avian paramyxovirus serotypes are also within the Rubulavirus genus (17).

The first isolate of APV was obtained in South Africa in 1979 (8) and later the virus was detected in other countries around the world (1). In the USA, APV was first isolated in 1996 in Colorado then in Minnesota (20). The APV and PMVs have been known to cause respiratory and reproductive problems in turkeys (1). One APV serotype has been identified and within this serotype there are three subgroups designated A, B, and C (11, 13, 19). These subgroups (A, B, and C) were identified based on molecular and antigenic differences (11, 13, 19). Cook et al showed that there are antigenic relationships between APV subgroups (11). The PMVs were grouped on the basis of their
antigenic relatedness in Hemagglutination inhibition (HI) tests to nine serotypes (1, 23).
Moreover, low antigenic cross reactions between the PMV serotypes have been reported
(2, 3, 4, 7, 10, 14, 21). One-way inhibition was reported among avian PMVs or between
avian PMVs and animal viruses such as bovine parainfluenza type-3 (14, 22).

Because of the reports on antigenic relatedness between viruses in the
paramyxoviridae family, we initiated this study to investigate possible antigenic
relationships between the PMV-1, PMV-2, PMV-3, and PMV-7 and the APV.

MATERIALS AND METHODS

Viruses. The APV (APV/Minnesota/turkey/2a/97) was obtained from D. Senne at the
National Veterinary Service Laboratories (NVSL), Ames, Iowa. The virus was passaged
in vero cells twelve times at the NVSL and four times in our laboratory. Other viruses
used in this study were PMV-1 (B1, Lasota vaccine), PMV-2 (Yucaipa, 59), PMV-3
(Ontario, 6661, 60), and PMV-7 (PMV-7/Ohio/turkey/97). The APV and PMVs were
passaged twice in specific pathogen free (SPF) chicken embryos, which were inoculated
via the allantoic route. The in vitro passage history of the PMVs prior to use in this study
is not known. Virus titrations were expressed as the mean embryo infective dose (EID50)
(Table 1) and calculated by the method of Reed and Muench (16).

Antisera. Thirty week-old turkeys were used to produce antisera to APV, PMV-1,
PMV-2, PMV-3, or PMV-7. Purified viruses were inactivated using 0.1% β-
propiolactone for 2 hours at 37 C. Turkeys were inoculated three times at two week
intervals. In the first inoculum, 0.5 ml of the inactivated purified viruses was mixed with

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an equal volume of Freund’s complete adjuvant. In the second and third inocula, 0.5 ml inactivated purified viruses was mixed with an equal volume of Freund’s incomplete adjuvant. Ten days after the last inoculation, the turkeys were bled and the sera were collected and heat inactivated at 56°C for 30 minutes.

**Hemagglutination (HA) and Hemagglutination inhibition tests.** The HA and HI tests were used for identification and detection of the avian PMVs as described earlier (6). Allantoic fluids, from chicken embryos inoculated with avian PMVs were used. Chicken red blood cells (RBCs) were used for both tests. The diluted-serum constant-virus method was used for the HI test with 4 HA units of virus for each dilution. Positive and negative controls for each virus were used with each test. The avian paramyxoviruses types 1, 2, 3, and 7 and turkey anti-sera for these viruses were tested against each other using HI tests. At the same time, turkey anti-APV hyperimmune serum was tested against the PMVs using HI test.

**Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR).** RT-PCR was used to detect APV in allantoic fluids containing the APV. The viral RNA was extracted with Trizol® LS Reagent (GibcoBRL) using manufacturer’s protocol. Two hundred fifty μl of the allantoic fluid was used for viral RNA extraction. The RNA pellet was suspended in 20 μl of diethylpyrocarbonate (DEPC) treated water and stored at -20°C until used.

The primers were designed from the M protein sequence and synthesized at Integrated DNA Technologies Inc, Coralville, Iowa, using published sequence (5). The RT-PCR was performed with the Titan™ One Tube RT-PCR System (Boehringer Mannheim) following the manufacturer’s recommendations. Ten μl of the viral RNA was
used for reverse transcription (cDNA synthesis). The RNA and primers mixture was incubated at 70°C for 10 minutes then chilled on ice. The reverse transcriptase (RT) reaction was started at 45°C for one hour followed by a denaturation step at 94°C for three minutes. Forty cycles of denaturation, annealing, and extension at 94°C for 30 second, 50°C for one minute, and 72°C for one minute, respectively, were performed. The final amplification was performed at 72°C for ten minutes. The resulting products were subjected to electrophoresis in 1.2% agarose gel (SeaKem LE Agarose Rockland, Maine). The gel was stained with ethidium bromide and product bands were visualized with an ultraviolet transilluminator.

ANTIGEN PREPARATION FOR ELISA. The preparation of APV and avian PMVs antigens for ELISA was similar to that described earlier (9) with some modifications. Briefly, the supernatant of APV infected vero cells or allantoic fluid from chicken embryos inoculated with PMVs were collected and centrifuged at 100,000×g for three hours at 4°C (Beckman L7-55 ultracentrifuge, rotor SW 41. Palo Alto, CA). The pellets were resuspended in PBS (pH 7.2) and placed on sucrose gradient (35% and 55%) and centrifuged at 100,000×g for three hours at 4°C. The viruses were harvested from the sucrose interphase and diluted with PBS (pH 7.2), then centrifuged at 100,000×g for three hours at 4°C. The concentration of the virus protein was determined by electrophotometry at wavelengths of 280 and 260 nm (12). Negative controls of Vero cells or allantoic fluids from SPF chicken embryos were treated the same way. Turkey antisera for all the viruses and a peroxidase conjugate directed to (H+L) chain of turkey
IgG were used in the ELISA. The ELISA procedures were performed as described earlier (15). For each ELISA plate positive and negative controls for each virus were set for each test.

**Virus neutralization test in vero cells.** The PMVs antisera were tested against the APV using vero cells. The procedure for conducting the VN test was described earlier (15) with some modification as follows: the avian paramyxoviruses antisera were serially diluted two fold in serum free tissue culture medium (MEM) starting from 1:10 up to 1:1280. A volume of 50 µl containing 100 tissue culture infective dose_{50} (TCID\textsubscript{50}) of APV (Minnesota/turkey/2a/97) was added to an equal volume of each avian paramyxovirus types 1, 2, 3, and 7 antiserum dilution contained in sterile 96-well flat-bottom plates (Corning Incorporated, Corning, NY) and incubated for one hour at room temperature. A volume of 50 µl of the virus/serum mixture of each dilution was transferred to a duplicate of monolayers of Vero cells contained in 96-well flat-bottom plates. The cells were incubated at 37 °C for 5-6 days and checked every day for cytopathic effect (CPE) consisting of large syncytial formation and rounded cells. APV antibody positive and negative sera were used as controls for each test.

**Virus neutralization test in chicken embryos.** Nine to 11 day old embryos were used for titration and virus neutralization of APV and avian paramyxoviruses 1, 2, 3, and 7. The antiserum for each virus was diluted two fold in BPS (pH 7.2) starting from 1:20 up to 1:320. An equal volume of each virus preparation containing 100 EID\textsubscript{50} was mixed with an equal volume of appropriate antiserum dilution and incubated for one hour at room temperature. In addition, for each test there were positive and negative controls for
each virus tested. The APV was tested against avian paramyxoviruses 1, 2, 3, and 7 antisera and avian paramyxoviruses 1, 2, 3, and 7 were tested against APV antiserum. Four SPF chicken embryos were used for each dilution and a volume of 0.1 ml of the virus/serum mixture of each dilution was injected to each embryo and the embryos were incubated at 37 C for three days. Allantoic fluids derived from PMVs inoculated embryos were collected and tested using HA and HI tests. The allantoic fluids that were derived from APV inoculated chicken embryos, were collected and tested using RT-PCR.

RESULTS and DISCUSSION

The present work was undertaken to examine the possible antigenic relatedness between APV and PMVs using ELISA, VN test, and HI test. The RT-PCR results indicated that the allantoic fluids from APV inoculated chicken embryos contained APV (data not shown). The results of the HI test, ELISA and VN tests are presented in Tables 1, 2, 3, respectively. No antigenic relationships were demonstrated between the four avian PMVs and APV using the above tests.

The paramyxoviridae family contains important avian pathogens such as avian PMVs and APV that cause similar respiratory diseases in turkeys (1, 11, 18). It was shown in earlier studies that there are low cross reactions among the avian PMVs using the HI test (2, 3, 4, 7, 10, 14, 21). In addition, one-way inhibition was reported between PMV-6 and PMV-7 (14), where the PMV-6 antiserum did not inhibit the PMV-7, while PMV-7 antiserum inhibited the PMV-6 using HI test (14).
Previous reports on antigenic relationships between PMVs serotypes using the HI test showed a cross reaction between NDV and PMV-3 (PMV-3/parakeet/Netherlands/449/75) at low dilutions (14). Moreover, one-way asymmetric inhibition was reported between PMVs and animal viruses such as a reaction between PMV-2 and bovine Parainfluenza type-3 (22).

Cross reactions between the PMVs were noticed in earlier studies when birds were vaccinated or challenged with one serotype virus and challenged with another serotype virus (1). Chickens infected with PMV-3 viruses were shown to be protected against challenge with a virulent NDV strain (4). Antibodies to both PMV-1 and PMV-3 may be detected in birds vaccinated with PMV-1 (1,3,7). In addition, birds vaccinated with PMV-1 and infected with PMV-3 had an increase in antibodies to PMV-1 and PMV-3 (1,3,7). The lack of antigenic relationships between the PMVs in the current study might be attributed to the use of one virus in each bird.

In conclusion, the results using ELISA, VN test, and HI test were in agreement confirming the lack of antigenic relatedness between APV and the four avian PMVs examined or between the four avian PMVs using HI test. The tests used provide useful serologic tools to distinguish APV from the PMVs.
REFERENCES


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<td>PMV-7</td>
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\(^A\)HI titer.

\(^B\)HI titer less than 10.

Table 4.1. Hemagglutination Inhibition Test Results.
| Viruses used to coat ELISA controls plates | Mean of Standard Deviation (SD) | Antisera PMV-1 | | Antisera PMV-2 | | Antisera PMV-3 | | Antisera PMV-7 | | Antisera APV |
|-----------------------------------------|--------------------------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| PMV-1                                   | .241                          | .15            | .15^a          | .17            | .17            | .12            | .41            | .44            | .45            |
| PMV-2                                   | .222                          | .09            | .28            | .28            | .25            | 1.01           | .95            | 1.1            | .3             |
| PMV-3                                   | .225                          | .11            | .36            | .35            | .39            | .33            | .39            | .31            | 1.2            |
| PMV-7                                   | .231                          | .16            | .41            | .45            | .45            | .47            | .43            | .39            | 1.22           |
| APV                                     | .215                          | .12            | .395           | .405           | .41            | .385           | .355           | .365           | .455           |

^a The cutoff point for the PMV-1, PMV-2, PMV-3, PMV-7 and APV is the mean of the negative controls + three SD = 0.619, 0.492, 0.555, 0.711, and 0.575, respectively.

Table 4.2. Enzyme Linked Immuno sorbent Assay results.
<table>
<thead>
<tr>
<th>VN tests</th>
<th>Viruses</th>
<th>Antisera</th>
</tr>
</thead>
<tbody>
<tr>
<td>VN test using</td>
<td>PMV-1</td>
<td>PMV-2</td>
</tr>
<tr>
<td>chicken</td>
<td>160</td>
<td>-</td>
</tr>
<tr>
<td>embryos</td>
<td>PMV-2</td>
<td>-</td>
</tr>
<tr>
<td>PMV-3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PMV-7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>APV</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

| VN test using | APV       |          |          |          | 160      |
| vero cells    |           |          |          |          |          |

^ VN test less than 20

Table 4.3. Virus neutralization test results.
GENERAL CONCLUSIONS

• The APV infection combined with *Bordetella avium* resulted in a severe disease and extended invasion of the respiratory tracts.

• The APV infection combined with another respiratory pathogen resulted in a higher antibody response than a single APV infection.

• Uncomplicated APV infection was restricted to respiratory tract tissues.

• Antibodies to APV could be detected using enzyme linked immunosorbent assay (ELISA) and virus neutralization test (VI) as early as 7 day post inoculation.

• The APV persists in birds with combined infection longer than in the single infection. In single infection the virus persists between 5 to 7 days.

• The APV is not highly transmissible in turkey poults.

• The turkey and ostrich sera from Ohio had no antibodies to APV and the infection has not yet been diagnosed in Ohio.

• The virus neutralization (VN) test and enzyme linked immunosorbent assay (ELISA) are sensitive and specific for diagnosis of the APV infection.

• ELISA’s using (APV/ Minnesota / turkey /2a/97) or (APV/Colorado/turkey/97) antigens have a similar sensitivity, specificity, and accuracy.

• No antigenic relationships were identified between APV and four avian paramyxoviruses (PMV-1, PMV-2, PMV-3, and PMV-7) using ELISA,
hemagglutination inhibition (HI) test, and VN test in vero cells or chicken embryos.
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