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Characterization and Epidemiology of Pigeon Paramyxovirus Type-1 Viruses (PPMV-1) Isolated in Macedonia

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SUMMARY. We have characterized in this study 10 PPMV-1 isolated from domestic pigeons and one PPMV-1 isolated from a feral pigeon in the period 2007–2012, using both classical methods (HI test and ICPI test) and molecular methods (RT-qPCR, RT-PCR, and nucleotide sequencing). Using phylogenetic analysis of partial fusion gene sequences, these viruses clustered with recent European PPMV-1 isolates (EU/re) within the genotype VIb/1. All isolates possessed virulent cleavage site motifs with variable morbidity and mortality in pigeons. The intracerebral pathogenecity indices of the five isolates ranged from 0.59 to 1.53. The repetitive isolation of PPMV-1 viruses for several consecutive years led toward establishing enzootic presence of the disease in pigeons. A high nucleotide sequence homology between the Macedonian isolates and EU/re isolates was shown. Co-circulation of different isolates in the same holdings was detected. This is the first study to extensively describe the molecular epidemiology of PPMV-1 isolated in Macedonia.

RESUMEN. Caracterización y epidemiología de Paramyxovirus de paloma tipo 1 (PPMV-1) aislados en Macedonia.

En este estudio se caracterizaron diez paramixovirus de paloma tipo 1 (con las siglas en inglés PPMV-1) aislados de palomas domésticas y un paramixovirus PPMV-1 aislado de una paloma silvestre en el período comprendido entre los años 2007-2012 utilizando métodos clásicos (prueba de inhibición de hemaglutinación y el índice de patogenicidad) y métodos moleculares (transcripción reversa y reacción en cadena de la polimerasa cuantitativa, transcripción reversa y reacción en cadena de la polimerasa y secuenciación de nucleótidos). Mediante el análisis filogenético de las secuencias parciales de los genes de fusión, estos virus se agruparon con los aislamientos europeos recientes de PPMV-1 (EU/re) dentro del genotipo VIb/1. Todos los aislamientos mostraron secuencias motivo del tipo virulento en el sitio de disociación con morbilidad y mortalidad variables en las palomas. Los índices de patogenia intracerebral de los cinco aislamientos variaron de 0.59 a 1.53. El aislamiento repetido de los virus PPMV-1 durante varios años consecutivos condujo a establecer la presencia enzoótica de la enfermedad en las palomas. Se demostró una alta similitud genética en las secuencia de nucleótidos entre los aislados macedonios y los aislados UE/re. Se detectó la co-circulación de diferentes aislamientos en las mismas explotaciones. Este es el primer estudio que describe ampliamente la epidemiología molecular de los paramixovirus de paloma tipo 1 aislados en Macedonia.

Key words: pigeon paramyxovirus type 1, pigeon, characterization, phylogeny, epidemiology, Macedonia

Abbreviations: APMV-1 = avian paramyxovirus type 1; CS = cleavage site; ECE = embryonated chicken eggs; F = fusion; HI= hemagglutination inhibition; ICPI = intracerebral pathogenecity index; M = matrix; MAb = monoclonal antibody; PPMV-1 = pigeon paramyxovirus type 1; RT-PCR = reverse transcriptase PCR; RT-qPCR = reverse transcriptase quantitative real-time PCR

Avian paramyxovirus type 1 virus (APMV-1) is widely distributed among the majority of bird species, in which it causes disease with varying intensity depending on the host, age and immune status, virulence of the virus, presence of a co-infection, and environmental conditions (3,21,38). Pigeons are also susceptible to APMV-1 infection. According to extensive scientific data about the properties of APMV-1 found in pigeons, they are classified as pigeon paramyxovirus type 1 (PPMV-1). These viruses are responsible for the ongoing panzootic in pigeons (1), originating back at least to 1978 in Iraq (20). A couple of years later PPMV-1 had already reached Europe (8), while in the mid-1980s their geographical circulation had become extensive, thus acquiring a panzootic character (6). International trade, races, and exhibitions of pigeons are stated as reasons for the global spread of PPMV-1 (6). Pigeon PMV-1 is antigenically distinct from other APMV-1s, which is proven by the use of monoclonal antibodies (7). The results of restriction enzyme and phylogenetic analysis showed that they form a separate clade of monophyletic descent, genotype VIb of Class II APMV-1 (34,35). Genotype VIb is further divided into VIb/1, containing the vast majority of pigeon isolates, which are grouped in four subgroups from all over the world, and VIb/2, originally containing isolates from Croatia and later including 70 isolates from Russia (28,35).

The disease is enzootically present in domestic pigeons and occasionally spreads to feral pigeons, doves, and exotic birds (4,19). This group of viruses represents a constant threat to poultry (4,19). In 2010, we isolated and characterized PPMV-1 from back-yard domestic chickens showing respiratory distress and low level of mortality. This isolate was identified as a pigeon variant using MAb. It possessed a virulent cleavage site (CS) motif of ^{112}RRQKR*F^{117} and had a mesogenic ICPI of 0.81. Phylogenetically, this isolate is clustered in genotype VIb/1 (13).

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Table 1. Anamnesis and clinical data and post-mortem findings related to isolates characterized in this study.

Isolate	Host	Sampling date (dd.mm.yyyy)[A]	Organs used in diagnosis[B]	Location coordinates	Anamnesis and clinical data	Post-mortem
Macedonia/234/2007	DP	30.11.2007	PVO	N 41°28′57 E 22°05′32	Diarrhea, cachexia, lethargy, torticollis	*Laryngotracheitis haemorrhagica, enteritis haemorrhagica*
Macedonia/1501/2007	DP	XX.XX.2007	PVO	N 41°28′57 E 22°05′32	Increased mortality[C]	*Tracheitis haemorrhagica, aerosacculitis, enteritis haemorrhagica*
Macedonia/1503/2007	DP	XX.XX.2007	PVO	N 41°28′57 E 22°05′32	Increased mortality[C]	*Tracheitis haemorrhagica, enteritis haemorrhagica*
Macedonia/230/2008	DP	18.9.2008	PVO	N 41°49′59 E 20°52′58	Not available	Not available
Macedonia/232/2008	DP	XX.XX.2008	PVO	N 41°38′09 E 22°28′00	Not available	*Tracheitis haemorrhagica, enteritis haemorrhagica*
Macedonia/962/2008	DP	XX.XX.2008	PVO	N 41°48′03 E 20°54′50	Emergency vaccination when first cases occurred in the holding with 120 pigeons (35 dead, 60 sick). Signs: unstable gait and flight, opistotonus. Antibiotic treatment.	*Laryngotracheitis haemorrhagica, hepatitis necroticans, enteritis haemorrhagica*
Macedonia/2810/2010	DP	12.3.2010	PVO	N 41°28′57 E 22°05′32	Mortality in holding with 100 pigeons. Clinical signs: hobbling, stiffness of legs and wings with edema of joints, cachexia	*Enteritis haemorrhagica*
Macedonia/233/2011	DP	4.4.2011	Intestines	N 41°26′15 E 22°38′33	Increased mortality	*Enteritis haemorrhagica*
Macedonia/415/2011	DP	XX.5.2011	PVO	N 41°58′51 E 21°31′37	Low mortality for prolonged period of time, intestinal ascaridosis	*Enteritis haemorrhagica*
Macedonia/419/2011	DP	XX.7.2011	PVO	N 41°55′32 E 21°37′49	Low mortality in adult birds, significantly higher in younger birds, lethargy, torticollis, uncoordinated locomotion	*Enteritis haemorrhagica, hyperemia meningi*
Macedonia/9701/2012	FP	17.10.2012	PVO	N 41°01′58 E 21°20′25	One dead bird found on-site	*Enteritis haemorrhagica*

[A]XX = date not known.
[B]DP = domestic pigeon; FP = feral pigeon; PVO = pool of visceral organs.
[C]Same holding.

The aim of our study was to characterize PPMV-1 isolates from domestic and feral pigeons using classical and molecular methods, as well as to describe the epidemiological scenario of these viruses isolated in Macedonia.

MATERIALS AND METHODS

Viruses and virus isolation. Eleven PPMV-1 isolates recovered from domestic and feral pigeons in Macedonia in the period from 2007 to 2012 were analyzed in this study. All viruses were isolated from dead birds from different parts of the country, after they were submitted to the laboratory at the Faculty of Veterinary Medicine in Skopje, Macedonia, because of clinical signs associated with APMV-1 and increased mortality (Table 1). The supernatant of homogenized organs (brain, trachea, lungs, liver, kidney, spleen, and intestines) was inoculated in 9–11-day-old embryonated chicken eggs (ECE) and incubated for a period of 5 days at 37 C. Allantoic fluid was harvested from the ECE and used as inoculum in the subsequent passage. After each passage, the allantoic fluid was tested for the presence of hemagglutination activity (38). Subsequently, a nomenclature of viruses was made according to the following scheme: serotype/host/country of origin/lab ID/year of collection, and as such it was submitted to GenBank for provision of accession numbers.

Hemagglutination inhibition test. Infective allantoic fluids were assayed in hemagglutination inhibition (HI) test with a polyclonal PMV-1 serum and monoclonal antibody (MAbs): 617/161 specific for pigeon variants of APMV-1, MAb U85 specific for classical strains and MAb 7D4 specific for La Sota and F strains (5,6,9), which were kindly provided by R. Manvell from APHA, Weybridge, UK. Additionally, infective allantoic fluids were tested using H5 avian influenza antisera (A/cygnus olor/1/Croatia/2005 H5N1 and A/turkey/Italy/1980 H5N2) and H7 avian influenza antisera (A/turkey/Italy/2732/99 H7N1 and A/turkey/Italy/8535/2002 H7N3). The test was done following the internationally recommended protocol (38).

Pathogenicity test. An intracerebral pathogenecity index test was used to determine the pathogenicity of the viruses. The test was done and the index was calculated following the internationally recommended protocol (38).

Extraction of RNA, RT-qPCR, and RT-PCR. Extraction of RNA was conducted using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's protocol. The purified RNA was amplified using a Qiagen OneStep RT-PCR Kit (Qiagen) in the RT-qPCR test using primers and probes for the detection of the matrix (M) gene and the fusion (F) gene (37). A 700 bp region of the F gene was amplified using MSF1 5′-GACCGCTGACCACGAGGTTA-3′ (forward primer) and no. 2 5′-AGTCGGAGGATGTTGGCAGC-3′ (reverse primer) (2). The reverse transcription

step in the two-step RT-PCR was performed using the forward primer and the QuantiTect Reverse Transcription Kit (Qiagen) for the preparation of cDNA at 42 C for 60 min followed by 95 C for 3 min. The second PCR step was performed using Taq PCR Master Mix Kit (Qiagen) at 94 C for 3 min, 42 cycles of 94 C for 1 min, 50 C for 1 min, and 72 C for 3 min, and a final elongation at 72 C for 10 min. The obtained PCR product was visualized in 1.5% agarose gel with the addition of ethidium bromide under ultraviolet light. The DNA was purified using the QIAquick Gel Extraction Kit (Qiagen).

Sequencing and phylogenetic analyses of the F gene. The sequencing reaction was performed using the Big Dye Terminator v3.1kit (Applied Biosystems, Foster City, USA) a different forward primer no. 7 5′-TTAGAAAAAACACGGGTAGAA-3′, and the same reverse primer no. 2 as in the two-step RT-PCR (2), with a thermal protocol of 96 C for 5 min, 25 cycles of 96 C for 10 sec, and 50 C for 5 sec and 60 C for 4 min. The sequencing reaction product was purified with ethanol precipitation and analyzed using the ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The analysis of the sequence data was done using the BioEdit Sequence Alignment Editor version 7.0.9.0 (18), and the alignment of the sequences was done using the Clustal W method in the MEGA 5.10 software (32). Nucleotide sequences were aligned to begin at the start codon ATG at the position 47 of the F gene and to end at position 420, encompassing the CS in total length of 374 bp. The phylogenetic analysis was done using MEGA 5.10, and the tree was constructed according to the maximum likelihood method based on the Kimura 2-parameter model with a bootstrap value of 1000. The accession numbers of the sequences submitted to the GenBank database and used for construction of the phylogentic tree are shown in parentheses (Fig. 1).

RESULTS

All isolates in this study were obtained from dead pigeons in the second ECE passage, except for isolate APMV-1/pigeon/Macedonia/1503/2007, which was isolated in the third passage. Serological typing revealed that all the isolates showed inhibition of hemagglutination in the presence of polyclonal PMV-1 antiserum and MAb 617/161, which is specific for pigeon variants of APMV-1, while they did not show any inhibition of hemagglutination in the presence of MAb U85 and 7D4, which are specific for classical strains and La Sota and F strains, respectively (Table 2). Additionally, all isolates were tested using H5 and H7 avian influenza antisera and were found to be negative (data not shown).

The ICPIs of five tested isolates ranged from 0.59 to 1.53 (Table 3). Based exclusively on ICPI, isolate APMV-1/pigeon/Macedonia/232/2008 is classified as a lentogenic pathotype, isolate APMV-1/pigeon/Macedonia/1501/2007 is classified as a velogenic pathotype, and all other isolates are classified as a mesogenic pathotype. The isolate APMV-1/pigeon/Macedonia/232/2008, despite having the ICPI of avirulent viruses, has an amino acid motif of CS typical for virulent viruses, which is not uncommon for PPMV-1.

All isolates were tested using RT-qPCR for detection of the M gene and the virulent F gene. They were found to be positive with both tests (data not shown), that is, they were proven to be velogenic or mesogenic strains of Class II APMV-1. In addition, the isolates were tested using a RT-qPCR recommended protocol for detection of the M gene of avian influenza virus (AIV) (30) and were found to be negative (data not shown). RT-PCR generating a product of 700 bp of the F gene was used to sequence isolates. All isolates produced an expected product size in gel electrophoresis and were subjected to nucleotide sequencing.

Sequences of all isolates were submitted to GenBank for provision of unique accession numbers, as shown in Table 3. The amino acid motif of CS of all isolates revealed a single typical pattern for virulent strains, ^{112}RRQKR*F^{117}, thus fulfilling the accepted molecular criteria for pathogenicity of virulent viruses. Nevertheless, the most recently isolated viruses APMV-1/pigeon/Macedonia/419/2011 and APMV-1/feral pigeon/Macedonia/9701/2012 differ from the other isolates at the nucleotide position 351 in the CS region, resulting in synonymous substitution (C to T) not affecting the CS motif (data not shown). Interestingly, all other global isolates used for the construction of the phylogenetic tree (Fig. 1) have this substitution except for the Belgian isolates.

For the purposes of phylogenetic analysis, a 374-bp fragment spanning over the CS of the F gene (nt 47–420) from studied and reference virus sequences was used for construction of the phylogenetic tree. Based on clustering in the phylogenetic tree, it can be concluded that the Macedonian isolates cluster in genotype VIb of class II viruses (Fig. 1). Despite being isolated over a 6 yr period, they clearly group with each other. However, isolate APMV-1/feral pigeon/Macedonia/9701/2012 has the highest nucleotide sequence identity of 99.5% with Serbian pigeon isolates. The isolate APMV-1/pigeon/Macedonia/419/2011 has the lowest nucleotide sequence identity ranging from 97.6% to 98.1% compared to the other Macedonian isolates.

DISCUSSION

Clinical signs of affected pigeons consisted mainly of nervous signs in the form of uncoordinated movement, hobbling, unstable flying, torticollis, and opistotonus. In our study, a variation in the mortality rate among pigeons was observed in one holding, whereas it was higher in young birds than in adult birds (Table 1). This confirms previous reports that young pigeons are more susceptible to infection. Their mortality and morbidity rate can reach up to 100% accompanied with neurological signs, while morbidity in adult pigeons is usually below 10% and death occurs in a chronic manner (33). Increased mortality, regardless of the age, was observed in holdings where co-circulation of two isolates was detected: APMV-1/pigeon/Macedonia/1501/2007 and APMV-1/pigeon/Macedonia/1503/2007. This could possibly be either due to simultaneous circulation of these two viruses or due to the high pathogenicity of the isolate APMV-1/pigeon/Macedonia/1501/2007, which has showed that it possesses an ICPI of 1.53. The majority of the gross lesions were consistent with previous reports and consisted of hemorrhagic enteritis and hemorrhages in the upper respiratory system, while macroscopic lesions were absent in the nervous system (29,33).

Isolation of investigated viruses was performed in the second ECE passage, except for the isolate APMV-1/pigeon/Macedonia/1503/2007, which was isolated in the third passage. Isolation of APMV-1 in the third passage is rare, and it happens in 5% of cases. Some strains of PPMV-1 show an inability to grow in the ECE, so instead they are isolated in chicken liver cells or chicken embryo cells (24). In the HI test, all isolates were typed as PPMV-1 using MAb 617/161, which is used for discrimination between pigeon and other classical or vaccine strains (5,7,9,29,31).

The pathogenicity indices of the five isolates in this study were varied and ranged between 0.59 and 1.53, despite having the same CS motif ^{112}RRQKR*F^{117}. The isolate APMV-1/pigeon/Macedo-

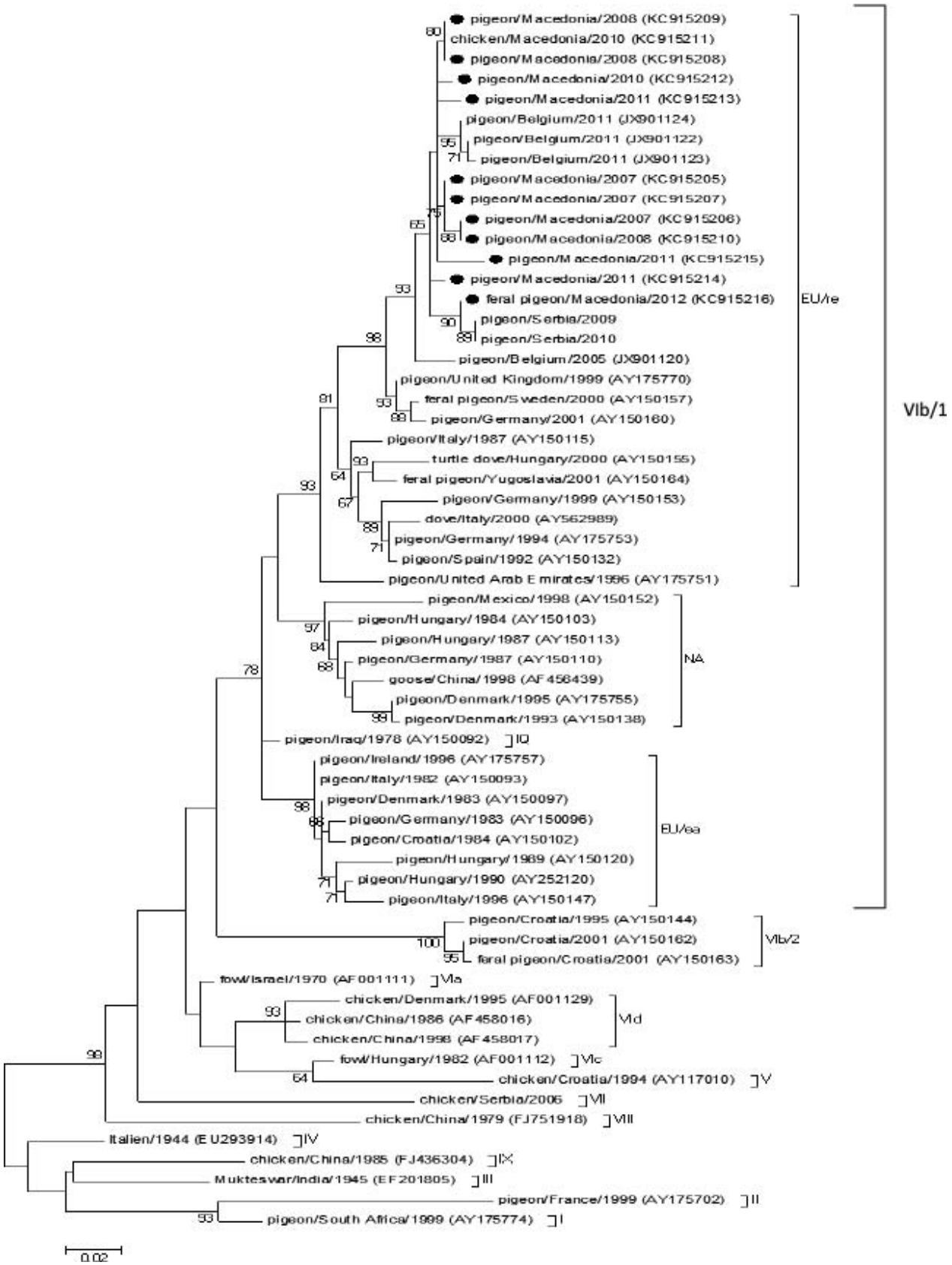

Fig. 1. Phylogenetic anaylsis of PPMV-1 isolates used in this study (marked with a black circle) and PPMV-1 strains representing other genotypes and subgenotypes was inferred by using the Maximum Likelihood method based on the Kimura two-parameter model. A discrete Gamma distribution was used to model evolutionary rate differences among sites (two categories [+*G*, parameter = 0.7886]). The analysis involved 61 nucleotide sequences. There was a total of 374 nucleotide positions in the final dataset.

Table 2. Hemagglutination titers and hemagglutination inhibition titers of isolates used in this study.

Isolate	HA titer (log_2)	HI titer (log_2) using 4 HA units			
		Polyclonal PMV-1	MAb 617/161	MAb U85	MAb 7D4
APMV-1/pigeon/Macedonia/234/2007	8	5	10	<2	<2
APMV-1/pigeon/Macedonia/1501/2007	6	4	9	<2	<2
APMV-1/pigeon/Macedonia/1503/2007	7	4	10	<2	<2
APMV-1/pigeon/Macedonia/230/2008	8	5	10	<2	<2
APMV-1/pigeon/Macedonia/232/2008	7	6	9	<2	<2
APMV-1/pigeon/Macedonia/962/2008	6	4	9	<2	<2
APMV-1/pigeon/Macedonia/2810/2010	7	4	9	<2	<2
APMV-1/pigeon/Macedonia/233/2011	8	7	8	<2	<2
APMV-1/pigeon/Macedonia/415/2011	7	8	9	<2	<2
APMV-1/pigeon/Macedonia/419/2011	6	6	11	<2	<2
APMV-1/feralpigeon/Macedonia/9701/2012	6	8	11	<2	<2

nia/232/2008 had an ICPI of 0.59, which is typical for lentogenic strains, but it also had the CS of the F gene typical for virulent viruses. However, this is not uncommon for PPMV-1 as was previously reported (10,25). This discrepancy of biological and molecular pathogenicity traits is not associated with the F protein (14), but with nucleoprotein, phosphoprotein, and polymerase in the form of a replication complex (16). In fact, although the F protein is a major determinant of pathogenicity (17,26,27) other proteins influence pathogenicity too (15). In a study of viruses isolated from dead racing pigeons manifesting nervous signs prior to their death, Meulemans et al. (25) reported that PPMV-1 with CS motif ^{112}RRQKR*F^{117} possesses highly variable but low ICPI values (average 0.69) and PPMV-1 with CS motif ^{112}GRQKR*F^{117} possesses high ICPI values (average 1.44). As previously mentioned, the isolate APMV-1/pigeon/Macedonia/1501/2007 isolated from a holding with increased mortality had an ICPI of 1.53, thus classifying the strain as a velogenic pathotype. Still, increased values of ICPI and IVPI of PPMV-1 passaged in chicks or ECE are reported, acknowledging that isolates adapted to other birds besides poultry may not show their potential virulence in chicks using classical tests for assessment of pathogenicity (7). This once again confirms the need that *in vivo* pathogenicity tests should always be accompanied with sequencing, especially in PPMV-1, because the CS motif does not always correspond to ICPI (14). Moreover, sensitivity of ICPI sometimes is not sufficient to detect subtle changes in virulence (15).

It has been previously reported that some PPMV-1 with a CS motif ^{112}RRQKR*F^{117} cannot be detected by the widely used and USDA validated F gene assay due to probe-site mismatches (22). After the alignment of the fusion probe F-4894 sequence with sequences of our isolates (from nucleotide position 4877 to 4900 of the whole genome), three mismatches were detected at positions 1 (T to A), 13 (A to G), and 19 (A to G) of the F probe binding site, but none at position 6, which is crucial for failure to detect PPMV-1 (23). Viruses with mismatches at positions 1 and 13 were readily detected using F gene assay, as was the case in our study (37).

Pigeon strains with an amino acid motif ^{112}RRQKR*F^{117} of the CS are being isolated since the 1990s (25). All isolates in this study that have been isolated during the 2007–2012 period have this motif of CS, whereas they were classified as virulent strains according to the internationally accepted definition (38). The position in the phylogenetic tree showed that all isolates group in the subgenotype VIb, according to the genotype classification of APMV-1 (11). Subgenotype VIb is divided into VIb/1 and VIb/2, with VIb/1 being further divided into four subgroups: Iraqi strain (IQ), early European strains (EU/ea), North American strains (NA), and recent European strains (EU/re), most probably originating in northeast Africa (35). The Macedonian strains investigated in this study belong to the subgroup EU/re representing recent European strains. According to the phylogenetic tree (Fig. 1), they form a clade together with Serbian PPMV-1 isolated in 2009 (pigeon/Serbia/2009) and 2010 (pigeon/Serbia/2010) and three Belgian PPMV-1s isolated in 2011 (accession numbers: JX901122, JX901123, JX901124). Following the phylogenetic analyses of the complete genomes of these three Belgian isolates and other earlier isolated PPMV-1 in Belgium and worldwide, it was found that they are the only members of a separate clade (36). Similarity between our and Serbian isolates can be expected due to the fact of geographical

Table 3. Biological and molecular characteristics of isolates used in this study.

Isolate	GenBank accession no.	Cleavage site motif	ICPI[A]	Virulence	Genotype
Macedonia/234/2007	KC915205	^{112}RRQKR*F^{117}	0.96	Virulent	VIb
Macedonia/1501/2007	KC915206	^{112}RRQKR*F^{117}	1.53	Virulent	VIb
Macedonia/1503/2007	KC915207	^{112}RRQKR*F^{117}	ND	Virulent	VIb
Macedonia/230/2008	KC915208	^{112}RRQKR*F^{117}	0.84	Virulent	VIb
Macedonia/232/2008	KC915209	^{112}RRQKR*F^{117}	0.59	Virulent	VIb
Macedonia/962/2008	KC915210	^{112}RRQKR*F^{117}	ND	Virulent	VIb
Macedonia/2810/2010	KC915212	^{112}RRQKR*F^{117}	ND	Virulent	VIb
Macedonia/233/2011	KC915213	^{112}RRQKR*F^{117}	1.19	Virulent	VIb
Macedonia/415/2011	KC915214	^{112}RRQKR*F^{117}	ND	Virulent	VIb
Macedonia/419/2011	KC915215	^{112}RRQKR*F^{117}	ND	Virulent	VIb
Macedonia/9701/2012	KC915216	^{112}RRQKR*F^{117}	ND	Virulent	VIb

[A]ND = not done.

proximity, although high similarity with the Belgian isolates is nevertheless an unusual finding. These Belgian isolates share higher similarity with Macedonian isolates then Belgian isolates isolated in 2005. It could be hypothesized that illegal trade and movement of birds are one of the reasons for these findings, or it may be presumed that an intermediate host, such as wild birds, acted as a link between these two pools of viruses. Considering that racing pigeons can fly long distances over a relatively short period, it is also likely that they could have played a role in the direct transmission of the virus, especially if pigeons have been in an incubation period shortly before and during their flight. This is supported by the findings that Macedonian and Belgian isolates possessing the highest similarity were isolated in the same year. However, there is a possibility that other closely related PPMV-1 have been circulating in other countries but have gone undetected or unreported, which would have contributed toward a better understanding of the epidemiological link. Pigeon PMV-1 isolated from geographically distant European countries form a distinct cluster supported by a high bootstrap value, indicating a circulation of these viruses on the European continent.

During the course of a period of 6 yr, 11 PPMV-1 with a close phylogenetic relationship were isolated from dead pigeons from different areas, thus establishing an enzootic presence in the country. Despite vaccination, these viruses are enzootic in domestic pigeons in many countries and can spread to feral pigeons and, in some instances, to zoo birds (1,4,23,25). The only isolation of PPMV-1 in 2012 was from a dead feral pigeon (APMV-1/feral pigeon/Macedonia/9701/2012). This isolate showed the highest degree of similarity with Serbian pigeon isolates from 2009 and 2010 but still is in the same group as other local isolates from domestic pigeons isolated in the previous years. Established enzootic circulation and grouping in the phylogenetic tree suggest a spillover of the virus between domestic and feral pigeons or vice versa. Enzootic circulation of PPMV-1 with virulent CS can lead to infection of backyard poultry as was previously reported, although with no indication whether domestic or feral pigeons have played a role in the introduction of this virus to poultry (13). It is evident in the phylogenetic tree that this isolate from 2010 (accession number KC915211) is closely related to PPMV-1 isolated from domestic pigeons from other parts of the country in 2008. The low level of mortality associated with this isolate could be attributed to the mandatory vaccination of backyard poultry, which is done twice per year, as well as to its moderate ICPI of 0.81. However, continuous circulation of PPMV-1 in pigeons and their transmission to poultry remains a permanent threat to the poultry industry. In European Union countries, the majority of outbreaks of PPMV-1 in poultry have occurred in backyard flocks (4). This category of poultry is highly susceptible to infectious diseases and can be responsible for the introduction of certain diseases in a region or a country. Furthermore, backyard poultry can facilitate the spread of virulent Newcastle disease virus by infecting wild birds, which in some instances can have a deadly outcome (12).

Three different PPMV-1 were isolated in 2007 from two holdings in one town (N 41°28′57. E 22°05′32). In the first holding two different isolates were isolated (APMV-1/pigeon/Macedonia/1501/2007 and APMV-1/pigeon/Macedonia/1503/2007), while in the second holding the isolate APMV-1/pigeon/Macedonia/234/2007 was isolated, which is identical to one of the isolates (APMV-1/pigeon/Macedonia/1503/2007) from the first holding. Moreover, the other isolate from the first holding (APMV-1/pigeon/Macedonia/1501/2007) is identical to APMV-1/pigeon/Macedonia/962/2008, isolated a year later in another part of the country (N 41°48′03. E 20°54′50). The isolate from 2008 though is different from APMV-1/pigeon/Macedonia/230/2008, which was isolated in the same year and in the same area (N 41°49′59. E 20°52′58). This intense co-circulation of different isolates in the same holdings or areas probably comes as a result of the nature of husbandry and breeding, which are characterized by continuous trade and mixing of pigeons with poorly known or unknown health status.

This study comprehensively describes the biological and molecular characteristics of PPMV-1 isolated in Macedonia and the epidemiological context of their circulation at the transboundary level.

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