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Murine susceptibility to Avian pneumovirus (APV) of turkey origin

Hyun-Jin Shin*

College of Veterinary Medicine, University of Minnesota, St. Paul, MN 55108, U.S.A.

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Abstract : The infectivity of an isolate of avian pneumovirus (APV) from turkeys to Balb/c mice was investigated to examine the transmission possibility to mammals. Three different age groups (3, 5 and 7 weeks old) were exposed by oculonasal route with a cell cultured APV of turkey origin. No clinical signs were observed from both APV-inoculated and commingled mice. However, all the tissue samples including blood from mice in the APV-inoculated group were positive for APV by polymerase chain reaction (PCR) up to 6 days postinoculation. At 14 days postinoculation, APV was not detected from blood samples by PCR, but sera showed the presence of APV-specific antibodies. In commingled mice, APV was detected from lung and rectal swap samples by PCR. These results suggest that an APV isolate from turkey could be transmitted to mice by direct contact or other ways.

Key words : Avian pneumovirus, transmission, PCR

Introduction

Pneumoviruses are members of the sub-family pneumovirinae under the family paramyxoviridae. Avian pneumovirus (APV) is classified under the genus Metapneumovirus (16). APV has a single stranded non-segmented RNA (18, 21), and has a close genetic homology with human and bovine respiratory syncytial viruses (22).

Avian pneumovirus (APV) is a newly emergent paramyxovirus belonging to the *Metapneumovirus* genus that causes acute rhinotracheitis in turkeys (hence the original name turkey rhinotracheitis virus) characterized by coughing, nasal discharge, tracheal rales, foamy conjunctivitis and sinusitis in turkeys of all ages (1, 2). In young turkeys, the disease can be severe with a morbidity of 100% and a mortality of up to 25% if accompanied by the secondary bacterial and/or viral infections (3). APV was first detected in South Africa in 1978, but quickly spread to the United Kingdom, France, Spain, Germany, Italy, the Netherlands, Israel, and Asia (3, 4). The United States was free from APV until 1996, when the 10-month outbreak of upper the respiratory system infection among turkeys in Colorado resulted in the isolation of APV (5, 6). The virus was detected in 1997 in Minnesota, the north central U.S. state, where the incidence of disease has increased over the past four years, in part, because Minnesota is the largest turkey producing state in U.S. A.(7). Recently, the disease has been detected in commercial turkeys in North Dakota and South Dakota (8). Analysis of genetic sequences from 15 APV isolates from U.S. turkeys between 1996 and 1999 revealed a homogenous subgroup (subgroup C) of viruses different from the European subgroups A and B (8). The description of a third subgroup of APVs and a recent report suggesting the emergence of a fourth subgroup indicates that the avian paramyxoviruses are more heterogenous than the mammalian pneumoviruses (9). This study was performed to evaluate the possibility of infection and transmission of a turkey isolate of APV in mice.

Materials and Methods

Mice. Two-week-old Balb/c mice negative for APV antibody were obtained from a commercial source in Minnesota and maintained in isolators under the negative pressure.

Virus. APV/MN2A isolate which originally isolated in Minnesota from turkeys showing the respiratory disease was used (4). The virus was propagated in Vero cells and the virus titer in cell culture harvest was determined. The virus titer for the experimental inoculation was adjusted to contain $10^{5.2}$

*Corresponding author : Phone:1-312-503-1354 e-mail: shin0089@hotmail.com

$TCID_{50}$ /ml (1, 17). The titrated virus was stored in the cell culture medium with 4 % fetal bovine serum, 1 % L-glutamine, 1 % non-essential amino acids, penicillin (0.5 U/ml) and streptomycin (0.5 g/ml).

Experimental design. Ten Balb/c mice of different age (3, 5 and 7 weeks old) were divided equally into two groups and placed in isolators. Mice in each age group 1 were inoculated with 200 $\mu\ell$ of APV per mouse by the oculo-nasal route. Mice in each age group 2 were kept as the un-inoculated controls. Mice in inoculated and un- inoculated groups were monitored daily for any clinical signs. On days 4, 6 and 14, 2 mice from each group were sacrificed and samples (blood, lung, trachea, mouth and rectal swabs) were collected and examined the presence of APV by RT-PCR. To monitor whether APV can be transmitted by contact exposure, two mice from each age were replaced with new two same aged mice on days 4 and 6. After 8 and 10 days commingling with the infected mice, same tissues were also collected from those contacted control mice.

Viral RNA extraction. Samples were homogenized and centrifuged at 10,000 xg. The supernatants were collected in sterile tubes and kept -70℃ until use. The supernatants were retained for viral RNA extraction for APV monitoring. Blood was subjected to RT-PCR analysis and serology. Viral RNA extraction for RT-PCR was performed using the RNA extraction kit (Qiagen, Valencia, CA) following the manufacturer's instruction.

PCR Primers. Two primers (C3 and C4) were designed to amplify the M gene of APV/MN2A isolate. C3 (positive sense) was composed of 5'-ACAGTGTGTGAGTTAAAAG-3' and C4 (negative sense) primer was composed of 5'-TGACTTCAGGACATATCTC-3' (19).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). The cDNA was prepared by using random hexamers (Perkin Elmer, Foster City, CA) in 10l of the reaction mixture. The conditions for cDNA synthesis included annealing at 20℃ for 10 min and extension at 42℃ for 20 min, followed by denaturation and the enzyme inactivation at 99℃ for 5 min.

Ten $\mu\ell$ of cDNA was used as a template for PCR amplification. The conditions for amplification included an initial denaturation at 94℃ for 5 min followed by repeated 35 cycles of annealing at 51℃ for 1 min, extension at 72℃ for 2 min and denaturation at 94℃ for 1 min, followed by the final extension at 72℃ for 10 min. PCR products were analyzed in 1.2% agarose gel electrophoresis. The Colorado strain was used for the positive control and cell culture medium was used for the negative control(13, 15, 18, 19).

Virus isolation(VI). Samples were homogenized with Minimum Essential Medium [(MEM), GIBCO BRL, Chagrin Falls, Ohio] at 1:10 ratio. One hundred microliters of homogenized suspension was put on Vero cell monolayer containing MEM supplemented with 1 % fetal bovine serum, 1 % amphotericin B, 1 % non-essential amino acids, 1 % L-glutamin, and 1 % streptomycin-penicillin. The Vero cells treated with the homogenized samples were incubated in 10 % CO_2 (4, 10, 14, 20). The formation of syncytia on Vero cells was considered to be caused by APV infection and the supernatants were tested by RT-PCR for confirmation.

Enzyme linked immunosorbent assay (ELISA). Wells of flat bottom 96 well microplates (Nunc, Naperville, IL) were coated with APV for 5 hrs at 4℃. Briefly, Vero cells infected with APV were removed from culture plates and freeze thawed twice. The Vero cell suspension was centrifuged at 8,000 xg for 15 min. The supernatant containing virus was suspended in carbonate coating buffer. The virus suspension in carbonate buffer was used to coat the microplates. One hundred microliters of virus suspension in carbonate buffer was dispensed into each well and allowed to set for 5 hrs. After 5 hrs of inoculation, the plates were washed with PBS containing 0.05 % Tween 20 and incubated with the blocking buffer (PBS containing 1 % fetal calf serum, KPL) for 1 hr at room temperature. The plates were drained off the blocking buffer and used for the assay of antibodies to APV. For assay of antibodies, one hundred $\mu\ell$ from each serial dilution of serum to be tested (1:20, 1:40, 1:100) in dilution buffer (PBS with 1 % fetal calf serum), was placed in each well and the plates were incubated at room temperature for 1 hr. The plates were washed three times with PBS and each well was added with 100 $\mu\ell$ of goat anti-mouse IgG (diluted 1:1000 in PBS) conjugated to horseradish peroxidase (KPL). The plates were incubated for 1 hr and washed as before with PBS. Each well was then dispensed with 100 $\mu\ell$ of the substrate -ABTS (KPL) and incubated for 10 min in the dark. At the end of 10 minutes, the reaction was stopped by adding 100 $\mu\ell$ /well of stop solution (PBS with 1 % SDS). The absorbance in each well was measured at 405 nm wave-length (3, 5, 6, 11, 12).

RESULTS

Clinical signs. Most mice, both control and infected mice, did not produce any significant clinical signs. No respiratory distresses as characterized in the APV-infected turkeys such as coughing, sneezing, nasal discharge was observed in mice exposed to APV of turkey origin. Contact control mice also didn't show any clinical signs.

RT-PCR results of collected tissue samples. Most tissue samples from mice in the APV-inoculated were positive for APV by RT-PCR up to 14 days post inoculation (Table 1). There were no differences in RT-PCR results in the different ages. All samples from mice in the APV un-inoculated control groups were negative for APV by RT-PCR (Table 1). APV was detected from lungs and rectal swab samples from the contact control mice at 8 and 10 days after commingling. Trachea was positive after 10 days commingling (Table 2).

Virus isolation. No infectious virus particles were re-isolated from any sample (Table 1).

Serology. ELISA results are described in Table 1. All the sera from the inoculated groups on day 14 showed the presence of APV-specific antibodies. Sera from the un-inoculated control groups did not show antibodies to APV throughout the study. Sera from commingled contact control mice did not contained the antibodies to APV.

Table 1. Results of RT-PCR, Virus Isolation and Serology from Tissues

Treatment	Age (weeks)	DPI	RT-PCR					Virus Isolation	Serology
			Bl	Lu	Tr	MS	RS		
Un-inoculated	3	4	-	-	-	-	-	-	-
	5		-	-	-	-	-	-	-
	7		-	-	-	-	-	-	-
	3	6	-	-	-	-	-	-	-
	5		-	-	-	-	-	-	-
	7		-	-	-	-	-	-	-
	3	14	-	-	-	-	-	-	-
	5		-	-	-	-	-	-	-
	7		-	-	-	-	-	-	-
Inoculated	3	4	+	+	+	+	-	-	-
	5		+	+	+	+	-	-	-
	7		+	+	+	+	-	-	-
	3	6	+	+	+	-	-	-	-
	5		+	+	+	-	-	-	-
	7		+	+	+	-	-	-	-
	3	14	-	+	+	-	-	-	+
	5		-	+	+	-	-	-	+
	7		-	+	+	-	-	-	+

DPI; Days Post-Infection, Bl; Blood, Lu; Lung, Tr; Trachea, MS; Mouth Swab, RS; Rectal Swab

Table 2. Transmission of APV from Infected Mice to Commingled Contact Mice

Age (weeks)	DPI	RT-PCR					Virus Isolation	Serology
		Bl	Lu	Tr	MS	RS		
3	8	-	-	-	-	+	-	-
5	8	-	-	-	-	+	-	-
7	8	-	+	-	-	-	-	-
3	10	-	-	+	-	-	-	-
5	10	-	+	-	-	+	-	-
7	10	-	+	-	-	+	-	-

DPI; Days Post-Infection, Bl; blood, Lu; lung, Tr; trachea, MS; Mouth Swab, RS; Rectal Swab

Discussion

In this study, we examined the ability of the avian pneumovirus of Minnesota turkey origin to infect the Balb/c mice. Mice experimentally infected with APV were monitored for any clinical signs of APV infection, for the persistence of virus in different tissues and for serological response to the inoculated APV. No mice exhibited signs of the respiratory illness including coughing, sneezing and nasal discharge which are characteristic in the APV infected turkeys.

RT-PCR results have shown the persistence of viral RNA in the different tissues of up to 14 days PI. There were no differences between the different ages. The fact that viral nucleic acid was found in the blood of mice exposed to APV raises the possibility of occurrence of viremia in these mice. Although no infectious virus particles were re-isolated from these RT-PCR positive samples, APV RNA was persistently detected from most of tissues.

Blood samples from the inoculated groups showed sero-positive for antibodies to APV at day 14 post-inoculation. None of the sera collected at days 4 and 6 from inoculated group had detectable antibodies to APV.

APV genome was detected from lungs, trachea and rectal swab samples of commingled contact mice based on RT-PCR results. However, any infectious virus particles were not re-isolated from these samples. The commingled contact mice did not produce the antibodies. Although they did not show any clinical signs of respiratory illness, they might probably get APV from the inoculated mice based on RT-PCR results.

References

1. Burleson. Virology, Laboratory Manual, 1992 Academic Press.
2. Buys, S. B., and J. H. Preez. The isolation and attenuation of a virus causing rhinotracheitis in turkeys in South Africa. J. Vet. Res. 1989, 56, 87-98.
3. Chettle, N. J., and P. J. Wyeth. Turkey rhinotracheitis: Detection of antibodies using an ELISA test. Brit. Vet. J. 1988, 144, 282-287.
4. Chiang, S. J., A. M. Dar, S. M. Goyal, K. V. Nagaraja, D. A. Halvorson, and V. Kapur. Isolation of Avian *pneumovirus* in QT-35 cells. Vet. Rec. 1998, 143, 596.
5. Chiang, S. J., A. M. Dar, S. M. Goyal, J. C. Pederson, B. Panigrahy, D. A. Halvorson, K. V. Nagaraja, and V. Kapur. Seroprevalence of Avian *pneumovirus* in Minnesota turkeys. J. Vet. Diag. Inv. (in press, 1999).
6. Cook, J. K. A., and C. A. Dolby. Demonstration of antibodies to turkey rhinotracheitis virus in serum from commercially reared flocks of chickens. Avian. Pathol. 1988, 17, 403-410.
7. Dani, M. A. C., E. L. Durigon, and C. W. Arns. Molecular characterization of Brazilian avian pneumovirus isolates: comparison between immunochemiluninescent Southern blot and nested PCR. J. Vir. Met. 1999, 79, 237-241.
8. Giraud, P., and G. Bennejean. Turkey rhinotracheitis in France: Preliminary investigations on ciliostatic virus. Vet. Rec. 1986, 119, 606-607.
9. Goyal, S.M., S. Chiang, A. M. Dar, K. V. Nagaraja, D. A. Halvorson, and V. Kapur. Isolation of Avian *pneumovirus* from an outbreak of respiratory illness in Minnesota Turkeys. J. Vet. Diagn. Invest. (in press, 1999).
10. Gough, R. E. Isolation of an avian pneunovirus from broiler chickens. Vet. Rec. 1994, 34(14),353-354.
11. Grant, M., and J. C. Baxter. An enzyme-linked immunosorbent assay for the serodiagnosis of turkey rhinotracheitis infection. Vet. Rec. 1987, 120, 279-278.
12. Heckert, R. A, D. J. Myers, A. Afshar, and J. Riva. Development and evaluation of an Enzyme-linked Immunosorbent Assay for the Detection of Antibodies to Avian Pneumovirus. Avian. Dis. 1994, 38, 694-700.
13. Li, J., K. A. Jane, T. Cook, K. David, S. Kathleen, and D. Cavanagh. Detection of turkey rhinotracheitis virus in turkeys using the polymerase chain reaction. Avian Pathol. 1993, 22, 771-783.
14. Maharaj, S. B. Isolation of an avian pneumovirus -like agent from broiler breeder chickens in South Africa. Vet. Rec. 1994, 134(20), 525-526.
15. Mase, M., A. Setsuo, I. Kunotoshi, N. Kikuyasu, and Y. Sigeo. Detection of turkey rhinotracheitis virus from chickens with swollen head syndrome by reverse transcriptase-polymerase chain reaction (RT-PCR). J. Vet. Med. Sci. 1996, 58(4), 359-361.
16. Pringle, C. R. Virus Taxonomy-1999. Arch. Virol. 1999, 144/2, 421-429.
17. Reed, L. S., and H. Muench. A simple method of estimating fifty percent end points. Amer. J. Hyg. 1938, 27. 493-497.
18. Seal, B. S. 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.
19. Shin, H. J., G, Rajashekara, F.F, Jirjis, D.P. Shaw, S. M. Goyal, D.A. Halvorson and K.V. Nagaraja. Specific detection of avian pneumovirus(APV) US isolates by RT-PCR. Arch Virol. 2000, 145(6), 1239-1246.
20. Tanaka, M. Turkey rhinotracheitis virus isolation from broiler chicken with swollen head syndrome in Japan. J. Vet. Med. Sci. 1995, 57(5),939-941.
21. Yu, Q., and P. J. Davis. Cloning and sequencing of the matrix protein gene of turkey rhinotracheitis virus reveal a gene order different from that of respiratory syncytial virus. Virology 1992, 186, 426-434.
22. Yu, Q., P. J. Davis, T. Barrett, M. M. Binns, M. E. G. Boursnell, and D. Cavanagh. Deduced amino acid sequence of the fusion glycoprotein of turkey rhinotracheitis virus has greater identity with that of human respiratory syncytial virus, a pneumovirus, than that of paramyxoviruses and morbilliviruses. J. Gen. Virol. 1991, 72, 75-81.

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칠면조에서 분리된 Avian pneumovirus(APV)의 쥐의 감염성에 대한 연구

신현진

미국 미네소타 수의과대학, Pathobiology과

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국문초록 : 칠면조에서 분리된 avian pneumovirus(APV)의 포유동물 전파 여부를 확인하기 위해 Balb/c 쥐에 감염을 시도하였다. APV를 3주, 5주, 7주령의 실험동물에 비강내 감염시킨 후 감염동물과 동거동물의 임상증상 및 조직내 바이러스 유전자 존재 여부등을 확인하였다. 감염 동물 및 동거 동물에서 임상증상은 나타나지 않았으나 감염 6일후에 혈액, 폐, 기도, 구강 및 직장 시료에서 RT-PCR 방법으로 APV의 유전자를 검출할 수 있었다.

14일 후에는 혈액에서는 유전자가 검출되지 않았으나 혈청내 항체를 확인할 수 있었다. 동거동물에서는 폐와 직장시료에서 PCR로 APV를 검출할 수 있었다.

이와 같은 결과로 칠면조 유래 APV는 실험쥐에 감염되어 직접 접촉에 의한 전파도 가능하다는 것을 알 수 있었다.