AVIAN DISEASES 56:243–248, 2012

Case Report—

Genetic Characterization of a Pigeon Paramyxovirus Type 1 Isolated from *Columba livia* in Uruguay

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Received 21 June 2011; Accepted and published ahead of print 22 September 2011

SUMMARY. The isolation and molecular characterization of pigeon paramyxovirus type 1 (PPMV-1) from a sick racing pigeon in Uruguay is reported for the first time. Hemagglutination inhibition (HI) tests were performed to detect antibodies against avian paramyxovirus serotype 1 (APMV-1), and a HI titer of 1/32 was obtained. Tracheal and cloacal swabs were processed by real-time reverse transcription–polymerase chain reaction (rRT-PCR) with the use of the National Veterinary Services Laboratory–U.S. Department of Agriculture validated matrix (M) gene assay and were positive for APMV-1. Viral isolation in embryonated chicken eggs confirmed the molecular detection of the isolate. A fragment corresponding to the 3′ region of the fusion (F) protein gene was amplified by RT-PCR, and subsequently sequenced. The deduced amino acid sequence at the F protein cleavage site displayed the motif ^{112}RRQKR/F^{117}. Phylogenetic analysis of this part of the genome allowed the isolated virus to be grouped in the lineage VIb/4b, which suggests that it shares the same ecologic niche with other PPMV-1 that were found in the region, and it is not imported as other European or North American viruses.

RESUMEN. *Reporte de Caso*—Caracterización genética de un paramixovirus de las palomas tipo 1 aislado de una paloma (*Columba livia*) en Uruguay.

Se aisló y se caracterizó molecularmente por primera vez un paramixovirus de las palomas de tipo 1 (PPMV-1) proveniente de una paloma mensajera enferma hallada en Montevideo, Uruguay. Se realizó la prueba serológica de inhibición de la hemaglutinación para detectar anticuerpos específicos contra este virus y se obtuvo un título de 1/32. Los hisopados traqueales y cloacales se procesaron mediante la prueba de transcripción reversa seguida por la reacción en cadena de la polimerasa en tiempo real validada por el Laboratorio Nacional de Servicios Veterinarios del Departamento de Agricultura de los Estados Unidos (NVSL-USDA) para detectar el gene que codifica la proteína de la matriz viral del paramixovirus de las palomas 1, resultando positivos a la misma. El aislamiento viral en huevos embrionados de pollo confirmó la detección molecular. Se amplificó y se secuenció el fragmento correspondiente al extremo 3′ del gene codificante para la proteína de fusión (F). La secuencia de aminoácidos deducida en el sitio de disociación de la proteína F presentó el motivo ^{112}RRQKR/F^{117}. El análisis filogenético de esta parte del genoma permitió agrupar al virus aislado dentro del linaje VIb/4b característico de la variante de paloma de paramixovirus tipo 1 (PPMV-1) y sugiere que el mismo comparte el mismo nicho ecológico con otros paramixovirus de las palomas tipo 1 hallados en la región y no fue importado como otros virus procedentes de Europa o de Norteamérica.

Key words: genetic characterization, phylogenetic analysis, pigeon paramyxovirus

Abbreviations: aa = amino acid; APMV-1 = avian paramyxovirus serotype 1; F = fusion; HA = hemagglutination; HI = hemagglutination inhibition; ICPI = intracerebral pathogenicity index; M = matrix; ND = Newcastle disease; NDV = Newcastle disease virus; NVSL-USDA = National Veterinary Services Laboratories–United States Department of Agriculture; OIE = World Organisation for Animal Health; PPMV-1 = pigeon paramyxovirus type 1; rRT-PCR = real-time reverse transcription–polymerase chain reaction; RT-PCR = reverse transcription–polymerase chain reaction

Virulent strains of avian paramyxovirus serotype 1 (APMV-1) or Newcastle disease virus (NDV) are responsible for Newcastle disease (ND), a highly contagious and severe disease with worldwide distribution that can cause important economic losses in the poultry industry (5). At least three major panzootics of ND have been reported in the past 80 yr. The first was recognized during the mild 1920s and affected birds in Indonesia and England (4), whereas the second was identified in Europe during the late 1960s but was thought to have originated in Asia. The third panzootic, involving a pigeon-adapted variant of avian paramyxovirus serotype 1 (PPMV-1), first emerged in the Middle East during the late 1970s, then spread throughout Europe in 1981 and now is found around the world (2) included neighboring countries of Uruguay such as Argentina (22).

PPMV-1 produces neurologic and viscerotropic disease, including mortality, in pigeons and doves (Columbiformes) (4). The PPMV-1 isolates represent a threat for poultry productions because they were shown to be pathogenic for poultry both in experimental infections (14) and natural outbreaks, as those described in Great Britain for chicken broilers, breeders, and layers, in 1984 (7) and for pheasants in 1996 (6).

Currently, the poultry industry in Uruguay is considered free of virulent NDV isolates. Disease control programs to prevent the

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reintroduction of pathogenic NDV strains into farms include vaccination and quarantine of imported birds and must be complemented with monitoring programs. Rapid diagnostic assays such as real-time reverse transcription–polymerase chain reaction (rRT-PCR) aid in the timely detection of potential outbreaks of ND and are very useful to keep the present health status of the commercial poultry populations.

Both wildlife pigeons and racing pigeons with symptoms characteristic of ND were reported by the first time in Uruguay in 2001; however, PPMV-1 was not isolated in that instance.

The present study involved the isolation and molecular characterization of a PPMV-1 strain isolated from a sick racing pigeon in Montevideo, Uruguay.

CASE REPORT

Materials and methods. *Samples*. Samples were collected at an urban area of Montevideo, Uruguay, from a sick racing pigeon (*Columba livia*), with typical signs of paramyxovirosis (diarrhea, torticolis, and ataxia). Serum blood was obtained from the axilar vein. Cloacal and tracheal swabs were collected and conditioned in transport media containing Dulbecco's modified eagle medium with antibiotic–antimycotic solution pH 7.2 and stored at −70 C until use. The sample was named Pigeon/Uruguay/Montevideo/02/08.

Hemagglutination (HA) and hemagglutination-inhibition (HI) tests. The HA and HI assays were performed by conventional microtiter methods as described by the World Organisation for Animal Health (OIE) (21). For HI, four HA units of NDV La Sota strain were used.

Virus isolation. Virus isolation was performed by propagation of the cloacal and tracheal swabs in 9–11-day-old embryonated chicken eggs obtained from birds negative for NDV antibodies. Aliquots of 0.2 ml of each swab were inoculated into the allantoic cavity of five embryonated eggs. After incubation for 4 days at 37 C, the eggs were chilled at 4 C, the allantoic fluids were collected and aliquots of those were reinoculated into chicken eggs. The allantoic fluids from the same sample obtained in the second passage were pooled and analyzed by HA test. The specificity of hemagglutinating activity was determined by HI assay with the use of an APMV-1–specific antiserum provided by National Veterinary Services Laboratories–United States Department of Agriculture (NVSL-USDA).

RNA extraction and rRT-PCR for amplification of matrix (M) gene. RNA from the cloacal and tracheal swabs examined in this study was obtained by a double extraction procedure with the use of the RNA extraction method described by Chomczynski and Sacchi (9), and then extracted again with the RNeasy Mini Kit according to the manufacturer's instructions (Qiagen, Valencia, CA). The extracted RNA was analyzed by rRT-PCR with the use of the USDA-validated M-gene assay protocol (20).

RT-PCR and nucleotide sequencing of the fusion (F) protein gene. The viral RNA was processed by RT-PCR with the use of the forward primer 5′TTAGAAAAAACACGGGTA3′ and reverse primer 5′AGTCGGAGGATGTTGGCAGC3′, described by Aldous et al. (2) to generate an amplicon of 518 base pairs corresponding to the 3′ region of the protein F gene. For this purpose, One-Step Reverse Transcriptase (RT)-PCR Kit (Qiagen, Hilden, Germany) was used. RT-PCR was performed by uninterrupted thermal cycling with the following program: 30 min at 50 C for RT; RT inactivation at 95 C for 15 min was followed by 40 cycles of denaturation at 94 C for 30 sec, annealing at 52 C for 1 min, extension at 72 C for 1 min, and final extension at 72 C for 10 min. The amplified product was analyzed by electrophoresis on a 1% agarose gel stained with ethidium bromide. The DNA fragment was purified with QIAEX II Gel extraction kit (Qiagen) according to the manufacturer's recommendation. The nucleotide sequencing was carried out by the Sanger dideoxinucleotide terminator method (16) with the use of the Big Dye® Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and oligonucleotide primers used for RT-PCR. Reactions were analyzed with an ABI PRIS® 310 Genetic Analyzer (Applied Biosystems, Tokyo, Japan).

Sequence analysis. Nucleotide sequence editing, analysis, prediction of amino acid (aa) sequences and alignment were conducted by using BioEdit v7.0.5 (12) and MEGA 5 (19) software's (www.bioedit.com and www.megasoftware.net). The deduced aa sequences of the F gene, including the F2/F1 cleavage site, were compared with the published sequences in the GenBank.

Phylogenetic analysis. A 375-nt fragment of the F gene, starting from the ATG and including de F2/F1 cleavage site, was aligned with the use of the Clustal W 5 method in MegAlign to determine the phylogenetic relationships between Pigeon/Uruguay/Montevideo/2/08 (gb/JF749831), Pigeon/Argentina/Tigre/6/99 (gb/AY734536.1), Pigeon/Argentina/Capital/3/97 (gb/AY734535.1), and other APMV-1 published in the GenBank. The results are presented as a phylogenetic tree with the neighbor-joining method with the use of the Tamura-Neil model with 1000 bootstrap replicates to assign confidence level to branches.

Results and discussion. Since 1984, a worldwide panzootic of PPMV-1 infection has been reported, causing a frequently fatal disease primarily associated with neurological signs in racing and shown pigeons. The presence of PPMV-1 in Uruguay is suspected, because different strains of this virus were isolated from free-living pigeons in Argentina between 1997 and 1999 (22) and an outbreak of ND was clinically detected in Columbiformes from our country in 2001 but without laboratory confirmation. In order to analyze this hypothesis, serologic and molecular assays were carried out from samples obtained from a racing pigeon with paramyxovirus symptoms. The serologic result showed the presence of anti-APMV-1 antibodies in the serum sample, with a HI titer of 1/32.

After virus isolation the allantoic fluids corresponding to the second passage of tracheal and cloacal swabs presented hemagglutinating activity with titers of 16 and 64 units hemagglutination, respectively. These HA values were similar to those reported for others isolates obtained from pigeons (8). The specificity of the hemagglutinating activity was confirmed by HI test with the use of a specific APMV-1 antiserum.

RNA was extracted directly from swab samples and processed with the use of the USDA-validated NDV M gene rRT-PCR assay. So, specific sequences of APMV-1 were detected in tracheal and cloacal samples with cycle threshold values of 30.43 and 28.89, respectively. In addition, a fragment of 518 nucleotides, corresponding to the 3′ end of the F gene, was amplified by RT-PCR with the use of the extracted RNA as template (data not shown). These results reinforce the concept that the molecular techniques are rapid, sensitive, and specific tools for virus diagnostic detection (2). The sample would be also tested by another USDA-validated rRT-PCR fusion gen test, which is directed at the fusion-cleavage site and was developed to differentiate virulent NDV from those of low virulence (1). But when the sequence of the virus isolated was compared with the sequences of the fusion test probes, five nucleotide mismatches were identified at the fusion test probe site. This test should produce false-negative results because more than three mismatches were sufficient to prevent the successful binding of the probe (13). Then, alignment and comparison of a sequence of 375 base pairs, which included the region encoding the cleavage activation site and signal peptide of the F protein gene, were performed with Pigeon/Uruguay/Montevideo/2/08 virus and representative APMV-1 strains, including PPMV-1

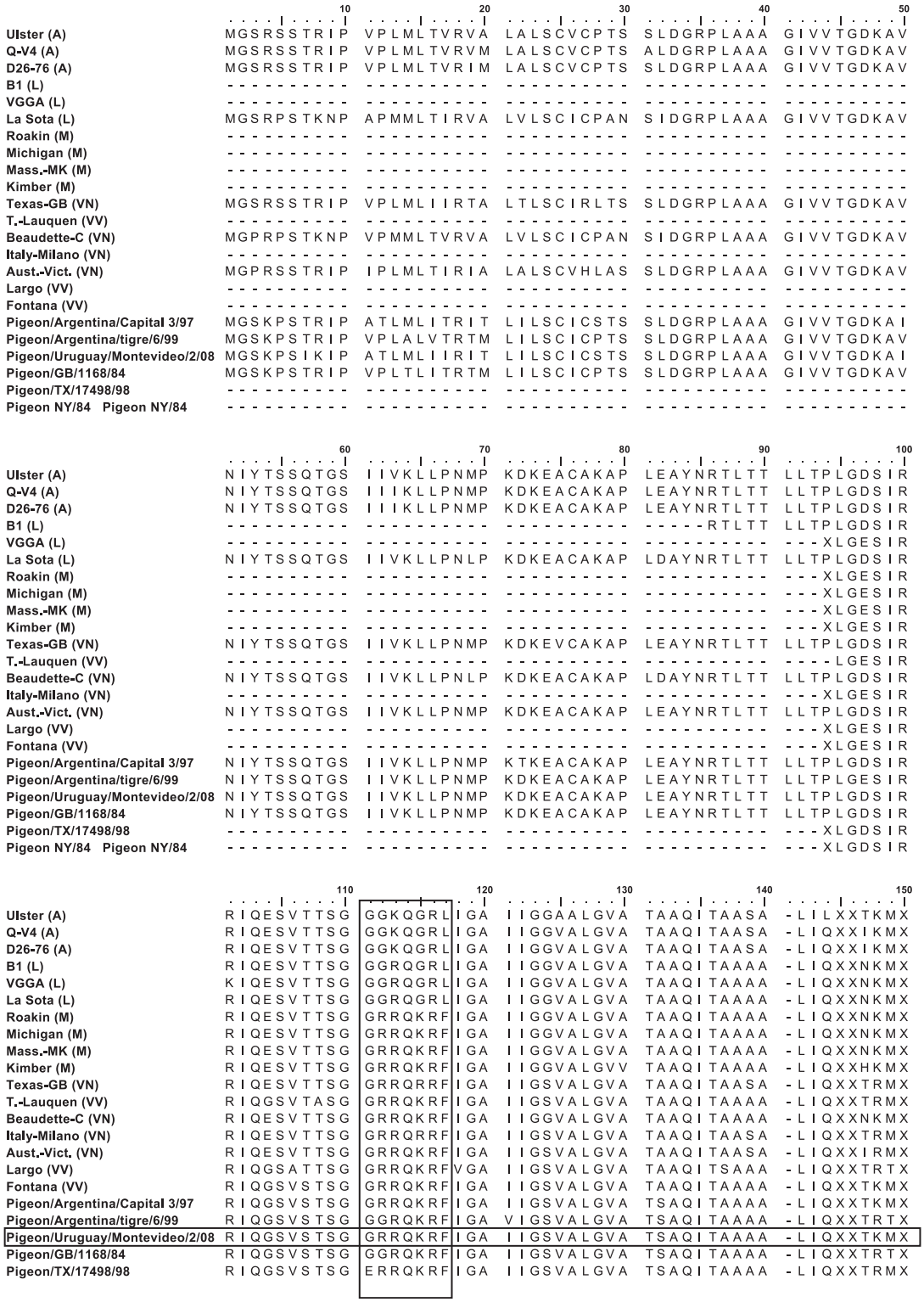

Fig. 1. Alignment of predicted amino acid sequences surrounding the F2/F1 protein cleavage site. The amino acids involved in the F protein cleavage site, from position 112 to 117, are underlined. The amino acid sequence from Pigeon/Uruguay/Montevideo/2/08 isolate is boxed.

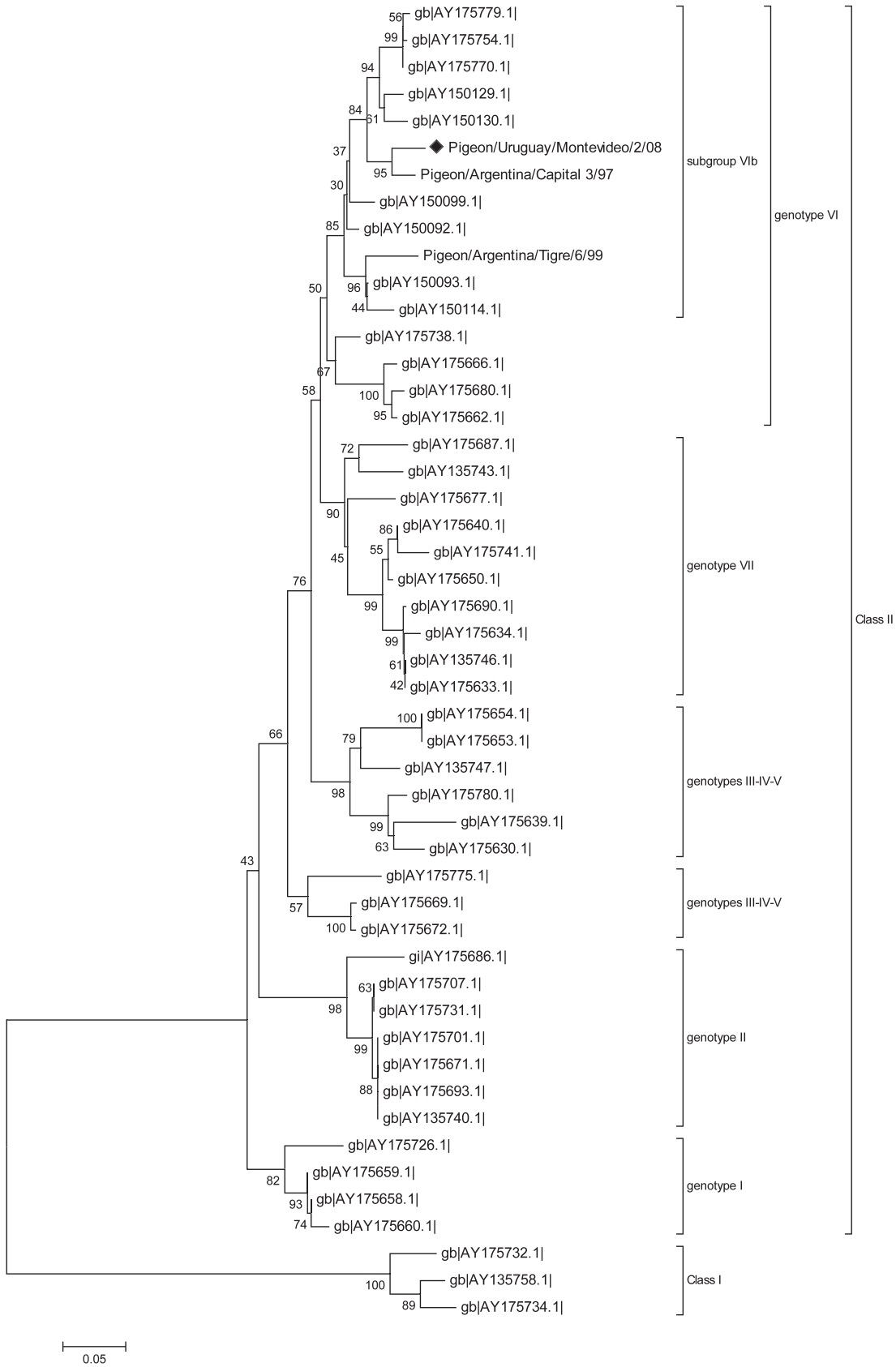

Fig. 2. Phylogenetic relationship among different lineages of APMV-1 isolates based on F gene nucleotide sequences between position 47 and 423. The phylogenetic tree was constructed with the use of the neighbor-joining method with 1000 bootstrap replicates on MEGA 5. Pigeon/Uruguay/Montevideo/2/08 isolate was indicated by the rotated black square.

isolates published in the GenBank (data not shown). Analysis showed that Pigeon/Uruguay/Montevideo/2/08 has higher nucleotide similarity with pigeon isolates (89.3% with Pigeon/Argentina/Tigre/6/99, 91% with Pigeon/TX/3503/04 and Pigeon/GB/1168/84, and 92% with Pigeon/NY/84) than with other APMV-1 stains such as Ulster and La Sota (81%) or Texas-GB (85%). The highest genetic identity (96.2%) was observed with Pigeon/Argentina/Capital 3/97.

The predicted amino acid sequences surrounding the F2/F1 protein cleavage site from the NDV strains analyzed above are presented in Fig. 1. Virulent NDV isolates (mesogenic and velogenic) possess two pairs of basic aa at residues 112–113 and 115–116 at the C terminus of the F2 protein, and phenylalanine at position 117 at the N terminus of the F1 protein, whereas low pathogenic strains (avirulent and lentogenic) have only two single basic aa at positions 113 and 116 and leucine at position 117 (15). Pigeon/Uruguay/Montevideo/2/08 virus displayed the motif ^{112}RRQKR/F^{117}, which is typical of virulent NDV isolates (10). However, in contrast to the APMV-1 strains, the pathogenicity of PPMV-1 isolates cannot be deduced by the proportion of basic aa present at the F2/F1 protein cleavage site. This is based on previous reports where PPMV-1 isolates with an identical aa sequence at F2/F1 protein cleavage site were characterized by biological tests and showed to have both high and low intracerebral pathogenicity index (ICPI) values (10,11,15,18). These data suggest that other genetics, environmental and/or host's own factors are involved in the pathogenicity of PPMV-1. In order to know the virulence of Pigeon/Uruguay/Montevideo/2/08 it will be necessary to carry out biological tests such as the ICPI.

As shown in Fig. 1, a serine at position 124 was predicted for almost virulent NDV strains and PPMV-1 isolates included Pigeon/Uruguay/Montevideo/2/08 virus. In addition, a cysteine at position 76 (an important aa in disulphide bond formation between F1 and F2), potential glycosylation sites (85–87 residues) and fusion inducing hydrophobic stretch (117–142 residues) were found to be conserved in Pigeon/Uruguay/Montevideo/2/08 isolate and in other NDV strains irrespective of pathotype, as reported earlier by other workers (17).

Sequences of 375 nucleotides corresponding to the 3′ end of F protein gene were used to determine the phylogenetic relationships of the Pigeon/Uruguay/Montevideo/2/08 isolate as compared with published reference strains (Fig. 2). Pigeon/Uruguay/Montevideo/2/08 isolated from a sick pigeon was classified, together with the other PPMV-1 viruses associated with the ongoing panzootic in pigeons, into the genotype VIb/4b (3). This virus clusters together with other representative PPMV-1 strains isolated from free-living and domestic pigeons in many countries of the world. The phylogenetic tree revealed that Pigeon/Uruguay/Montevideo/2/08 and Pigeon/Argentina/Capital/3/97 viruses were placed in a distinct clade within subgroup VIb, and the geographical proximity of theses isolates allows us to suspect that these viruses may have a common origin different from North American and European strains.

Further investigation into the effect of sequence variation at probes and primer sites on the performance of rapid diagnostic tests such as rRT-PCR may assist in improving their sensitivity and specificity to prevent the failure of detection of PPMV-1. The present study reports the first isolate of PPMV-1 from a sick racing pigeon in Uruguay. The presence of this virus in Columbidae should be a warning for public and company veterinarians, and for farmers who should improve biosecurity and control measures to avoid potential sources of reintroduction of ND in commercial poultry populations.

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ACKNOWLEDGMENTS

The authors thank Claudio Afonso, Christian Mathieu, and Alicia Dib for critical reading of this manuscript, Juan Piquerés for providing the clinical samples, and Ruth Santestevan for preparing the manuscript. This work was supported by funding from Facultad de Veterinaria-UDELAR-Programa de Posgrados.