Molecular Epidemiology of Subgroup C Avian Pneumoviruses Isolated in the United States and Comparison with Subgroup A and B Viruses

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The avian pneumovirus (APV) outbreak in the United States is concentrated in the north-central region, particularly in Minnesota, where more outbreaks in commercial turkeys occur in the spring (April to May) and autumn (October to December). Comparison of the nucleotide and amino acid sequences of nucleoprotein (N), phosphoprotein (P), matrix (M), fusion (F), and second matrix (M2) genes of 15 U.S. APV strains isolated between 1996 and 1999 revealed between 89 and 94% nucleotide sequence identity and 81 to 95% amino acid sequence identity. In contrast, genes from U.S. viruses had 41 to 77% nucleotide sequence identity and 52 to 78% predicted amino acid sequence identity with European subgroup A or B viruses, confirming that U.S. viruses belonged to a separate subgroup. Of the five proteins analyzed in U.S. viruses, P was the most variable (81% amino acid sequence identity) and N was the most conserved (95% amino acid sequence identity). Phylogenetic comparison of subgroups A, B, and C viruses indicated that A and B viruses were more closely related to each other than either A or B viruses were to C viruses.

The *Turkey rhinotracheitis virus*, commonly referred to as avian pneumovirus (APV), is a member of the *Paramyxoviridae* family that causes acute rhinotracheitis in turkeys that is characterized by coughing, nasal discharge, tracheal rales, foamy conjunctivitis, and sinusitis in turkeys of all ages. In laying birds, there is a transient drop in egg production, along with mild respiratory tract illness (11). 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 (11). APV was first detected in South Africa in 1978 but was isolated soon thereafter in the United Kingdom, France, Spain, Germany, Italy, The Netherlands, Israel, and Asia (1, 11). The United States was free of APV infection until 1996, when APV was isolated from turkeys in Colorado during a 10-month outbreak of upper respiratory disease (14, 35). The APV outbreak in Colorado was controlled by intense biosecurity measures, and the disease has not been reported there since early 1997 (14). However, APV was detected in 1997 in Minnesota, where the incidence of disease has increased over the past 4 years despite the establishment of biosecurity measures similar to those used in Colorado (3, 6, 10).

Mammalian pneumoviruses encode 10 proteins that include nonstructural (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 (29, 31) and has a smaller L gene than

mammalian pneumoviruses (33). The gene order of APV is 3'-N-P-M-F-M2-SH-G-L-5' (20, 46). Based on these differences, the APVs were classified in the genus *Metapneumovirus*, whereas their mammalian counterparts belong to the genus *Pneumovirus* (29). Two subgroups (A and B) of APV were defined in Europe; these subgroups were initially based on the level of genetic variations in the attachment (G) glycoproteins of APV strains (12). Subsequent studies showed significant heterogeneity in M and F gene nucleotide sequences between the two subgroups so that the genetic composition of these genes could also be used to distinguish between the subgroups (32, 44, 45). However, there has been no comparative genetic analysis based on P and M2 genes across the subgroups because these genes have never been sequenced from subgroup B viruses.

Initial studies suggested that APV isolates from the United States were different antigenically and genetically from the European A and B subtypes (14, 35, 36). For example, an enzyme-linked immunosorbent assay (ELISA) developed with European APV isolates as antigen failed in detecting antibodies against the U.S. strain (14). In addition, sequence comparison of M and F genes from three 1997 APV isolates from the United States revealed that these genes shared 98% nucleotide sequence identity among one another, but only 60 to 70% nucleotide sequence identity with APV subgroup A and B viruses (35, 36). As a result of these differences, it was suggested that the U.S. isolates should be classified as subgroup C (35, 36). Anecdotal evidence from APV outbreaks in Minnesota suggested that turkeys infected early (i.e., at 1 to 3 weeks of age) could develop a second APV infection later, raising the possibility that multiple strains of virus may be responsible for the disease (16). To better understand the epidemiology of

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TABLE 1. History of the APVs isolated in the United States^a

Isolate	Month	Yr	State
APV/CO	November	1996	Colorado
APV/MN1A	November	1997	Minnesota
APV/MN1B	November	1997	Minnesota
APV/MN2A	November	1997	Minnesota
APV/MN2B	November	1997	Minnesota
APV/MN4A	April	1999	Minnesota
APV/MN4B	April	1999	Minnesota
APV/MN4C	April	1999	Minnesota
APV/MN5	April	1999	Minnesota
APV/MN6	July	1999	Minnesota
APV/MN7	October	1999	Minnesota
APV/MN8	November	1999	Minnesota
APV/MN9	November	1999	Minnesota
APV/MN10	November	1999	Minnesota
APV/MN11	December	1999	Minnesota

^a All viruses were isolated from turkeys. Viruses isolated from the same farm were assigned the same number, e.g., MN4A, MN4B, and MN4C. The APV/MN3 was lost.

APV in the United States, the annual incidence of the disease and the genetic composition of 15 viruses isolated from naturally infected commercial turkeys over 4 years were investigated. In order to perform genetic comparison between the three subgroups of APV, several APV subgroup B (Hungary/657/4) genes were isolated and sequenced for the first time.

MATERIALS AND METHODS

Viruses. Fourteen APV strains were isolated from naturally infected turkeys in the state of Minnesota between 1997 and 1999 (Table 1). Virus isolation was attempted from 110 samples collected from commercial turkeys showing clinical signs of APV infection. Nasal turbinate tissues or swabs from the choanal cleft and trachea were collected from exposed asymptomatic birds (in barns where other birds were showing overt clinical signs of APV) or from birds with early clinical signs (rales, mild infraorbital sinus swellings, etc.) and processed for APV isolation as described previously (7). Briefly, samples were homogenized and cultured in chicken embryo fibroblasts for five blind passages and then cultured in Vero cells. In Vero cells, cytopathic changes could be observed as early as in the second passage. Isolates were confirmed by immunofluorescence with APV-specific polyclonal antibody. The APV/Colorado isolate (APV/CO) and Hungary/657/4 (subgroup B APV) were obtained from National Veterinary Services Laboratory, Animal Plant Health Inspection Service, U.S. Department of Agriculture in Ames, Iowa. The subgroup B virus was propagated in Vero cells under biosafety level 3 conditions at the Southeast Poultry Research Laboratory, U.S. Department of Agriculture, Athens, Ga. After isolation, the Minnesota viruses were passaged for between 5 and 10 times before being sequenced, whereas the passage history of the European A and B viruses used in sequence comparisons was not known.

Monitoring the APV outbreak. To determine the annual incidence of APV outbreaks in United States, 72 privately owned turkey farms located in central and southern regions of Minnesota were monitored for APV outbreaks. Each farm had between 30,000 and 280,000 commercial turkeys housed in one to several barns. Turkey flocks were inspected daily for signs of respiratory disease. Upon development of clinical signs, turbinate tissues were collected from 10 birds for reverse transcription-PCR (RT-PCR) and serum samples were collected for ELISA (3, 37). The ELISA was performed routinely by using whole

APV/CO lysate and turkey sera at a 1:40 dilution as described previously (7). An absorbance reading of at least 0.2 at a 490-nm wavelength in at least 5 of the 10 samples constituted a positive flock, whereas two to four positive samples constituted a suspect flock that was reanalyzed a week later for confirmation. The RT-PCR detection was performed as described previously (37). In addition, turkey sera from six counties with the highest incidence of APV (Kandiyohi, Meeker, Morrison, Stearn, Swift, and Todd) were collected from the rendering plant (10 samples per flock) and tested for APV antibodies. For the rendering plant flocks, an absorbance reading of at least 0.2 at a 490-nm wavelength in at least 5 of the 10 samples constituted a positive flock, whereas fewer than 5 positive samples was considered negative for the purpose of statistical analysis. Typically, APV-positive flocks were taken for slaughter 4 to 6 weeks after the outbreaks. The monthly incidence of APV outbreaks (number of farms) was compared by using Student's *t* test.

RNA extraction, RT-PCR, cloning, and sequencing. The N, P, M, F, and M2 genes of each of the 15 viruses were amplified and sequenced for nucleotide and predicted amino acid comparison. RNA from the virus-infected Vero cells was extracted by using guanidinium thiocyanate-phenol-chloroform reagent (Gibco-BRL, Rockville, Md.) and reverse transcribed with random hexamer oligonucleotides (Gibco-BRL) with Moloney murine leukemia virus reverse transcriptase at 42°C for 1 h (4, 15). Primers for amplification of N, P, M, F, and M2 genes of APV were designed from consensus sequences of APV (36) (GenBank accession numbers AF187152, AF187153, AF176590, AF176591, and AF176592) as presented in Table 2. After RT, 5 to 10 µl of the reaction mixture was used for PCR. The cDNA was denatured at 95°C for 5 min, followed by 35 cycles of denaturation (95°C for 1 min), annealing (55°C for 1 min), and elongation (72°C for 1 min). The PCR products were gel purified (Qiagen, Valencia, Calif.) and cloned into PCR2.1 plasmid vector (Invitrogen, San Diego, Calif.) (22). The subcloned APV genes were sequenced with T7 forward and M13 reverse primers. Two independent clones of each gene plus the PCR products were sequenced for each of the 15 viruses with automated DNA sequencers (40).

Nucleic acid sequence analysis. Nucleotide sequence editing, prediction of amino acid sequences, and protein computer structure predictions were performed using Intelligenetics GeneWorks 2.5.1TM (Mountain View, Calif.) and DNASTar (Madison, Wis.) software. Alignments were performed by using the CLUSTALW method (42). The nonsynonymous and synonymous substitutions were determined by the method of Nei and Gojobori (24) in a molecular evolutionary genetics analysis program (17). To determine relationships among APV isolates, phylogenetic analysis of the aligned sequences was performed by using PAUP* software (41) by both parsimony and neighbor-joining analyses. Both analyses resulted in duplicate phylogenetic relationships and were evaluated by testing 2,000 bootstrap replicates. The nucleotide sequences of the five genes of APV subgroup A (N, P, F, M, and M2) and two genes of subgroup B (M and F) were as published (GenBank accession numbers Y14291 to Y14294, U37586, U22110, and U39295 to U39296). The bovine respiratory syncytial virus sequences were used as outgroups for comparative analysis across subgroups (21, 26, 34) (GenBank accession numbers M35076 and M82816). The nucleotide sequences for the 15 U.S. viruses have been submitted to GenBank with the following accession numbers: F genes, AAF01768 to AAF01772; M genes, AAG30828 to AAG30846; P genes, AY028569 to AY028582; N genes, AY028556 to AY028568; and M2 genes, AY028542 to AY028555. The accession numbers for APV/B virus (Hungary/647/4) N, P, and M2 gene nucleotide sequences are AF325442, AF325443, and AF356650, respectively.

RESULTS AND DISCUSSION

Seasonal occurrence of APV outbreaks. To determine the seasonal pattern of APV outbreaks in United States, 72 turkey farms were monitored daily for clinical signs of APV over a 2-year period (January 1998 to December 1999), and out-

TABLE 2. Primers used for isolation of APV genes

Gene	5' Primer (5'-3') sequence	3' Primer (5'-3') sequence
N	GGGACAAGTGAAAATGTCTCTTCAGGGGATTCAGCTTAGT	ACTCATAATCATTCTGGCCT
P	GGGACAAGTCAAAAATGTCCTTTTC	GTTTTTTATTAACATAGTAAGGGAGTATAGGTCATC
M	GGGGACAAGTIAAIIATGGAGTC	GGGAGGCCCTTTTTTTTTTTTTTTT
F	GGGACAAGTGAAAATGTCTTTGG	GGGAGGCCCTTTTTTTTTTTTTTTT
M2	GGGACAAGTGAAGATGTCTCGCAAGGCT	AATTTTTTTATTAACCTAGAAATGTTTAAACATAAATGAT

FIG. 1. High incidence of APV in spring and autumn. The monthly incidence of APV was analyzed in 72 turkey farms (black bars) by daily clinical evaluation over 2 years, and disease outbreak was confirmed by ELISA. The number of birds at each farm ranged from 30,000 to 280,000 turkeys. Data were based on ELISA tests of 10 samples from each flock showing clinical signs of APV. A flock was declared positive when at least 5 of the 10 samples gave a positive ELISA reading (optical density of ≥ 0.2). Flocks with two to five positive samples were retested a week later to confirm the result. In addition, turkey sera from flocks taken for slaughter from six Minnesota counties that had the highest incidence of APV (10 samples per flock) were tested for APV antibodies (shaded bars). Data are shown as the percent APV-positive flocks.

breaks were confirmed by ELISA for APV antibodies (detected 2 weeks after the onset of clinical signs) or by RT-PCR for viral RNA (detected 3 to 10 days after onset of clinical signs). Approximately the same numbers of birds were maintained in the farms throughout the year. There were outbreaks on 55 farms during 1998 and on 109 farms during 1999, with some farms reporting more than one outbreak in a year. Over the 2-year period, 39% of the outbreaks occurred between April and May, whereas 40.8% occurred between October and December (Fig. 1), which was significantly higher than in the other 7 months ($P < 0.001$). The mean ($\pm$ the standard deviation) number of farms that were positive for APV outbreaks in these high-incidence months (April to May and October to December) was 13.2 ± 7.5 farms per month, whereas the mean in the low-incidence months was 2.4 ± 1.3 farms per month. To further monitor the seasonal trend of APV outbreaks, APV tests (ELISA) were performed in all turkey flocks received for slaughter from six Minnesota counties previously shown to have the highest incidence of APV outbreaks (Kandiyohi, Meeker, Morrison, Stearns, Swift, and Todd counties). Turkey sera were collected at the rendering plant (10 samples per flock), which was typically 4 to 6 weeks after clinical APV disease because farmers waited this long in an effort to minimize the incidence of APV-induced air sacculitis detectable at slaughter, which resulted in bird condemnation. On average, 93 and 76 flocks per month were tested from the six counties in 1999 and 2000, respectively. The mean ($\pm$ the standard error) percent APV-positive flocks from May to June and from November to January (a total of 5 months) was $83\% \pm 2.3\%$

flocks per month, which was significantly higher than the $60\% \pm 3.6\%$ APV-positive flocks per month in the other 7 months (February to April and July to October) of the year ($P < 0.005$, Fig. 1).

The high incidence of APV in spring (April to May) and autumn (October to December) may have been due to the migratory patterns of wild birds, which according to recent studies may be involved in spreading the virus (38, 39). RNA was isolated from nasal turbinate samples of wild sparrows, geese, swallows, and starlings (39). More recently, infectious APV (APV/MN12) was isolated from sentinel ducks placed in a pond neighboring a turkey farm (200 m away) and shown to have high nucleotide sequence identity with four APV strains (APV/MN4A, APV/MN4B, APV/MN4C, and APV/MN5) isolated from the neighboring turkey farm (38) (GenBank accession numbers AF368170 to AF368174). There are no studies documenting the seasonal trends of APV outbreaks in Europe. However, in both human and bovine respiratory syncytial viruses, seasonal activities have been observed, with most outbreaks being recorded during the rainy or colder months (5, 8, 25, 43).

Homogeneity of U.S. viruses. To determine whether the outbreaks in the United States were due to the emergence of genetically different APV strains, 14 viruses were isolated from the outbreaks in Minnesota over 3 years (1997 to 1999; Table 1) and the sequences of N, P, M, F, and M2 genes compared with the Colorado isolate (APV/CO). A total of 110 (59 turbinate pools and 51 swab pools) turkey samples were processed over the 3 years, resulting in 14 isolates.

TABLE 3. Comparison of N, P, M, F, and M2 proteins of subgroup A, B, and C APVs

Protein	Size (no. of amino acids)			% Identity				
	Subgroup A	Subgroup B ^a	Subgroup C (U.S. viruses)	Among subgroup C viruses ^b	Between subgroups A and B	Between subgroups A and C	Between subgroups B and C ^a	Between subgroups A, B, and C ^a
N	391	385	394	95	91	70	70	67
P	278	279	294	81	71	53	52	46
M	274	272	254	88	89	78	77	77
M2	259	259	255	86	89	70	65	68
F	538	538	537	91	84	72	71	71

^a The APV/CO virus was used as the representative for subgroup C strains in comparisons among subgroups and compared with strains of A and B viruses available in GenBank. The N, P, and M2 genes of European subgroup B viruses that were not previously available were isolated from the Hungary/647/N strain (GenBank accession numbers AF325442, AF325443, and AF356650).

^b GenBank accession numbers AAF01768 to AAF01772, AAG30828 to AAG30846, and AY028542 to AY028582. Identity comparison was based on predicted amino acids from nucleotide sequences.

The protein sizes and amino acid sequence comparison among the U.S. viruses and between U.S. viruses and European viruses are summarized in Table 3. Each gene contained one to two open reading frames, as well as noncoding sequences. The N gene consisted of 1,207 nucleotides in a single open reading frame encoding a protein of 394 amino acids. Both the nucleotide and the predicted amino acid sequence identity among the 15 U.S. isolates was 95%. The P gene contained 910 nucleotides encoding a single protein of 294 amino acids. The P gene was the most variable of those analyzed among the U.S. APV isolates, consisting of 89% nucleotide sequence identity and 81% predicted amino acid sequence identity (Table 3). The M genes from U.S. viruses had

871 nucleotides and encoded a single protein of 254 amino acids. The gene had a nucleotide sequence identity of 92% and a predicted amino acid sequence identity of 88% among the U.S. isolates. The M2 gene was 750 nucleotides in size with two open reading frames. The major open reading frame (positions 14 to 569) encoded a 184-amino-acid protein, whereas the second minor open reading frame (positions 525 to 740) encoded a small protein of 71 amino acids. The M2 nucleotide sequence identity was 91% and predicted amino acid sequence identity 86% among the U.S. isolates.

The F gene sequences of U.S. viruses had 1,666 nucleotides, with a single open reading frame encoding a protein of 537 amino acids. The F gene protein had a cleavage site located

FIG. 2. Comparative amino acid sequence alignment and phylogenetic relationships among APVs based on fusion (F) protein. Sequences aligned and amino acid differences denoted by the single-letter code with consensus below are shown for amino acids 315 to 421 in order to compare viruses in subgroup A, B, C, and D viruses. After alignment, a rooted phylogram was generated by maximum-parsimony analysis by using bovine respiratory syncytial virus (BRSV) as an outgroup. Absolute distances are listed above each branch, with bootstrap confidence levels given in parentheses below. APV/MN1 to -11 and APV/CO represent viruses isolated from Minnesota (14 viruses) and Colorado (1 virus), respectively. The European subgroup A, B, and D viruses are also indicated, along with the country where they were isolated.

FIG. 3. Phylogenetic relationships among APVs isolated in the United States. After alignment of contiguous nucleotide coding sequences for the nucleocapsid (N), phosphoprotein (P), matrix (M), and second matrix (M2) protein genes for 15 APVs isolated from United States, a cladogram was generated by maximum parsimony. Absolute distances are presented above each branch with bootstrap confidence levels in parentheses. The U.S. viruses were compared with subgroup A (APV/A) and B (APV/B) viruses from Europe.

between amino acids 103 and 104, whose connecting peptide (7 amino acids) was conserved among all U.S. isolates consisting of Arg-Lys-Ala-Arg/Phe-Val-Leu amino acids. In comparison, the connecting peptide for subgroup A viruses consists of Arg-Arg-Arg-Arg/Phe-Val-Leu, whereas that for subgroup B viruses consists of Arg-Lys-Lys-Arg/Phe-Val-Leu amino acids. The resultant F1 and F2 subunits were 103 and 431 amino acids long, respectively, for the U.S. isolates, a finding which is comparable to the corresponding F protein subunits of subgroup A and B viruses. The overall F protein amino acid sequence identity among the U.S. isolates was 91%, with the F1 subunit being more conserved than the F2 subunit, as observed in subgroup A and B viruses (23). Utilizing recently published partial F gene amino acid sequences (amino acids 315 to 421) from a potential subgroup D APV in France (GenBank accession numbers AF368170 to AJ400730), we compared these F protein amino acid sequences with the 15 U.S. isolates (subgroup C) and the viruses from subgroups A and B (2). As shown in Fig. 2, sequence alignment and phylogenetic analysis indicated that subgroups A, B, and D viruses were more closely related to each other than any of them was to the subgroup C viruses, reaffirming that the U.S. viruses are different from European viruses.

Analysis of nucleotide and amino acid sequence relatedness of various strains within a subgroup for bovine respiratory syncytial virus and human respiratory syncytial virus indicate that the percent identities ranged from 83.7 to 100% within a subgroup, whereas the nucleotide sequence identities between the two different subgroups ranged from 41 to 70% (27, 30).

Our data indicate that there is high homogeneity among U.S. isolates (89 to 94% nucleotide sequence identity). On the other hand, U.S. isolates had 41 to 77% nucleotide identities compared to European (subgroup A and B) viruses, thus supporting their classification into a single new subgroup (35). Interestingly, the ratio of nonsynonymous nucleotide changes in the surface F gene between the APV/CO and 1997 isolates from Minnesota (APV/MN2A and APV/MN1B) was between 1.8 and 2.00 (9), suggesting that there was positive selection early as the virus spread within the United States (Fig. 3). In contrast, the ratio of nonsynonymous to synonymous changes in recent (i.e., 1999) Minnesota isolates (APV/MN9 to APV/MN11) compared to the 1997 isolates (APV/MN2A) was between 0.4 and 0.54, indicating minimal accumulation of nonsynonymous mutations after the establishment of APV in Minnesota (13).

Comparison of U.S. and European viruses. The U.S. isolates had between 62 and 65% nucleotide sequence identity with subgroup A viruses and 60 to 63% nucleotide sequence identity with subgroup B viruses (18, 19, 20, 32, 44, 45, 46). The predicted amino acid sequence identity among U.S. viruses was between 81 and 95%, whereas the identity between subgroups A, B, and C was between 46 and 77%, indicating that all of the U.S. viruses are relatively homogeneous but distinctly different from their European counterparts and supporting their classification into subgroup C (35, 36). The predicted N, P, M, and F proteins had between 84 and 91% identity between subgroup A and B viruses but 53 to 78% identity between subgroup A

and C viruses and 52 to 77% identity between subgroup B and C viruses.

In conclusion, the data demonstrate a seasonal occurrence of APV outbreaks in the United States, with most outbreaks being reported in the spring and autumn. Analysis of genetic sequences of 15 APV isolates from the United States revealed a homogeneous subgroup of viruses different from the European viruses but with relatively few mutations over 4 years. An intriguing question is determining the level of homogeneity in the highly variable and immunogenic attachment G genes among U.S. viruses, which is the immediate focus of our research efforts. The mammalian pneumoviruses, including human respiratory syncytial virus and bovine respiratory syncytial virus have two subgroups of viruses: A and B (27, 28, 30). Therefore, the description of a third subgroup of APVs in our study and a recent report suggesting the emergence of a fourth subgroup may suggest that the avian paramyxoviruses are more heterogeneous than their mammalian counterparts (2).

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