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CHARACTERIZATION OF VELOGENIC NEWCASTLE DISEASE VIRUSES ISOLATED FROM DEAD WILD BIRDS IN SERBIA DURING 2007

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ABSTRACT: Avian paramyxoviruses type 1 or Newcastle disease viruses (NDV) are frequently recovered from wild birds and such isolates are most frequently of low virulence. Velogenic NDV are usually recovered from poultry and only occasionally from wild birds. Five NDV isolates were obtained from carcasses of four wild bird species during 2007 in Serbia: Mallard (*Anas platyrhynchos*), Eurasian Sparrowhawk (*Accipiter nisus*), feral Rock Pigeon (*Columba livia*), and Eurasian Collared Dove (*Streptopelia decaocto*). All the isolates have a typical fusion protein cleavage site motif of velogenic viruses (^{112}R-R-Q-K-R-F^{117}). The highest homology (99%) for the nucleotide sequences spanning the M and F gene of the studied isolates was with the genotype VII NDV isolate Muscovy duck/China(Fujian)/FP1/02. Phylogenetic analysis based on a partial F gene sequence showed that the isolates from wild birds cluster together with concurrent isolates from poultry in Serbia within the subgenotype VIId, which is the predominant pathogen involved currently in Newcastle disease outbreaks in poultry worldwide. It is unlikely that the wild birds played an important role in primary introduction or consequent spread of the velogenic NDV to domestic poultry in Serbia, and they probably contracted the virus from locally infected poultry.
Key words: Genotype, Newcastle disease virus, Serbia, wild birds.

INTRODUCTION

Newcastle disease (ND) is a highly contagious disease that causes severe economic losses in poultry, especially chickens, worldwide (Alexander, 2000). It is caused by Newcastle disease virus (NDV), also called avian paramyxovirus type 1 (APMV-1), a member of the genus *Avulavirus*, family *Paramyxoviridae*. The genome of NDV is an approximately 15.2 kb long, negative-sense, single-stranded RNA that codes for RNA-directed RNA polymerase (L gene), hemagglutinin-neuraminidase (HN gene), fusion (F gene), matrix (M gene), phosphoprotein (P gene), and nucleocapsid (NP gene) proteins, in that order, from the 5′ terminus to the 3′ terminus (Kattenbelt et al., 2006). Recent analysis of the genome sizes and sequences of the F and L genes identified two major classes (I and II; Czeglédi et al., 2006). Class I isolates are generally of low virulence and have been recovered primarily from waterfowl of the family Anatidae; whereas, class II viruses comprise the vast majority of isolates of diverse virulence recovered from poultry (gallinaceous birds), pet, and wild birds. Each class has nine genotypes, genotypes 1–9 in class I and I–IX in class II (Kim et al., 2007). Severity of NDV infection varies from subclinical to 100% mortality, depending on the susceptibility of the host and the virulence of the virus. Strains of NDV are therefore categorized into highly virulent (velogenic), intermediate (mesogenic), or nonvirulent (lentogenic) based on their pathogenicity in chickens (Alexander, 2000). The molecular basis of pathogenicity seems to be mainly determined by the amino acid sequence motif at the protease cleavage site of the precursor fusion protein and the ability of host proteases to cleave the fusion protein of different pathotypes (Collins et al., 1993).

Wild aquatic birds are considered the natural reservoirs of NDV of both classes,

but they mostly harbor lentogenic strains (Alexander, 2000; Kim et al., 2007). However, such lentogenic strains apparently have the potential to become velogenic after transmission to and circulation in chicken populations (Shengqing et al., 2002). An antigenic and host variant of the class II NDV is a pigeon-type NDV, often called pigeon PMV-1 (PPMV-1), which causes a serious disease in domesticated, feral, and wild birds of the family Columbidae (Collins et al., 1989; Ujvári et al., 2003). These isolates may differ widely in their virulence for birds, especially chickens. Some have high virulence, and others low virulence for chickens, which in some cases increases on passage through chickens (King, 1996). PPMV-1 has also been responsible for ND outbreaks in poultry (Alexander et al., 1985). In this study velogenic NDV isolates from a Mallard (*Anas platyrhynchos*), wild and feral Columbidae, and a Eurasian Sparrowhawk (*Accipiter nisus*) in Serbia were antigenically and genetically analyzed and compared with concurrent velogenic NDV isolates from poultry.

MATERIALS AND METHODS

Virus isolation and identification

Five NDV were isolated from dead wild birds found in early 2007 in the vicinity of infected poultry during an outbreak of velogenic ND in Serbia. Of these, two viruses were isolated from Eurasian Collared Doves (*Streptopelia decaocto*; 44°49′N, 20°28′E; 44°35′N, 20°22′E), and one virus was isolated from a feral Rock Pigeon (*Columba livia*; 44°30′N, 21°18′E), a Mallard (45°26′N, 20°17′E), and a Eurasian Sparrowhawk (44°49′N, 20°28′E). To compare these isolates with concurrent isolates from domestic poultry, three NDV isolated from chickens during late 2006 and early 2007 were included in this study. All viruses were isolated in chicken embryos using standard procedure (Anonymous, 1992), and allantoic fluid was used as a source of the virus for further studies. Preliminary identification and characterization of virus isolates were carried out by hemagglutination (HA) and hemagglutination inhibition (HI) tests with APMV-1 polyclonal antiserum and three monoclonal antibodies (mAbs) as described previously (Anonymous, 1992). Monoclonal antibodies U85 specific for classical NDV strains (Alexander et al., 1997), 7D4 specific for La Sota and F strains (Meulemans et al., 1987), and 161/617 specific for PPMV-1 strains responsible for the pigeon panzootic (Collins et al., 1989) were used. The mean death time (MDT) in chicken embryos was determined for isolates from the Rock Pigeon and the Eurasian Sparrowhawk using a standard procedure (OIE, 2004).

Reverse transcription PCR

Viral RNA was extracted from allantoic fluids using QIAamp Viral RNA Mini Kit (Qiagen GmbH, Hilden, Germany). Two-step reverse transcription (RT) and PCR were carried out for all isolates. cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, California, USA) and random primers according to the manufacturers instructions. The PCR amplifications were carried out using AmpliTaq Gold PCR Master Mix (Applied Biosystems) with 1 μl of cDNA in final volume of 50 μl.

To obtain the complete M and F gene sequences for the isolates from wild birds, seven partially overlapping fragments were amplified by PCR using seven primer pairs (Table 1), each at a final concentration of 0.4 μM. For the three isolates from poultry, only a partial sequence of the F gene was amplified using MV1 and B2 primers. The thermocycling conditions for all primer pairs except for the MV1 and B2 primer pair consisted of initial denaturation at 95 C for 5 min, followed by 40 cycles of 94 C for 45 sec, 55 C for 45 sec, 72 C for 2 min, and a final extension at 72 C for 7 min. The thermocycling conditions for the MV1 and B2 primer pair differed only in the annealing temperature, which was 50 C.

All PCR products were analyzed by electrophoresis in 1% agarose gel stained with ethidium bromide. Specific bands were excised and purified using QIAquick Gel Extraction Kit (Qiagen) according to manufacturer's instructions.

Sequencing and analysis of sequence data

Sequencing of all PCR amplicons was performed in both directions with Big Dye Terminator v3.1 Kit (Applied Biosystems) according to the manufacturer's instructions in a 3130 Genetic Analyzer (Applied Biosystems) and with the same primers used for PCR. After sequencing, the primer sequences were trimmed. The trimmed sequences for each virus isolate were assembled with MEGA

TABLE 1. Primers used for PCR amplification and nucleotide sequencing of the M and F gene of genotype VII Newcastle disease viruses (NDV) isolates from wild birds and domestic poultry from Serbia in 2006 and 2007.

Primer	Sequence (5′–3′) or reference	Target gene	Position in the gene[a]	Expected product size (base pairs)
NDVP 1301–1320F	TCACTTTAGCGTCACACGGA	Phosphoprotein	1,301–1,320	542
NDVM 371–390R	TTCTTGCAAGTTACCACCAT	Matrix	371–390	
NDVM 292–311F	ACTCTCTTCCGCAATGCTCT	Matrix	292–311	549
NDVM 818–840R	CCGACAGATAGATTGAGTCTCCT	Matrix	818–840	
NDVM 738–757F	TTTTTCTGCATATCGGGCTT	Matrix	738–757	553
NDVF 29–48R	ATATTGCGTCCTGAATGTGC	Fusion	29–48	
MV1	Lomniczi et al. (1998)	Matrix	1,163–1,187	572
B2	Lomniczi et al. (1998)	Fusion	470–492	
ONDV1aa	Lomniczi et al. (1998)	Fusion	334–362	540
NDVF 847–873R	TACAGTATAGGGTAACCAGTGATCAGG	Fusion	847–873	
NDVF 744–771F	TGACCATCCAGGCACTTTATAATTTAGC	Fusion	744–771	570
NDVF 1283–1313R	AATGTCTATCTATCAGGGATACAGCTTCTCC	Fusion	1,283–1,313	
NDVF 1250–1273F	ACAGACCCTCCTGGTATCATATCG	Fusion	1,250–1,273	521
NDVF 1753–1770R	GACAGGTTATCGGAATTG	Fusion	1,753–1,770	

[a] Based on GenBank accession number DQ839397 (isolate KBNP-4152).

TABLE 2. Characterization of Serbian Newcastle disease viruses (NDV) isolated from wild birds and poultry in 2006 and 2007 using hemagglutination inhibition (HI) with polyclonal and monoclonal antibodies.

Isolate	HI titer			
	Polyclonal PMV-1 serum	Monoclonal antibodies[a]		
		U85	161/617	7D4
NDV/Serbia/Collared Dove/749/2007	512	256	<2	<2
NDV/Serbia/Collared Dove/915/2007	128	64	<2	<2
NDV/Serbia/feral pigeon/108/2007	512	256	<2	<2
NDV/Serbia/Mallard/765/2007	256	256	<2	<2
NDV/Serbia/Sparrowhawk/1038/2007	512	128	<2	<2
NDV/Serbia/chicken/717/2007	256	256	<2	<2
NDV/Serbia/chicken/749/2007	256	256	<2	<2
NDV/Serbia/chicken/7721/2006	128	128	<2	<2

[a] Monoclonal antibody U85 is specific for classical NDV strains, 161/617 for Pigeon PMV-1 strains and 7D4 for La Sota and F strains.

software version 4 (Tamura et al., 2007). Phylogenetic analysis was performed with the same software using the Neighbor Joining algorithm with 1,000 bootstrap replicates. In addition to M and F gene sequences of the five NDV isolates from wild birds analyzed in this study, 14 previously reported, corresponding NDV sequences representative of genotypes I–VII were included for comparison. The nucleotide sequence GenBank accession numbers and the genotype of these NDV were chicken/N. Ireland/Ulster/67, AY562991 (I); LaSota, AF077761 (II); Mukteswar, EF201805 (III); Herts/33, AY741404 (IV); cormorant/Canada/98CNN3-V1125/1998, GQ288382 (V); anhinga/U.S.(Fl)/44083/93, AY562986 (V); PPMV-1 IT-227/82, AJ880277 (VI); chicken/U.S.(CA)/1083(Fontana)/72, AY562988 (VI); cockatoo/Indonesia/14698/90, AY562985 (VIIa); Sterna/Astr/2755/2001, AY865652 (VIIb); KB-NP-4152, DQ839397 (VIId); JSD0812, GQ-849007 (VIId); Goose paramyxovirus SF02, AF473851 (VIId), Muscovy duck/China(Fujian)/FP1/02, FJ872531 (VIId). To further clarify the subgrouping of analyzed viruses of genotype VII, a second phylogenetic tree was constructed by comparing the nucleotide sequences of the F gene from nt 47 to 420. Sequences of three NDV from poultry analyzed in this study, sequences of two concurrent NDV from poultry in Bulgaria, and 14 reported NDV sequences representative of different VII subgenotypes were also included in the second phylogenetic tree. The nucleotide sequence GenBank accession numbers and the subgenotype of these NDV were RI-1/88, AF001134 (VIIa); chicken-2415-580-Burkina Faso-2008, FJ772463 (VIIb); TW-84-486, DQ898540 (VIIc); TW/96P, AF083971 (VIIc); Chicken/South Africa/171/06, FJ985977 (VIId); ZA331/B/99, AF532739 (VIId); Kr-owl/7/05, EF63-5439 (VIId); Kr-017/00, AY630428 (VIId); Kr-005/00, AY630423 (VIId); K-2, DQ296069 (VIId); AV 237/00 M, AY135754 (VIId); NDV08-027, FJ810453 (VIId); NDV05-066, DQ439921 (VIId); and NDV/Ch/Ivano-Frankivsk/01/07, EU780892 (VIId).

Nucleotide sequences of NDV isolates analyzed in this study were also translated into amino acid sequences to predict the virulence of each isolate according to the F0 protein cleavage site.

RESULTS

Characterization with polyclonal and monoclonal antibodies

All eight isolates from wild birds and poultry were characterized by polyclonal serum as APMV-1 strains. The mAb-binding pattern for the analyzed isolates obtained using HI tests was typical of classical NDV strains. None of the isolates was inhibited with either 7D4 mAb or 161/617 mAb, which are specific for the lentogenic La Sota and F strains and PPMV-1 strains responsible for the pigeon panzootic, respectively. The HI titers are shown in Table 2.

Pathogenicity of the isolates

From the pathotype prediction based on the cleavage site of the fusion protein, all eight NDV isolates were placed in the velogenic group with the motif ^{112}R-R-Q-

K-R-F^{117}. Mean death times of 50.4 hr and 48.4 hr for the isolates from Rock Pigeon and Eurasian Sparrowhawk, respectively, confirmed their velogenic phenotype.

Phylogenetic analysis

We compared the M and F gene sequences (2,949 nucleotides) among the NDV isolates from wild birds obtained in Serbia in 2007 and other representative NDV isolates. All five isolates from wild birds were closely related, belonging to the genotype VII (Fig. 1). The highest nucleotide homology (99%) for the 2,949 nucleotides of the studied isolates was with Muscovy duck/China(Fujian)/FP1/02. Additional phylogenetic study, including three concurrent isolates from poultry in Serbia, was based on sequence analysis of the variable region of the F gene (nt 47–420) and their comparison with genotype VII representative isolates. All eight isolates from Serbia clustered with the concurrent isolates from Bulgaria in genotype VIId (Fig. 2), which is currently the predominant pathogen involved in ND outbreaks worldwide.

DISCUSSION

Genetic studies between 1979 and 2002 of NDV strains from Serbia, Bosnia-Herzegovina, Croatia, and Slovenia, identified only the V genotype, which became endemic in the region (Wehmann et al., 2003). In our study, the five isolates from wild birds and three from domestic poultry collected during an outbreak of velogenic ND in Serbia during late 2006 and early 2007 belonged to the VII genotype (Figs. 1, 2), and possessed cleavage sites of the fusion protein typical for velogenic viruses (^{112}R-R-Q-K-R-F^{117}). Nevertheless some PPMV-1 isolates with this amino acid motif at the cleavage site can be of low or moderate pathogenicity (Collins et al., 1994). Therefore we determined MDT for two isolates from wild birds representing two branches in the phylogenetic trees. Both isolates had MDTs of less than 60 hr, indicating that they were velogenic.

Most NDV strains circulating in wild birds are lentogenic (Alexander, 2000; Kim et al., 2007), but phylogenetic analyses have identified virulent NDV isolates from Rock Pigeons and Double-crested Cormorants (*Phalocrocorax auritus*) as the likely source of some NDV outbreaks in poultry (Alexander et al., 1985; Banerjee et al., 1994). Although disputable (Alfonso, 2008), there are indications that recombination can occur between lentogenic and velogenic NDV strains, involving the region of the F gene, which is the main determinant of NDV pathogenicity (Qin et al., 2008). Because of this uncertainty, we compared the nucleotide sequences of the NDV isolates from wild birds spanning the region from the first nucleotide of the matrix gene to the last coding nucleotide of the fusion gene (2,949 nucleotides) with the corresponding sequences of NDV available in GenBank. All five isolates from wild birds in Serbia had the highest homology (99%) with the genotype VII isolate Muscovy duck/China(Fujian)/FP1/02 and none of them showed evidence of recombination with other genotypes of NDV within the analyzed region (data not shown). Most nucleotide sequences of the genotype VII NDV that are publicly available comprise a shorter sequence, mainly of the F gene. Therefore, we constructed another phylogenetic tree based on a 374 nucleotide sequence of a variable portion (nt 47–420) of the F gene. The tree revealed a close similarity of Serbian isolates with NDV isolates from poultry in China and isolates of subgenotype VIId from other countries in the Far East, Middle East, South Africa, and Europe (Fig. 1). Nevertheless, the Serbian isolates grouped most closely with the concurrent isolates from Bulgaria and Ukraine. The isolates from poultry in Serbia, Bulgaria, and Ukraine are most likely part of the NDV epizootic in the Balkan peninsula and eastern Europe

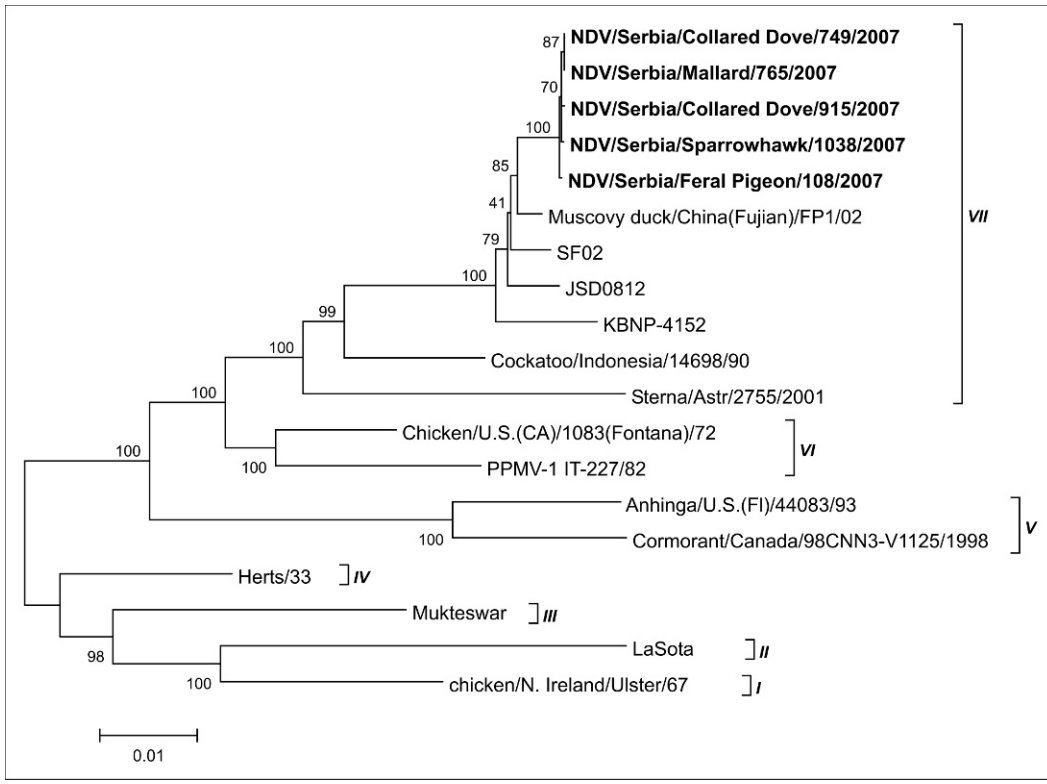

FIGURE 1. Phylogenetic tree (unrooted) based on 2,949-nucleotide sequences comprising the whole M gene and continuing portion (nt 1–1,707) of the F gene of representative class II Newcastle disease virus strains and isolates from wild birds from Serbia in 2007. The tree was constructed using Neighbor Joining algorithm with 1,000 bootstrap replicates. The percentage of replicate trees is shown next to the branches. The length of the horizontal lines is proportional to the genetic distance among isolates. The scale bar indicates the branch length based on the number of nucleotide substitutions per site. Isolates from Serbia are indicated in bold. Genotype groupings of class II Newcastle disease viruses are indicated on the right. GenBank accession numbers and other background information of the virus sequences used for the phylogenetic analysis are given in Materials and Methods section.

from 2006 to 2008 (OIE, 2010). Unfortunately, nucleotide sequences of other NDV from this epizootic are not available and the NDV genotype is not known.

Nucleotide sequences of the NDV isolates from wild birds and domestic poultry in Serbia were genetically highly similar, indicating interspecies transmission of the virus. Three of five isolates from wild birds came from Columbidae (two Eurasian Collared Doves and a Rock Pigeon), which was the likely source of some NDV outbreaks in poultry (Alexander et al., 1985). However, usually NDV isolates from Columbidae belong to the monophyletic VIb genotype (Ujvári et al., 2003), which is antigenically (Collins et al., 1989) and genetically distinct from NDV isolates from poultry. None of the Serbian isolates was inhibited by 161/617 monoclonal antibody, which is specific for pigeon NDV strains responsible for the pigeon ND panzootic. This result is in agreement with the phylogenetic study, which placed the isolates in the subgenotype VIId. A few recent isolates from pigeons in China were also of genotype VIId, a predominant genotype responsible for most ND outbreaks in chickens and geese since the end of last century (Liu et al., 2006). It is likely, therefore, that the doves and the pigeon from Serbia were

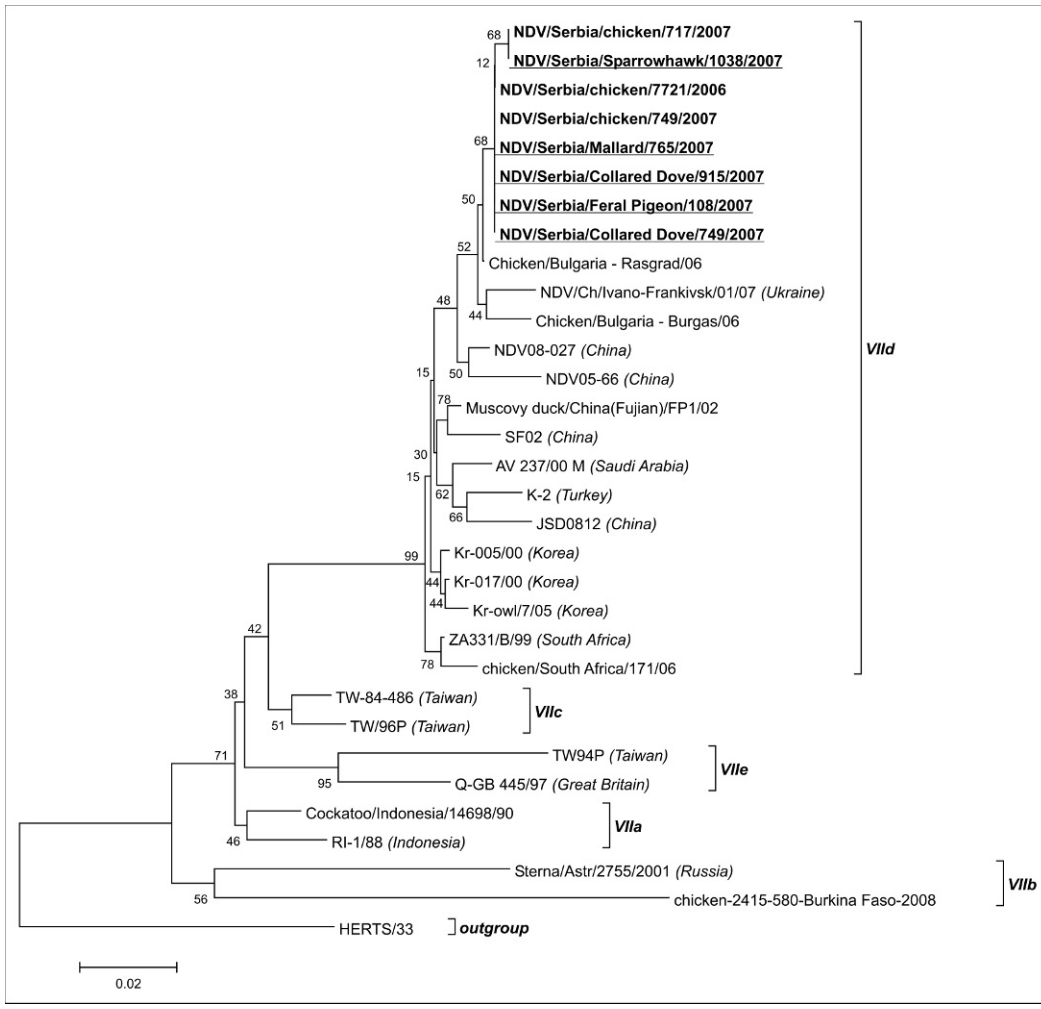

FIGURE 2. Phylogenetic tree of the nucleotide sequences of genotype VII NDV strains and isolates from wild birds and domestic poultry from Serbia in 2006 and 2007 based on a variable portion (nt 47–420) of the F gene. The tree was constructed using Neighbor Joining algorithm with 1,000 bootstrap replicates. The percentage of replicate trees is shown next to the branches. The length of the horizontal lines is proportional to the genetic distance among isolates. The scale bar indicates the branch length based on the number of nucleotide substitutions per site. All isolates from Serbia are indicated in bold, while isolates from wild birds are also underlined. Countries of origin are added in parentheses next to isolate designations where considered necessary. The tree is rooted with Herts/33 strain. Genotype subgroupings and the outgroup are indicated on the right. GenBank accession numbers and other background information of the virus sequences used for the phylogenetic analysis are given in Materials and Methods section.

most likely infected by other species, probably poultry, and were not the primary source for infection with the VIId genotype NDV.

Virulent NDV are occasionally isolated from birds of prey, but usually from those that died in captivity (Chu et al., 1976; Arias-Ibarrondo et al., 1978). The Eurasian Sparrowhawk was a free-living bird whose carcass was found in the area of infected poultry. This suggests infection of the sparrowhawk by eating infected poultry or doves, which would have been easy prey. As a migratory species, the Mallard could play an important role in spread of NDV over long distances and perhaps

introduced the virus locally. There is strong evidence that migratory birds have spread highly pathogenic avian influenza virus (H5N1) over long distances (Normile, 2006). Nevertheless, we do not know if the Mallard we tested was a feral Mallard or a farm Mallard raised for hunting. If the latter, the Mallard was probably infected locally, either at the mallard farm or during contact with domestic poultry, particularly domestic ducks, which are the same species as Mallards. However, infection of the mallard from other free-living birds in the vicinity cannot be ruled out.

Alexander et al. (1999) conducted epidemiologic investigations that suggested migratory birds may have been responsible for the primary introduction of a velogenic NDV into Great Britain in 1997 after unusual patterns of their movement in late 1996 and early 1997. Nevertheless, we found no evidence, except a single possibly migrating bird, that wild birds played an important role in the primary introduction or subsequent spread of velogenic NDV to domestic poultry. Moreover, the velogenic NDV isolated from each of four wild bird species could be associated with the ND outbreaks in domestic poultry in Serbia. Therefore, we assume that infection of wild birds with velogenic NDV of the VIId subgenotype occurred locally as part of an epizootic in poultry. The infected wild birds could have further infected susceptible poultry locally, but it is unlikely that these species spread the virus in the region. Systematic surveillance of wild birds for ND, similar to that for avian influenza, would provide additional insight into infection and spread of velogenic NDV by wild birds. However, there is still a need to focus on anthropogenic introduction and spread of this virus.

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