Susceptibility of ducks to avian pneumovirus of turkey origin

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Objective—To determine the susceptibility of ducks to avian pneumovirus (APV) of turkey origin.

Animals—30 Pekin ducks that were 2 weeks old.

Procedure—Ducks were assigned to 3 groups (10 ducks/group). Ducks of groups 1 and 2 were inoculated (day 0) with 200 µl of cell-culture fluid containing APV of turkey origin (10^3 median tissue-culture infective dose/ml) by the oculonasal (group 1) or oral (group 2) route. Ducks of group 3 served as noninoculated control birds. Two ducks from each group were euthanatized 3, 6, 9, 15, and 21 days after inoculation. Blood samples, tissue samples from the lungs, trachea, nasal turbinates, duodenum, diverticulum, vitellineum (Meckel’s diverticulum), and cecum, and swab specimens from the choana, cloaca, and trachea were obtained from all birds during necropsy and examined for APV by use of reverse transcriptase-polymerase chain reaction (RT-PCR), virus isolation, and histologic examination. Blood samples also were examined for APV antibodies, using an ELISA.

Results—Tissue samples obtained up to 21 days after inoculation had positive results when tested by use of RT-PCR. Virus was isolated from nasal turbinates of birds inoculated via the oculonasal route. Serum samples obtained 15 and 21 days after inoculation had positive results when tested for APV-specific antibody. Clinical signs of disease were not observed in ducks inoculated with APV of turkey origin.

Conclusions and Clinical Relevance—Ducks inoculated with APV of turkey origin may not develop clinical signs of disease, but they are suspected to play a role as nonclinical carriers of APV. (Am J Vet Res 2001;62:991-992)

Pneumoviruses are members of the subfamily Pneumovirinae in the family Paramyxoviridae. Avian pneumovirus (APV) is classified in the genus Metapneumovirus.1 Avian pneumovirus is a single-stranded nonsegmented RNA virus and has close genetic similarity with respiratory syncytial virus in humans and cattle.1 Avian pneumovirus has been isolated from turkeys or chickens in several countries.2,3,13 Clinical signs in APV-infected turkeys include coughing, nasal discharge, and swollen sinuses, and infection can result in death.6,14,17 Avian pneumovirus can cause experimentally induced rhinotracheitis in turkeys.5,13-17 This disease is severe in young turkeys. Within a flock, morbidity is often 100%, and mortality ranges from 1 to 90%.

Avian pneumovirus was first isolated in the United States in 1997 from turkeys in Colorado and subsequently from turkeys in Minnesota.4,5 Infection with APV continues to be a major concern to turkey producers in Minnesota. In chickens, APV appears to cause mild respiratory tract infection and has been associated with swollen head syndrome.18,19 Our laboratory group has been conducting studies to investigate the role of migratory waterfowl in transmission of APV to domestic turkeys. The APV isolates detected in birds in the United States can be distinguished phylogenetically from European subgroup A and B isolates20 and are classified as members of subgroup C.2,21 It is difficult to attribute the spread of APV within states in the north-central United States to bird migration alone, because Canada and states in the southern United States have not reported outbreaks of APV. However, it is important to mention that Minnesota, which has the highest incidence of APV, has many lakes that provide sanctuary for wild birds. This may provide an ideal environment for maintaining APV in a natural environment. A study22 that used ducks as sentinel animals revealed APV antibodies in sera from mallard ducks; subsequently, APV was isolated from those birds. However, susceptibility of ducks to infection with APV of turkey origin is unknown.

The objective of the study reported here was to determine whether ducks can become infected with a turkey isolate of APV. Furthermore, we wanted to determine whether ducks have clinical signs of disease after exposure to APV of turkey origin and whether ducks develop antibodies to turkey-origin APV.

Materials and Methods

Ducks—Pekin ducks were obtained from a commercial vendor. Ducks were obtained when they were 1 day old. All ducks were seronegative for APV antibody. Ducks were maintained in isolators with negative-pressure ventilation.

Virus for inoculation—The APV isolated from turkeys with respiratory tract disease in Minnesota was used. The virus was titrated in Vero cells prior to inoculation.23,24 Virus concentration was adjusted to contain 10^3 median tissue-culture infectious dose/ml. Titrated virus was stored in cell-culture medium supplemented with 4% fetal bovine serum, 1% l-glutamine, 1% nonessential amino acids, penicillin (0.5 U/ml), and streptomycin (0.5 µg/ml) until inoculated.

Experimental design—Two-week-old ducks were assigned to 3 groups (10 ducks/group) and placed in isolators. Each duck in group 1 was inoculated via the oculonasal route with 200 µl of cell-culture media containing APV. Each duck in group 2 was inoculated orally with 200 µl of cell-culture media containing APV. Ducks in group 3 served as noninoculated control birds. Day of inoculation was designated day 0. All ducks were monitored for clinical signs of disease.

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Two ducks from each group were euthanatized 3, 6, 9, 15, and 21 days after inoculation. During necropsy, blood samples, samples of tissue from the lungs, trachea, nasal turbinates, duodenum, diverticulum vitellinum (Meckel's diverticulum), and cecum, and swab specimens from the choana, trachea, and cloaca were collected for evaluation by use of virus isolation, histologic examination, and reverse transcriptase-polymerase chain reaction (RT-PCR).

Tissue samples from the lungs, trachea, nasal turbinates, and intestines were placed in neutral-buffered 10% formalin. Tissue samples then were embedded in paraffin, mounted on slides, and stained for histologic examination.

**RT-PCR of tissue samples**—Tissues were homogenized and centrifuged (11,400 × g). Supernatant was collected in sterile tubes and used for viral RNA extraction for RT-PCR. Viral RNA extraction for RT-PCR was performed, using an RNA extraction kit in accordance with the manufacturer's instructions.

Two primers (C3 and C4) were designed to amplify the M gene of the APV Minnesota isolate. Primer C3 had a nucleotide sequence of 5'-TGACTTCAGGATAACCAAA-3', and primer C4 had a nucleotide sequence of 5'-TGACTTCAGGACATACTCTT-3'. The expected size of the RT-PCR product for these primers was 438 base pairs.

The cDNA was prepared, using a cDNA synthesis kit with hexamers as primer. The cDNA synthesis was performed in a reaction mixture of 10 µl containing 5 mM MgCl2, 1 mM of each deoxynucleoside triphosphate (dNTP), 1 unit of RNase inhibitor, 2.5 µM of murine leukemia virus reverse transcriptase, and 2.5 µM random hexamer. Conditions for cDNA synthesis were annealing at 20 °C for 10 minutes and reverse transcription at 42 °C for 20 minutes, followed by enzyme inactivation at 99 °C for 5 minutes.

The PCR was performed in a 50-µl volume of 0.2 µM of each primer, 1 mM of each dNTP, 2 mM MgCl2, and 2.5 units of Taq DNA polymerase. The reaction was performed in a thermal cycler, and conditions for amplification were initial denaturation (94 °C for 1 minute), 35 cycles of annealing (51 °C for 1 minute), extension (72 °C for 2 minutes), and denaturation (94 °C for 1 minute), and final extension (72 °C for 10 minutes). The RT-PCR products were then analyzed on a 1.2% agarose gel.

**Virus isolation**—Virus isolation was attempted for all tissues from all ducks, using Vero cell culture. Briefly, samples from tissues were homogenized with minimum essential medium (MEM) at a ratio of 1:10. An aliquot (100 µl) of homogenized suspension was placed on a Vero cell monolayer containing MEM supplemented with 1% fetal bovine serum, 1% amphotericin B, 1% nonessential amino acids, 1% l-glutamine, and 1% streptomycin-penicillin. Cultures were incubated in 10% CO2. Samples were cultured in Vero cells for 5 blind passages. At the end of the fifth passage, cultures that had syncitia were tested for APV by use of RT-PCR and indirect fluorescent antibody assay. Each sample was cultured for 3 additional passages and tested again before it was considered to be negative.

**Serologic examination**—The procedure for conducting the ELISA has been described elsewhere. Briefly, Vero cells infected with APV were removed by centrifugation at 7,310 × g for 5 minutes. Supernatant that contained virus was diluted with coating buffer. An aliquot (100 µl) of virus suspension in coating buffer was dispensed into each well of a 96-well plate and allowed to sit undisturbed for 4 hours to coat the wells. Plates were washed with PBS solution containing 0.05% Tween 20 and incubated with blocking buffer at 37 °C for 1 hour. Blocking buffer was drained from each plate, and plates were used for assay of antibodies to APV.

A 100-µl volume of each serial dilution of serum to be tested (1:20, 1:40, and 1:100) in dilution buffer was placed in each well, and plates were incubated at 37 °C for 1 hour. Positive- and negative-control sera were included in each plate. Plates were washed 3 times with PBS solution, and 100 µl of goat anti-duck IgG (diluted 1:1,000 in PBS solution) conjugated to horseradish peroxidase was added to each well. Plates were incubated for 1 hour and washed with PBS solution. A 100-µl volume of substrate-2-azino-di (3-ethylbenzthiazoline-6-sulfonate) solution was added to each well, and plates were incubated for 10 minutes in the dark. The reaction was stopped by adding 100 µl of stop solution to each well. Absorbance in each well was measured on a spectrophotometer at a setting of 405 nm.

Forty serum samples obtained from Pekin ducks maintained in isolation facilities at the University of Minnesota and known to be free of APV infection were tested in duplicate for APV antibody, using the ELISA. Mean value of the optical density for the sera from these ducks was 0.15. Thus, the cutoff value for a positive result for the ELISA was based on a value of 0.15 + 2 SD.

**Results**

**Clinical signs**—Ducks exposed to APV of turkey origin via the oculonasal or oral route of administration did not have free of APV infection were tested in duplicate for APV antibody, using the ELISA. Mean value of the optical density for the sera from these ducks was 0.15. Thus, the cutoff value for a positive result for the ELISA was based on a value of 0.15 + 2 SD.

**Results of RT-PCR for tissue samples**—Most tissue samples collected from inoculated ducks for up to 21 days after inoculation had positive results for APV, as determined by use of RT-PCR (Tables 1 and 2). Samples of blood from ducks inoculated via the oculonasal and oral routes had positive results when tested for APV.

In the oculonasally inoculated group, blood samples and samples of lungs, trachea, and nasal turbinates collected for up to 21 days after inoculation had positive results. Positive results were obtained only for swab samples of the choana on days 3 and 6 after inoculation (Table 1). In the orally inoculated group, RT-PCR results were similar to those of the oculonasally inoculated group (Table 2). All samples from ducks in the control group had negative results for APV when tested by use of RT-PCR.

**Virus isolation**—Avian pneumovirus was isolated only from turbinate samples obtained on day 3 after inoculation from ducks that were inoculated via the oculonasal route. Results of virus isolation were confirmed by use of RT-PCR and indirect fluorescent antibody tests. We were unsuccessful in isolating APV from samples of other organs or swab specimens.

**Serologic examination**—Serum samples obtained 15 and 21 days after inoculation from ducks of groups 1 and 2 had antibodies to APV (Table 3). Optical density values for sera considered positive for APV antibody ranged from 0.5 to 0.6. All serum samples collected from oculonasally infected ducks on days 15 and 21 after infection yielded positive results. For ducks inoculated via the oral route, only 1 of the 2 serum samples obtained on day 15 after inoculation was seropositive, but both samples obtained on day 21 after inoculation were seropositive. Serum samples obtained from ducks of both inoculated groups on days 3, 6, and
Histologic examination—Abnormalities were not detected in tissues obtained from ducks 3 and 6 days after inoculation with APV. In samples obtained from inoculated ducks 9 and 15 days after inoculation, the tracheal mucosa was infiltrated by a small number of lymphocytes and macrophages, whereas the mucosal lining of the nasal cavities and sinuses was infiltrated by a mildly increased number of lymphocytes and macrophages. The lungs were congested and edematous, but substantial changes were not detected in the intestines. In samples obtained 21 days after inoculation, the mild inflammatory response observed in the tracheal mucosa at 9 and 15 days after inoculation had resolved. Also, lungs of ducks euthanatized 21 days after inoculation appeared normal. Tissues from noninoculated ducks appeared normal throughout the study.

Discussion

In the study reported here, we examined the ability of APV of turkey origin to infect ducks obtained from a commercial vendor. Ducks inoculated with APV by the oculonasal or oral route were monitored for clinical signs of APV infection, persistence of virus in various tissues, and serologic response to the inoculated APV. The ducks did not have any signs of respiratory tract disease. Little is known about the virulence or pathogenicity of the turkey isolates of APV in ducks. To our knowledge, this is the first report indicating the infectivity of a turkey isolate of APV in ducks.

Results of the RT-PCR indicated the persistence of viral RNA in various tissues for 3 to 21 days after inoculation. Histologic examination of the tracheal mucosa revealed infiltration of a small number of lymphocytes and macrophages, an indication of a mild inflammatory response. Mucosal lining of the nasal cavities and sinuses was infiltrated by a mildly increased number of lymphocytes and macrophages. Results of RT-PCR confirmed APV in these tissues. The fact that viral RNA was found in the blood of ducks inoculated with APV raises the possibility of viremia in these ducks.

In this study, turbinate tissue obtained 3 days after inoculation from birds exposed by the oculonasal route yielded APV on Vero cell culture. The reason we failed to isolate APV from internal organs other than turbinate samples could not be ascertained. However, it has been our experience that turbinate samples of turkeys are ideal tissues for use in isolation of APV, compared with other internal organs. It should be mentioned that it has been extremely difficult for investigators to isolate APV from infected birds. Our laboratory group has had minimal success when attempting to isolate APV from tissues. We have isolated APV from only a few of several hundred samples obtained from turkeys suspected of having APV infection. Both inoculated groups were seropositive for antibodies to APV in samples obtained 15 and 21 days after inoculation.

Minnesota has many lakes and ponds and therefore provides an ideal habitat for migratory waterfowl to breed during the early spring. Minnesota also is in a flyway for waterfowl during the late fall. The contribution of migratory waterfowl as a source of avian

Table 1—Results for tissue samples and swab specimens obtained from ducks inoculated with avian pneumovirus (APV) via the oculonasal route that were tested by use of reverse transcriptase-polymerase chain reaction (RT-PCR)

<table>
<thead>
<tr>
<th>Day*</th>
<th>Blood</th>
<th>Lungs</th>
<th>Trachea</th>
<th>Choana†</th>
<th>Cloaca†</th>
<th>Duodenum</th>
<th>Jejunum</th>
<th>Cecum</th>
<th>Diverticulum vitellimum</th>
<th>Nasal turbinates</th>
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<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<td>9</td>
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<tr>
<td>21</td>
<td>+</td>
<td>+</td>
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</table>

*Day of inoculation = Day 0. †Swab specimen.
+ = APV viral nucleic acid detected. – = APV viral nucleic acid not detected.

Table 2—Results for tissue samples and swab specimens obtained from ducks inoculated with APV via the oral route that were tested by use of RT-PCR

<table>
<thead>
<tr>
<th>Day*</th>
<th>Blood</th>
<th>Lungs</th>
<th>Trachea</th>
<th>Choana†</th>
<th>Cloaca†</th>
<th>Duodenum</th>
<th>Jejunum</th>
<th>Cecum</th>
<th>Diverticulum vitellimum</th>
<th>Nasal turbinates</th>
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<tbody>
<tr>
<td>3</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<td>21</td>
<td>+</td>
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</table>

See Table 1 for key.

Table 3—Results for serum samples obtained from noninoculated (control) ducks and ducks inoculated with APV and tested by use of an ELISA

<table>
<thead>
<tr>
<th>Group (n = 10 ducks/group)</th>
<th>Day*</th>
<th>Control</th>
<th>Oculonasal inoculation</th>
<th>Oral inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
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<tr>
<td>6</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
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<tr>
<td>9</td>
<td>0/2</td>
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<tr>
<td>21</td>
<td>0/2</td>
<td>2/2</td>
<td>2/2</td>
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</tbody>
</table>

Values represent No. with positive results/No. of samples tested. See Table 1 for key.
influenza to domestic turkeys in Minnesota has been documented. In Minnesota, there are many facilities where turkeys are not maintained in total confinement. Bird-proofing efforts at many facilities are not totally effective. Many cases of APV infection in turkeys have been reported in Minnesota. It is important to mention that the number of APV-infected turkeys dramatically increases in the spring and fall. Ongoing studies conducted by our laboratory group have resulted in isolation of APV from sentinel ducks placed in lakes that harbor wild ducks. Genetic similarities between APV isolates from sentinel ducks and isolates of APV from turkeys are being investigated.

Analysis of the results of this study clearly documented the susceptibility of ducks to APV of turkey origin. The fact that ducks exposed to APV of turkey origin did not have clinical signs of disease may indicate the avirulent nature of this isolate to cause infection in ducks. One could also speculate the possible existence of host specificity for isolates obtained from various species. However, a large number of isolates would need to be tested to prove or disprove this speculation. In the study reported here, the RT-PCR revealed viral nucleic acid of turkey-origin APV in several tissues from ducks after inoculation. The virus was isolated from tissues, and ducks seroconverted. Despite their lack of clinical signs of disease, these ducks may represent nonclinical carriers of APV and serve as a reservoir of infection in other species. However, a large number of isolates from sentinel ducks and isolates of APV from wild ducks harbor wild ducks. Genetic similarities between APV isolates from sentinel ducks and isolates of APV from turkeys are being investigated.

References

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