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Persistent Infection of Avian Pneumovirus (APV) in Turkeys

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Abstract : The persistent infection of avian pneumovirus (APV) was studied in turkeys. After APV infection to turkeys, healthy birds were transferred to infected birds and commingled at 4 days, 3 weeks, 6 weeks, 9 weeks, and 15 weeks post-infection. The genome of APV was detected up to 10 days from two birds transferred and commingled with infected birds at 3 weeks post-infection. A bird transferred at 6 weeks post-infection was positive in RT-PCR detection after 5 days. From then on, no bird showed positive results of persistency of APV infection. These findings suggest that persistent APV infection may spread to the healthy birds from the infected birds until several weeks.

Key words : Avian pneumovirus, persistent infection, turkey, RT-PCR

Introduction

The turkey rhinotracheitis virus, commonly referred to as avian pneumovirus (APV), is a member of the *Paramyxovirdae* family that causes acute rhinotracheitis in turkeys characterized by coughing, nasal discharge, tracheal rales, foamy conjunctivitis and sinusitis in turkeys of all ages. In laying birds, there is transient drop in the egg production along with mild respiratory tract illness (Jones, 1996). Uncomplicated cases have low mortality (2 to 5%) but APV infections accompanied by secondary infections (bacterial and/or viral) can result in up to 25% mortality (Jones, 1996). APV was first detected in South Africa in 1978, but quickly spreaded to the United Kingdom, France, Spain, Germany, Italy, the Netherlands, Israel, and Asia (Jones 1996; Alexander, 1997). The United States was free of APV infection until 1996, when a 10-month outbreak of upper respiratory system infection among turkeys in Colorado resulted in isolation of APV (Kleven, 1997; Seal, 1998). However, the APV outbreak in the western state of Colorado was controlled by intense biosecurity measures and the disease has not been reported there since early 1997 (Kleven, 1997). APV was detected in 1997 in the north-central state of Minnesota where the incidence of disease has increased over the past four years despite the establishment of biosecurity measures similar to those used in Colorado (Chiang *et. al*, 2000; Goyal *et al*, 2000; Jirjis *et al*, 2000).

Mammalian pneumoviruses encode ten gene proteins that include non-structural (NS1 and NS2), nucleocapsid (N), phosphoprotein (P), matrix (M), small hydrophobic (SH), surface glycoprotein (G), fusion (F), second matrix (M2) and RNA-dependent RNA polymerase (L) genes. In contrast, APV lacks the NS1 and NS2 genes (Pringle 1996, Randhawa *et al*, 1997) and has a smaller L gene than that of mammalian pneumoviruses (Randhawa *et al*, 1996b). The gene order of APV is 3'N-P-M-F-M2-SH-G-L5' (Ling *et al*, 1992; Yu *et al*, 1992b). Based on these differences, the avian pneumoviruses were classified in the genus *Metapneumovirus*, whereas their mammalian counterparts belong to the genus *Pneumovirus* (Pringle 1996). Two subgroups (A and B) of APV were defined in Europe, initially based on the level of genetic variations in the attachment (G) glycoproteins of APV strains (Juhasz & Easton, 1994). Subsequent studies showed significant heterogeneity in M and F gene nucleotide (nt) sequences between the two subgroups so that the genetic composition of these genes could also be used to distinguish between the subgroups (Yu *et al*, 1991; Yu *et al*, 1992a; Randhawa *et al*, 1996a; Seal, 1998; Seal *et al*, 2000). However, there has been no comparative genetic analysis based on N, P, and M2 genes across the subgroups because these genes have never been sequenced from subgroup B viruses.

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Initial studies suggested that APV isolates from the United States were different antigenically and genetically from the European A and B subtypes (Kleven 1997; Seal, 1998; Seal *et al*, 2000). For example an enzyme linked immunosorbent assay (ELISA) developed using European APV isolates as antigen failed in detecting antibodies against the US strain (Kleven, 1997). APV virus has an acute phase for the first week of disease (Jirjis *et al*, 2000). However, paramyxoviruses may cause immunosuppression and enhance susceptibility to extraneous diseases and reduce the ability of animals to respond to routinely used vaccines (Sharma, 1994).

In the present study, I examined the persistency of APV infection in turkey up to 15 weeks postinfection. The transmission of APV from infected birds to healthy birds experimentally was monitored by clinical observation, RT-PCR for genome detection of APV and serology for antibody detection.

Materials and Methods

Animals. Eighty 2-week-old APV antibody free commercial turkeys were obtained from a turkey farm in Minnesota and used for this experiment.

Virus. Avian pneumovirus APV/MN6A isolate was used.

Experimental design. Sixty 2 week-old commercial turkeys, were divided into two groups APV-exposed and unexposed. At day 0, APV/MN6A isolate ($10^{5.3}TCID_{50}$/0.2 ml) was inoculated to turkeys in the exposed groups oculo-nasally. Clinical signs were monitored daily post APV infection. At 4 days and 3, 6, 9, 12, 15 weeks PI, 5 birds from the unexposed group were transferred into the exposed group and commingled. At day 0 (before transfer) and at days 5, 10 and 14 after commingling, choanal swabs were collected from control birds and examined for the presence of APV by RT-PCR. At days 0 and 14, blood from the birds were collected and monitored for APV antibody presence.

RT-PCR of tissue samples. Tissues were homogenized and then centrifuged (11,400 X 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'-ACAGTGTGTGAGTTAAAAG-3', and primer C4 had a nucleotide sequence of 5'-TGACTTCAGGACATATCTC-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 random hexamers as primer. The cDNA synthesis was performed in a reaction mixture of 10 ul containing 5 mM $MgCl_2$, 1 mM of each deoxynucleoside triphosphate (dNTP), 1 unit of RNase inhibitor, 2.5 units of murine leukemia virus reverse transcriptase, and 2.5 uM random hexamer. Conditions for cDNA synthesis were annealing at 20℃ for 10 minutes and reverse transcription at 42℃ for 20 minutes, followed by enzyme inactivation at 99℃ for 5 minutes. The PCR was performed in a 50-*ul* volume of 0.2 μM of each primer, 1 mM of each dNTP, 2 mM $MgCl_2$, and 2.5 units of *Taq* DNA polymerase. The reaction was performed in a thermal cycler, and conditions for amplification were initial denaturation (94℃ for 5 minutes), 35 cycles of annealing (51℃ for 1 minute), extension (72℃ for 2 minutes), and denaturation (94℃ for 1 minute), and final extension (72℃ for 10 minutes). The RT-PCR products then were analyzed on a 1.2% agarose gel.

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 X g for 15 minutes. Supernatant that contained virus was diluted with coating buffer. An aliquot (100 $\mu\ell$) 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 then were washed with PBS solution containing 0.05% Tween 20 and incubated with blocking buffer at 22℃ for 1 hour. Blocking buffer was drained from each plate, and plates were used for assay of antibodies to APV.

A 100 $\mu\ell$-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 22℃ for 1 hour. Positive- and negative-control sera were included in each plate. Plates were washed 3 times with PBS solution, and 100 $\mu\ell$ of goat anti-turkey IgG (diluted 1:1,000 in PBS solution) conjugated to horse-radish peroxidase was added to each well. Plates were incubated for 1 hour and washed with PBS solution. A 100 *ul* volume of substrate-2'-azino-di (3-ethyl-benzthiazoline-6-sulfonate) solution was added to each well, and plates were incubated for 10 minutes in the dark. The reaction then was stopped by adding 100 $\mu\ell$ of stop solution to each well. Absorbance in each well was measured on a spectrophotometer at a setting of 405 nm.

Results

Clinical signs. APV infected turkeys showed signs of respiratory diseases such as coughing, sneezing, swollen sinus between 3 and 8 days PI. After 7 days, no bird showed any clinical signs of those. All birds transferred and commingled at day 4 showed similar clinical signs at days 5 and 10. Only one out of 5 birds transferred at week 3 showed clinical signs only at day 5. No bird transferred at weeks 6, 9, 12 and 15 showed any clinical signs.

RT-PCR. Choanal swab samples from transferred birds at day 4 were positive at days 5, 10 and 14. Especially 4 out of 5 (80 %) swabs from these were positive at day 10, and 4 birds showed clinical signs at the same time. Forty percent were positive at days 5 and 10.

Samples collected from birds transferred at week 3 were positive at days 5 and 10 (40 %). Only one sample was positive at day 5 from birds transferred at week 6. No sample was positive from birds transferred at weeks 9, 12 and 15.

Serology. Sera were collected from all transferred birds at day 0 (before transfer) and 14 days after commingling. Only 3 birds (60 %) from transferred at day 4 produced antibodies for APV at day 14. Any other transferred birds at weeks 3, 6, 9, 12 and 15 did not produce antibodies.

Discussion

In this study, I have shown that APV persists in the choanal swabs. I did not examined the mechanisms of persistence. However, it is well known that viruses with high mutation rates, especially RNA viruses persist by generating attenuated variants or altering epitopes in response to immune pressure (Narayan *et al*, 1988; Pewe *et al*, 1996).

Chary *et al* reported that APV was immunosuppressive, which could be a possible mechanism for persistence. For these viruses to persist they must assume a non-cytolytic phenotype to escape the immune surveillance. The control of long term or persistent viruses has been shown to be complex in LCMV by antibody mediated mechanisms, CD8 cells and cytokines all contributing in the clearance of the virus (Thomsen *et al*, 1996; Planz *et al*, 1997). The elevated antibody titers in the APV infected birds could be a possible mechanism of clearance of the virus (Kagi *et al*, 1995). I have shown the APV tropism in non-lymphoid cells of trachea and turbinates. This is in collaboration with the results from the prior studies (Chary *et al*, 2001). The genome of APV was not detected by RT-PCR, and the clinical and serological responses were not observed from the birds transferred at weeks 9, 12 and 15.

Table 1. Clinical, serological responses and the APV genome detection by RT-PCR at day 4

Days post infection (DPI)[1]	Clinical signs[2]	RT-PCR (n=5)	Serology
0	0	0	0
5	5	2	ND[3]
10	4	4	ND
14	0	2	3

[1]None of the APV infected birds showed clinical signs after 14 days post infection (DPI).
[2]Control birds did not show any clinical signs and remained negative monitored by RT-PCR and serologically.
[3]ND=Not Done

Table 2. Clinical, serological responses and the APV genome detection by RT-PCR at week 3

Days post infection (DPI)[1]	Clinical signs[2]	RT-PCR (n=5)	Serology
0	0	0	0
5	1	2	ND[3]
10	0	2	ND
14	0	0	0

[1]None of the APV infected birds showed clinical signs after 14 days post infection (DPI).
[2]Control birds did not show any clinical signs and remained negative monitored by RT-PCR and serologically.
[3]ND=Not Done

Table 3. Clinical, serological responses and the APV genome detection by RT-PCR at week 6

Days post infection (DPI)[1]	Clinical signs[2]	RT-PCR (n=5)	Serology
0	0	0	0
5	0	1	ND[3]
10	0	0	ND
14	0	0	0

[1]None of the APV infected birds showed clinical signs after 14 days post infection (DPI).
[2]Control birds did not show any clinical signs and remained negative monitored by RT-PCR and serologically.
[3]ND=Not Done

Table 4. Clinical, serological responses and the APV genome detection by RT-PCR at week 9

Days post infection (DPI)[1]	Clinical signs[2]	RT-PCR	Serology
	(n=5)		
0	0	0	0
5	0	0	0
10	0	0	0
14	0	0	0

[1]None of the APV infected birds showed clinical signs after 14 days post infection (DPI).
[2]Control birds did not show any clinical signs and remained negative monitored by RT-PCR and serologically.

Table 5. Clinical, serological responses and the APV genome detection by RT-PCR at week 12

Days post infection (DPI)[1]	Clinical signs[2]	RT-PCR	Serology
	(n=5)		
0	0	0	0
5	0	0	0
10	0	0	0
14	0	0	0

[1]None of the APV infected birds showed clinical signs after 14 days post infection (DPI).
[2]Control birds did not show any clinical signs and remained negative monitored by RT-PCR and serologically.

Table 6. Clinical, serological responses and the APV genome detection by RT-PCR at week 15

Days post infection (DPI)[1]	Clinical signs[2]	RT-PCR	Serology
	(n=5)		
0	0	0	0
5	0	0	0
10	0	0	0
14	0	0	0

[1]None of the APV infected birds showed clinical signs after 14 days post infection (DPI).
[2]Control birds did not show any clinical signs and remained negative monitored by RT-PCR and serologically.

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칠면조에서 Avian Pneumovirus(APV)의 지속 감염에 대한 연구

신현진

미국 미네소타 수의과대학, Pathobiology과

(2001년 12월 3일 게재승인)

국문초록 : 칠면조에서 avian pneumovirus(APV)의 지속감염성 여부를 조사하였다. 칠면조에 APV를 감염시킨 후 4일, 3주, 6주, 9주 후에 건강한 칠면조와 합사하였다. 감염 3주 후에 합사한 건강한 칠면조에서 10일까지 2수에서 APV 유전자를 검출할 수 있었다. 또한 감염 6주 후에 합사한 경우에서도 5일까지 RT-PCR법으로 유전자를 검출할 수 있었다. 그 이후로는 감염칠면조에서 건강한 동물로 전파되었거나 지속감염을 관찰할 수 없었다. APV는 감염 후 6주까지 지속적인 감염을 통해 건강한 동물에게 질병을 전파시킬 수 있다.

중심어 : Avian pneumovirus, 지속감염, 칠면조, RT-PCR