Biological and Phylogenetic Characterization of Pigeon Paramyxovirus Serotype 1 Circulating in Wild North American Pigeons and Doves

L. Mia Kim, Daniel J. King, Hilda Guzman, Robert B. Tesh, Amelia P. A. Travassos da Rosa, Rudy Bueno, Jr., James A. Dennett, and Claudio L. Afonso

USDA-ARS Southeast Poultry Research Laboratory, 934 College Station Rd., Athens, Georgia 30605; Department of Pathology, University of Texas Medical Branch, Galveston, Texas; and Mosquito Control Division, Harris County Public Health and Environmental Services, Houston, Texas

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As part of West Nile virus surveillance programs in Rhode Island and eastern Texas between 2000 and 2007, brain tissue was collected from 5,608 dead birds representing 21 avian orders found in public places or reported by homeowners. Fifteen Newcastle disease virus isolates were recovered only from birds of the order Columbiformes and were positively identified by the USDA-validated real-time reverse transcription-PCR assay targeting the matrix gene and more specifically as pigeon paramyxovirus serotype 1 (PPMV-1) by hemagglutinin inhibition with monoclonal antibodies. Based upon partial genomic sequencing and phylogenetic analysis, the newly isolated viruses represent a distinct sublineage within class II genotype VIb. All of the viruses (15/15) were classified as virulent based upon their fusion cleavage site motif (112RRKKRF117) and intracerebral pathogenicity indices of >0.7 (ranging from 0.98 to 1.35); however, these viruses escaped detection by the fusion gene-based real-time PCR test for virulence. Modifications introduced to the probe site of the fusion gene-based assay allowed rapid virulence detection within this distinct sublineage.

Virulent forms of Newcastle disease (ND) virus (NDV; also known as avian paramyxovirus serotype 1) are a major economic concern for poultry producers worldwide (6). While there have been previous outbreaks of disease due to virulent NDV infections, poultry in the United States is currently considered free of ND. Disease control programs to prevent the reintroduction of virulent NDV into domestic poultry flocks include vaccination and quarantine of imported birds and must be complemented with monitoring programs. Rapid diagnostic assays such as real-time reverse transcription (RT)-PCR aid in the timely detection of potential outbreaks and are a crucial part of these efforts (15, 40).

At least three major panzootics of ND have been reported in the past 80 years. The first was recognized during the mid 1920s and affected birds in Indonesia and England (5), the second was identified in Europe during the late 1960s but was thought to have originated in Asia (18), and a third panzootic involving a pigeon-adapted variant of avian paramyxovirus serotype 1 that likely originated in the Middle East was detected during the 1980s (19) and continues around the world (2, 5, 32). Pigeon paramyxovirus serotype 1 (PPMV-1) affects pigeons and doves (Columbiformes) and is known to infect poultry (9, 12, 23, 25, 39). Widespread in racing pigeon populations, the disease is now considered to be endemic (2, 31). For example, in the United States the virus is believed to be endemic and outbreaks were reported in 1998 in Texas and Georgia (25); however, PPMV-1 strains from the United States have rarely been phylogenetically characterized. Although many countries maintain compulsory vaccination of racing pigeons, there is no form of disease control in wild pigeons, which frequently have contact with backyard and free-range poultry (8, 9, 36). The virulence of PPMV-1 has been reported to be variable (14, 22, 31), and at times the only clinical sign of PPMV-1 infection in layer chickens was a drop in egg production, misshaped eggs, and soft egg shells (7). However, increased pathogenicity in chickens has been identified when PPMV-1 is passaged in chickens or embryonated eggs, indicating that viruses currently circulating among pigeon populations could lead to ND outbreaks (7, 22, 24). A recent study demonstrated that 11 (78.5%) of 14 virulent poultry NDV isolates from China obtained between 1996 and 2005 were typical of PPMV-1 strains that clustered into a single genetic lineage, 4b (2), or genotype VIb (28).

Rapid diagnosis of PPMV-1 is achieved with the USDA-validated real-time RT-PCR assay targeting the matrix gene (M-gene assay) (15, 40). The M-gene assay detects most class II NDV strains, including members of the PPMV-1 subgroup. Another real-time assay is employed that allows discrimination between virulent and avirulent isolates and is directed at the fusion gene (F-gene assay) (40). Upon initial testing of the F-gene assay, one member of the PPMV-1 subgroup was found to escape detection (Dove/Italy/2736/2000 [DoveIT]) (20, 40). When the sequence of the DoveIT isolate was compared to the sequences of the fusion test primers and probe, several mismatches were identified in the primer sequences, as well as 4-nucleotide (nt) mismatches at the fusion test probe site (20). The genetic differences between the DoveIT isolate and the fusion test probe appeared to be responsible for the test failure. Data from this study also revealed that the DoveIT isolate...
It was phylogenetically related to a subset of PPMV-1 strains belonging to lineage 4bii (genotype VIb1) (2, 38). Eighty-six percent of these isolates (32/37) contained identical mismatches with DoveIT, and it was predicted that viruses from this sublineage were unlikely to be detected by the F-gene assay.

To improve our understanding of the distribution and evolution of PPMV-1, we examined representative strains of the
Characterization of PPMV-1 in U.S. Pigeons and Doves

**MATERIALS AND METHODS**

Isolates and sequence data. NDVs were obtained as part of West Nile virus surveillance programs in Rhode Island and the Houston metropolitan area from 2000 to 2007. Brain tissue was collected from dead birds and cultured in Vero cells as described previously (27, 37). Initial virus identification as NDV was made by a complement fixation (CF) test performed on fluids of cultures showing a viral cytopathic effect. NDV was isolated only from birds of the family Columbidae (Table 1).

Lyophilized Vero cell culture material was received at the Southeast Poultry Research Laboratory, reconstituted in 500 μl nuclease-free autoclaved water, and propagated in embryonated eggs. RNA was extracted from allantoic fluids and propagated in embryonated eggs. RNA was extracted from allantoic fluids with Trizol LS (Invitrogen, Carlsbad, CA) according to manufacturer instructions. Five MAbs with different NDV specificities were used for differentiating isolates by the HI assay as previously described (22). Four HA units of viral test antigen was used in completing the HI assay.

Hemagglutination (HA) and HA inhibition (HI) assays. The HI assays were completed by microtiter methods. The HA assay of allantoic fluids harvested from inoculated embryonating eggs was used to identify NDV-positive fluids and antigenic characterization of virus isolates was conducted by HI with microtiter methods as previously described (22). Four HA units of viral test antigen was used in completing the HI assay with monoclonal antibodies (MAbs) and polyclonal antiserum.

**TABLE 2. MAbs reactivity patterns of the PPMV-1 isolates used in this study**

<table>
<thead>
<tr>
<th>Identification no.</th>
<th>GenBank accession no.</th>
<th>Isolate description</th>
<th>Reactivity with</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>AVS</td>
</tr>
<tr>
<td>447</td>
<td>EU477200</td>
<td>Mourning Dove/US/TX/4048/2004</td>
<td>-</td>
</tr>
<tr>
<td>435</td>
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<td>Eurasian Collared Dove/US/TX/817/2004</td>
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<td>436</td>
<td>EU477195</td>
<td>Eurasian Collared Dove/US/TX/6295/2006</td>
<td>-</td>
</tr>
<tr>
<td>437</td>
<td>EU477196</td>
<td>Eurasian Collared Dove/US/TX/6306/2007</td>
<td>-</td>
</tr>
<tr>
<td>438</td>
<td>EU477197</td>
<td>Eurasian Collared Dove/US/TX/6338/2007</td>
<td>-</td>
</tr>
<tr>
<td>440</td>
<td>EU477188</td>
<td>Dove/US/TX-B2580/2004</td>
<td>-</td>
</tr>
<tr>
<td>446</td>
<td>EU477191</td>
<td>Eurasian Collared Dove/US/TX/988/2004</td>
<td>-</td>
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<td>Pigeon/US/TX4125/2005</td>
<td>-</td>
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<td>Eurasian Collared Dove/US/TX/4156/2005</td>
<td>-</td>
</tr>
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<td>-</td>
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<td>Pigeon/US/TX337/2004</td>
<td>-</td>
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<td>Pigeon/US/TX350/2004</td>
<td>-</td>
</tr>
</tbody>
</table>

* n = 14.

b Polyclonal chicken antiserum served as the positive control and produced titers of ≥60 for each virus; the negative control titers were <2.

**FIG. 1.** Comparison of PPMV-1 sequences which encode the amino acid motif ^112^RRKKRF^117^ at the fusion cleavage site (n = 53) to the fusion gene real-time RT-PCR assay probe and the pigeon-specific probe. Mismatches with the fusion probe sequences are in bold; changes made to the pigeon-specific probe are underlined. *, Sequence identical for viruses not shown (n = 41), i.e., those with accession no. EU477189 to -202, AB070419, AB070420, AB070422, AB070426, AY175753, AY150129, AY150132, AY150133, AY150134, AY150137, AY150138, AY150139, AY150140, AY150141, AY150143, AY445669, AY471757, AY471759, AY471760, AY471761, AY471763, AY471764, AY471765, AY471766, AY471768, and AY471770. †, Sequence identical for viruses not shown (n = 3), i.e., those with accession no. AY150150, AY150151, and AY471771.
and their reactivity has been described previously (21). A positive result was defined as antibody-inhibited HA, and a negative result was defined as no HI.

**Pathogenicity assessment.** The pathogenic potential of selected pigeon and dove isolates was evaluated by using standard assay methods to determine the intracerebral pathogenicity index (ICPI) in 1-day-old chicks (4).

**Real-time RT-PCR.** Alternate probes were designed for the F-gene assay from the consensus of an alignment (Fig. 1) which included the PPMV-1 strains reported here (n = 15), as well as other reference sequences encoding the amino acid motif 112RRKKRF117 at the fusion cleavage site (n = 38). All pigeon viruses (n = 15) were tested by using the USDA-validated M-gene and F-gene assay protocols (40) and a modified F-gene assay with a degenerate probe and a PPMV-1-specific probe following the original M-gene assay protocol.

**Phylogenetic analysis.** Maximum-likelihood (ML) phylogenetic analysis with bootstrap values for 100 replicates was performed with Phylm under the general time-reversible model of nucleotide substitutions, ML estimates of base frequencies, the estimated transition/transversion ratio, and proportions of invariable sites with four categories of substitution rates (17). The full coding region of the F gene from pigeon isolates (n = 5) was compared to reference sequences representing known clades and genotypes (n = 74). The 374-bp region of the F gene, which has commonly been used for phylogenetic analysis of NDV (3), was sequenced to localize the U.S. pigeon viruses among other class II genotype VI isolates (Table 3).

**Serologic comparison of two isolates.** Immune sera to two of the NDV isolates (Pigeon/US/R1166/2000 and Eurasian Collared Dove/US/TX3377/2004) were prepared in adult mice at the University of Texas. The immunizing antigens were homogenates of brains of newborn mice inoculated intracerebrally with the respective viruses. CF tests were performed by the microtiter technique as previously described (11). Neutralization tests were performed by a plaque reduction neutralization method as previously described (33) in 24-well microplate cultures of Vero cells. Fifteen NDV isolates were cultured in Vero cells. Fifteen NDV isolates were recovered from 1,416 birds of the order Columbiformes, family Columbidae (Table 1). Isolates were serologically characterized with a panel of five different MABs. The reactivity of PPMV-1 strains in the MABs tested is typically as follows: AVS, negative; B79, positive; 15C4, negative; 10D11, negative; 617/161, positive. Nine of the isolates tested demonstrated the typical pattern of PPMV-1, with reaction to B79 and 617/161 (Table 2; n = 14). One isolate, Mourning Dove/US/TX4048/2004 (04US447MDV), exhibited a normal variation for PPMV-1, binding only to the 617/161 MAB. The remaining four isolates presented an atypical pattern, binding to 15C4, as well as B79 and 617/161.

All isolates were determined to be virulent according to the World Organization for Animal Health standard (10), which states that virulent viruses have an ICPI of $\geq 0.7$ or encode multiple basic amino acids at the C terminus of the F2 protein and have phenylalanine at residue 117. Each virus encoded a virulence fusion cleavage site motif ($^{112}$RRKKRF$^{117}$) and the ICPIs of selected isolates (n = 11) ranged from 0.98 to 1.35 (Table 3).

**RESULTS**

As part of a West Nile virus surveillance program in the Houston metropolitan area and in Rhode Island from 2000 to 2007, brain tissue from 5,608 dead birds representing 21 avian orders was cultured in Vero cells. Fifteen NDV isolates were recovered from 1,416 birds of the order Columbiformes, family Columbidae (Table 1). Isolates were serologically characterized with a panel of five different MABs. The reactivity of PPMV-1 strains in the MABs tested is typically as follows: AVS, negative; B79, positive; 15C4, negative; 10D11, negative; 617/161, positive. Nine of the isolates tested demonstrated the typical pattern of PPMV-1, with reaction to B79 and 617/161 (Table 2; n = 14). One isolate, Mourning Dove/US/TX4048/2004 (04US447MDV), exhibited a normal variation for PPMV-1, binding only to the 617/161 MAB. The remaining four isolates presented an atypical pattern, binding to 15C4, as well as B79 and 617/161.

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Initial testing demonstrated that all of the isolates were positively identified by the USDA-validated real-time RT-PCR M-gene assay (cycle threshold $[C_T]$ range, 15.36 to 19.24); however, none were detected with the F-gene assay after duplicate attempts ($C_T$; Table 3). To determine whether mismatches at the probe site were responsible for the F-gene assay failure, a PPMV-1-specific probe was designed by evaluating a 24-nt alignment of PPMV-1 sequences (positions 4871 to 4894) which encode the $^{112}$RRKKRF$^{117}$ motif compared to the F-gene assay probe site (Fig. 1). This region was identical in the 15 PPMV-1 strains described here and 27 other viruses from Japan, South Africa, Spain, Austria, Germany, Denmark, France, Ireland, and the United Kingdom. Three mismatches that were present in the PPMV-1 sequences compared to the 24-nt F-gene probe sequence (position 6, G to T; position 13, A to G; position 14, C to A) were chosen to make a new probe that successfully detected all PPMV-1 isolates (Table 3; n = 15). Based on results from a previous study (20), an additional probe was designed with a single degenerate site at position 6

**TABLE 3.** $C_V$ values from three real-time RT-PCR assays

<table>
<thead>
<tr>
<th>Identification no.</th>
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<th>Isolate description</th>
<th>Matrix</th>
<th>Fusion</th>
<th>Revised fusion</th>
<th>ICPI</th>
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<td>434</td>
<td>EU477193</td>
<td>Eurasian Collared Dove/US/TX2334/2003</td>
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<td>24.66</td>
<td>ND</td>
</tr>
<tr>
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<td>EU477196</td>
<td>Eurasian Collared Dove/US/TX6306/2007</td>
<td>15.36</td>
<td>0</td>
<td>21.63</td>
<td>1.31</td>
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<tr>
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<td>Eurasian Collared Dove/US/Tx6338/2007</td>
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<td>24.59</td>
<td>1.26</td>
</tr>
<tr>
<td>440</td>
<td>EU477188</td>
<td>Dove/US/TX-B2580/2004</td>
<td>16.53</td>
<td>0</td>
<td>22.3</td>
<td>0.98</td>
</tr>
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<td>441</td>
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<td>17.23</td>
<td>0</td>
<td>22.78</td>
<td>1.35</td>
</tr>
</tbody>
</table>

$^a$ The matrix gene assay, the fusion gene assay, and the revised fusion gene assay with a pigeon-specific probe (n = 15) were used. The associated ICPIs for selected PPMV-1 isolates (n = 11) are shown. $C_V$ values of 1 to 35 are considered positive. Virulent isolates have ICPIs of $>0.7$ and phenylalanine (F) at position 117 (all isolates exhibited $^{112}$RRKKRF$^{117}$).

$^b$ ND, not done.
(G to K = G/T); however, none of the isolates tested in this study were detected with the degenerate probe (data not shown).

To determine the distribution of these viruses in comparison to known clades and genotypes, sequencing and analysis of the full coding region of the F gene were performed (Fig. 2). The pigeon viruses clearly fall within the other genotype VI virus group. Among the PPMV-1 strains reported here, the 00US441PGN virus forms a separate branch likely because it is geographically separated (Rhode Island) from the other viruses, which were isolated in Texas. The most closely related isolates are a chicken and a dove isolate from Italy (00IT005CKN and 00IT003DVE) and a pigeon isolate from Belgium (98BE100PGN). The 00IT003DVE isolate (20) is included among isolates that were
not detected by the F-gene assay (Fig. 2; denoted by asterisks). Results of the plaque reduction neutralization assay comparing two PPMV-1 strains, Pigeon/US/RI166/2000 (00US441PGN) and Eurasian Collared Dove/US/TX2334/2003 (03US434ECD), demonstrate that these two isolates were distinct and support the results of a phylogenetic analysis (Table 4); however, the isolates were indistinguishable by the CF assay (data not shown).

Closer phylogenetic evaluation of genotype VI viruses with the 374-bp fragment suggests that the pigeon and dove viruses reported here belong to a subgroup, VIb/4bii (VIb refs; 4b refs), many of the members of which encode the\textsuperscript{112}RRKKRF\textsuperscript{117} motif at the fusion cleavage site (Fig. 3). Fourteen of the pigeon and dove viruses described here form a distinct cluster (designated TX, Fig. 3). While these viruses likely represent a geographical separation, it is interesting that the dates of isolation span 5 years (2003 to 2007). Viruses collected in 16 countries all over the world from 1992 to 2007 (51/52) are found among the VIb/4bii subgroup, suggesting broad geographic dispersal of this relatively recent subgroup. As with the full coding sequence analysis, the pigeon isolate 00US441PGN (Rhode Island) clusters separately from the TX viruses and groups together with a recent pigeon isolate from Quebec, Canada (06CA047PGN).

### DISCUSSION

Monitoring efforts in the Houston metropolitan area identified PPMV-1 in dead pigeons and doves from 2003 to 2007, suggesting that these viruses may be endemic and circulating in the United States. These findings are consistent with previous reports suggesting that, despite widespread vaccination efforts, the PPMV-1 identified during the mid 1980s panzootic appears to have become endemic in areas maintaining large populations of Columbiformes birds (2, 5, 31). The majority (67\% [951/1,416]) of the bird samples were received during the warmer months of May to September (average temperatures, 75.8 to 78.9°F); however, 10 of the 15 NDV isolations were from samples obtained during the cooler months (October to April; average temperatures, 51.8 to 70.4°F). The reason for the lower rate of isolation during warmer months is unknown; high temperatures may affect the stability of the virus in the environment, resulting in lower rates of transmission, or may cause inactivation of the virus in bird carcasses during the summer months.

Previous efforts to characterize the pathogenicity of various PPMV-1 strains in poultry revealed variability in the results of pathogenicity assays performed with chickens, even though the isolates typically had the recognized virulence fusion protein cleavage site motif \textsuperscript{112}G/RRQKRF\textsuperscript{117} (14, 22, 31). The isolates described in the present report encode a virulence fusion cleavage site motif \textsuperscript{112}RRKKRF\textsuperscript{117} and exhibit ICPIs ranging from 0.98 to 1.35 (Table 3), and these results are in agreement with previous studies characterizing isolates which encode the \textsuperscript{112}RRKKRF\textsuperscript{117} cleavage site (31, 35, 39).

A USDA-validated real-time RT-PCR assay (F-gene assay) directed at the fusion cleavage site of NDV differentiates virulent strains from those of low virulence (40). During the initial evaluation of the F-gene assay, one virulent PPMV-1 isolate, DoveIT, escaped detection and it was postulated that

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### TABLE 4. Results of plaque reduction neutralization assay with PPMV-1 isolates Pigeon/US/RI166/2000\textsuperscript{a} and Eurasian Collared Dove/US/TX2334/2003\textsuperscript{b}

<table>
<thead>
<tr>
<th>Identification no.</th>
<th>GenBank accession no.</th>
<th>Virus</th>
<th>Antibody dilution\textsuperscript{c}</th>
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<td>EU477189</td>
<td>Pigeon/US/RI166/2000</td>
<td>1:160 1:20</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Identification no. 441.  
\textsuperscript{b} Identification no. 434.  
\textsuperscript{c} Highest antibody dilution giving >90\% plaque reduction.

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![FIG. 3. Phylogenetic analysis of the 374-bp fusion gene fragment of PPMV-1 (n = 15; underlined) with reference sequences from class II genotype VI (n = 47). Asterisks represent isolates known to escape detection by the fusion gene assay. Names in bold also appear in the 374-bp fusion gene fragment phylogenetic analysis (Fig. 2). The tree was constructed by Phyml ML with 100 bootstrap replicates. The scale indicates the branch length based on the number of nucleotide substitutions per site. Virus designations represent a 10-character name containing the two-digit year of collection, the two-letter ISO country code abbreviation, the three-digit unique virus identification number, and the three-letter species abbreviation (refer to Table 1).](image-url)
viruses encoding the \text{112}RRKKKR\text{117} motif would escape detection because of the presence of three mismatches along the probe site sequence which prevented successful binding of the probe (20). This hypothesis held true for the PPMV-1 isolates reported here. The redesigned pigeon-specific probe successfully identified the PPMV-1 isolates as virulent, whereas the original F-gene probe failed. Further analysis suggested that a single nucleotide change (position 6 of the F-gene probe) may allow the probe to bind (20); however, the degenerate probe designed for the present study failed to bind. While these isolates were correctly identified by the M-gene assay, the accurate identification of virulent isolates is important to continued monitoring efforts. Therefore, inclusion of additional primers may need to be considered as part of the USDA surveillance plan.

The class II genotype VI group contains viruses from around the world collected as early as 1978 (19). This is a diverse group of viruses that have been phylogenetically characterized by many different authors into various sublineages (2, 16, 28–30, 38). Four isolates obtained from racing pigeons (Columba livia) fell outside the major grouping and encoded an unusual amino acid fusion cleavage site motif with lysine replacing glutamine at residue 114, producing the motif \text{112}RRKKKR\text{117} (31). Viruses characterized by the \text{112}RRKKKR\text{117} motif have been reported by several authors and include viruses from every major continent (1, 2, 31, 35, 38, 39). In each case, viruses encoding the \text{112}RRKKKR\text{117} motif tend to cluster together separately from other genotype VI viruses but clearly remain within the genotype VI domain.

An outbreak described in ECD in Italy in 2000 and 2001 found 18/20 ECD isolates to cluster together regardless of geographic origin and postulated that these viruses represented a distinct sublineage circulating within a species (35); however, the present study found viruses with 100% identity along the 374-bp fragment of the fusion gene circulating in both pigeons and ECD clustered according to geographic origin. Our results show that viruses found within the Vb/vbii subgroup are temporally clustered from 1992 to 2007, which is in agreement with Aldous et al., who postulated that subgroup 4b (1990s to 2000) was becoming the predominant sublineage over 4b (representing older viruses from the 1980s to the 1990s) and may reflect selective pressure from vaccine usage (2).

PPMV-1 represents a significant ongoing threat to domestic and wild bird populations, and further understanding of the natural ecology and the effect of selective pressures on these viruses is needed. Additionally, PPMV-1 detected in U.S. urban pigeon and dove populations deserves continued investigation since previous outbreaks in the United Kingdom were thought to originate from feed contaminated with pigeon feces and introduced into naive (unvaccinated) populations (8). The presence of these viruses in U.S. urban pigeons and doves, in addition to concerns about increased virulence of PPVM-1 upon replication in poultry, emphasizes the importance of control methods such as vaccination and monitoring in preventing PPMV-1 outbreaks in domestic poultry populations.

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**REFERENCES**