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Molecular detection and characterization of velogenic Newcastle disease virus in common starlings in Macedonia

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ABSTRACT

The objective of this study was to assess virulence using molecular methods and to phylogenetically characterize Newcastle disease virus (NDV) detected in common starlings (*Sturnus vulgaris*) in Macedonia. Nucleotide sequencing of the cleavage site of the F gene revealed an amino acid pattern specific for virulent strains of NDV with phenylalanine at position 117 and multiple basic amino acids between positions 112 and 116, i.e. ^{112}*RRQKR*FIG*^{119}. The 374 bp region of the fusion (F) gene used for phylogenetic analyses revealed that the detected strain belongs to subgenotype VIId of the class II NDV, and it is similar to virulent viruses detected in back-yard poultry in Macedonia in 2005 and 2006, as well as to viruses from poultry and wild birds detected in Serbia and Bulgaria in 2006 and 2007. Epidemiological data suggest that the common starlings were probably infected as a result of an ongoing epizootic in domestic poultry and they did not have a role in the primary introduction and spread of the virus to domestic poultry. This study represents the first report of the detection and molecular and phylogenetic characterization of virulent NDV from wild birds in Macedonia.

Key words: common starling, Newcastle disease virus, molecular characterization

Introduction

Viruses of Newcastle disease (ND) can be present in 241 species belonging to 27 of 50 orders in birds, either as natural or experimental hosts (KALETA and BALDAUF, 1988).

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It may be expected that all birds are susceptible to infection, with varying intensity of clinical signs and outcome (KALETA and BALDAUF, 1988; ALEXANDER, 2009). Clinical signs in birds infected with ND viruses (NDV) depend on the virulence of the virus, host, age, co-infection, immune status of the host and environmental conditions (ALEXANDER, 2009).

Newcastle disease viruses are often isolated from migratory wild birds and from other water birds (ALEXANDER et al., 2004). Wild water birds represent natural reservoirs of NDV, especially for lentogenic strains (ALEXANDER et al., 1992; ALEXANDER, 2000) although they may also harbor mesogenic strains (LIU et al., 2003). Consequently, the majority of these isolates from migratory wild birds and from waterfowl are of low virulence for poultry (ALEXANDER et al., 2004; ALEXANDER, 2001).

Results of different studies across the world show low prevalence of NDV in waterfowl regardless of virulence (SHENGQING et al., 2002; WOBESER et al., 1993; STANISLAWEK et al., 2002; PEROULIS and O'RILEY 2004). In Europe, lentogenic strains are more frequently detected than virulent ones, the latter belonging to genotypes VI, VII and VIII (ALEXANDER, 2011). Isolation of virulent viruses with low frequency from wild birds shows that mutations of low virulence to high virulence probably occur after the introduction of the virus to poultry (ALEXANDER et al., 2004).

Significant outbreaks of virulent NDV's in wild birds occurred in cormorants in North America in the 1990's (WOBESER et al., 1993). In all circumstances viruses isolated from dead birds were antigenetically similar, despite geographical distance (KUIKEN, 1998). There were outbreaks of virulent NDV's in wild birds in Europe: in pheasants in Denmark in 1996, in geese in Finland in 1996 and in cormorants in Denmark in 2001. All isolated viruses in these outbreaks were antigenetically similar (JORGENSEN et al., 1999; ALEXANDER et al., 1999).

Wild birds have always been considered as potential reservoirs for NDV, but it has been proven only in rare cases that they are able to spread the disease when infected. Only in recent years have wild birds been found to be responsible for introduction of virulent viruses to poultry (ALEXANDER et al., 2004). Wild migratory birds were found responsible for the outbreak of NDV in chickens and turkeys in Great Britain in 1997 (ALEXANDER et al., 1999). Suspicion has been raised about the persistence of the virulent virus in certain species of wild birds in Europe after the introduction of a similar virus to pheasants in Great Britain and France in 2005 (ALDOUS et al., 2007).

The majority of isolates of virulent viruses from wild birds are from dead birds found in the vicinity of infected poultry holdings (ALEXANDER, 2001). The susceptibility of passerine birds (order *Passeriformes*) is highly variable, ranging from no signs of disease with excretion of the virus to severe clinical signs (OIE, 2013). Because of their nature, these birds can easily come into contact with poultry and they can become infected with

either avirulent or virulent strains of NDV (SIMMONS, 1967; ZHU et al., 2010). House sparrows living around farms can carry the same virulent genotype as the one circulating in poultry (ZHU et al., 2010).

The objective of this study was to assess virulence using molecular methods, and to phylogenetically characterize NDV detected in common starlings (*Sturnus vulgaris*) in Macedonia.

Materials and methods

Sample collection and processing. Thirty carcasses of dead common starlings were received at the laboratory, where necropsy of all carcasses was performed. Birds were found in the central part of Macedonia (N 41°24'32. E 22°14'39.), were collected on 14.01.2006 and transported to the lab. After necropsy, the visceral organs were archived in the freezer. Six years later, the pool of visceral organs was homogenized with pestle and mortar, with the addition of sterile sand and 2 mL of antibiotic solution, according to the recommended protocol (OIE, 2012). After incubation of two hours at room temperature, the suspension was centrifuged for 15 minutes at 3000 rpm and the supernatant was used for extraction of viral RNA. Isolation of the virus on embryonated chicken eggs, (ECE) according to the international recommended protocol (OIE, 2012), was also attempted. The detected strain was labeled NDV/starling/Macedonia/068/2006 according to the serotype/host/location/laboratory number/sampling date scheme.

Extraction of RNA, reverse transcriptase PCR (RT-PCR) and real time RT-PCR (RT-qPCR). Extraction of viral RNA from clinical samples was done using RNeasy Mini Kit (Qiagen, USA). Briefly, 400 µL of supernatant was added to the lysis buffer and, after incubation at room temperature, 70 % ethanol was added. Then RNA was washed with buffers and, after final drying of the column, RNA was eluted with 50 µL of RNA-ase free water. One step RT-qPCR for the detection of M gene was performed with iScript One-Step RT-PCR Kit for Probes (Bio-Rad), five µL of RNA were added in 25 µL of final volume. The primers and hydrolysis probe used were forward primer M+4100 (5'-*AGTGATGTGCTCGGACCTTC*-3'), reverse primer M-4220 (5'-*CCTGAGGAGAGGCATTTGCTA*-3') and hydrolysis probe M+4169 (5'- *[FAM] TTCTCTAGCAGTGGGACAGCCTGC[TAMRA]*) (WISE et al., 2004). The thermal protocol was 50 ºC for 10 minutes, 95 ºC for 5 minutes and 40 cycles of 95 ºC for 10 seconds and 55 ºC for 30 seconds. The procedure for detection of the F gene was the same as previously used, with the use of different primers and hydrolysis probe: forward primer F+4839 (5'-*TCCGGAGGATACAAGGGTCT*-3'), reverse primer F-4939 (5'-*AGCTGTTGCAACCCCAAG*-3') and hydrolysis probe F+4894 (VFP-1) (5'-*[FAM] AAGCGTTTCTGTCTCCTTCCTCCA[TAMRA]*-3') and annealing temperature of the primers of 58 ºC (WISE et al., 2004).

In order to obtain PCR product for sequencing, two step RT-PCR was performed. Synthesis of cDNA was done using a QuantiTect Reverse Transcription Kit (Qiagen, USA), with forward primer used as a RT primer. Amplification of cDNA was done in semi-nested PCR with a Taq PCR Master Mix Kit (Qiagen, USA) using forward primer *MSF1* (5'-*GACCGCTGACCACGAGGTTA*-3') and reverse primer #2 (5'-*AGTCGGAGGATGTTGGCAGC*-3') with 2.5 µL of cDNA in final volume of 25 µL in the first PCR. A different forward primer was used in the second PCR, #7 (5'-*TTAGAAAAAACACGGGTAGAA*-3') and the same reverse primer, thus generating an amplicon of 500 bp (ALDOUS et al., 2003). The thermal protocol in both PCR's was 94 ºC - 3 min, 42 cycles of 94 ºC - 1 min, 50 ºC - 1 min and 72 ºC - 3 min and 72 ºC - 10 min. The amplified PCR products were analyzed in 1.5 % agarose gel, stained with ethidium bromide. The specific bands containing DNA were excised and purified using a QIAquick Gel Extraction Kit (Qiagen, USA).

Nucleotide sequencing and phylogenetic analysis. Purified DNA was sequenced using a Big Dye Terminator v3.1 kit (Applied Biosystems) with the same primers as in the second PCR, in the ABI PRISM 310 Genetic analyzer (Applied Biosystems). The obtained sequence was edited by BioEdit Sequence Alignment Editor Version 7.0.9.0. Alignment of the sequences and construction of the phylogenetic tree was done in MEGA 5.10 (TAMURA et al., 2007). Sequences used for construction of the phylogenetic tree were aligned to begin at nucleotide position 47 and to end at 420, encompassing the cleavage site of the F gene. GenBank accession numbers of the sequences used for construction of phylogenetic tree, where available, are shown in parentheses in Fig. 3.

Results

Post-mortem findings of carcasses of dead starlings revealed hemorrhagic laryngotracheitis, congestion and edema of the lungs, and hemorrhagic enteritis. We could not isolate the virus even after three passages on ECE, i.e. haemagglutinating activity of allantoic fluid was not present. Real-time RT-PCR for detection of the M gene of class II NDV's, regardless of virulence, was positive with a Ct value of 33.7. Real-time RT-PCR for detection of the F gene of virulent viruses was positive, with a Ct value of 38.8. Validity of the RT-qPCR assays was checked using virulent NDV with high Ct values (30 - 35) and the La Sota strain as controls. In order to confirm the results from the RT-qPCR, and to obtain PCR product for sequencing, the sample was tested with RT-PCR with an expected band size of 700 bp, and it was negative (Fig. 1). In the second PCR, i.e. "semi-nested RT-PCR", which is a slight modification of the protocol used, the sample was positive, with the expected size of the PCR product of 500 bp (Fig. 2). Nucleotide sequencing of the cleavage site of the F gene revealed an amino acid pattern specific for virulent strains of NDV, with phenylalanine at position 117 and multiple basic amino acids between positions 112 and 116, i.e. ^{112}*RRQKR*FIG*^{119}. Phylogenetic analysis was

done by constructing a phylogenetic tree on the 374 bp region encompassing the cleavage site of the F gene. In terms of its position in the phylogenetic tree, the detected virulent strain clustered in class II genotype VII, more specifically sub genotype VIId (Fig 3). The GenBank accession number of the partial F gene nucleotide sequence of the strain NDV/starling/Macedonia/068/2006 is KC915224.

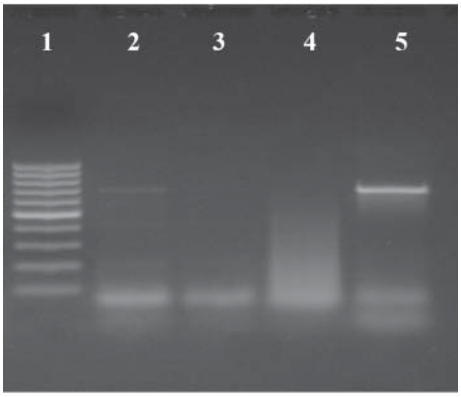

Fig. 1. Visualization of the gel after electrophoresis of 700 bp RT-PCR products using Newcastle disease virus specific primers (*MSF1* and #2; ALDOUS et al., 2003). Lane 1 - molecular marker 100-1000 bp, lanes 2, 3 - samples from various hosts that were previously positive in RT-qPCR (WISE et al., 2004) with high Ct values, lane 4 - investigated strain NDV/starling/Macedonia/068/2006 (negative result), lane 5 - positive control.

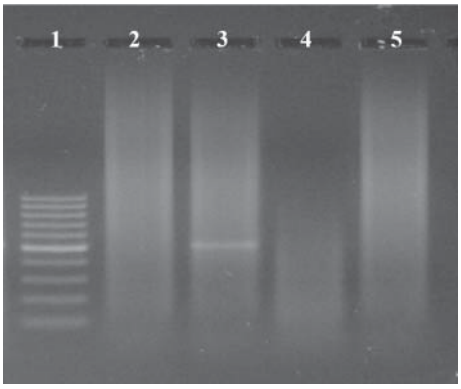

Fig. 2. Visualization of the gel after electrophoresis of 500 bp RT-PCR products using Newcastle disease virus specific primers (#7 and #2; ALDOUS et al., 2003). Lane 1- molecular marker 100-1000 bp, lanes 2, 4, 5 - samples from various hosts that were previously positive in RT-qPCR (WISE et al., 2004) with high Ct values, lane 3 - investigated strain NDV/starling/Macedonia/068/2006 (positive result).

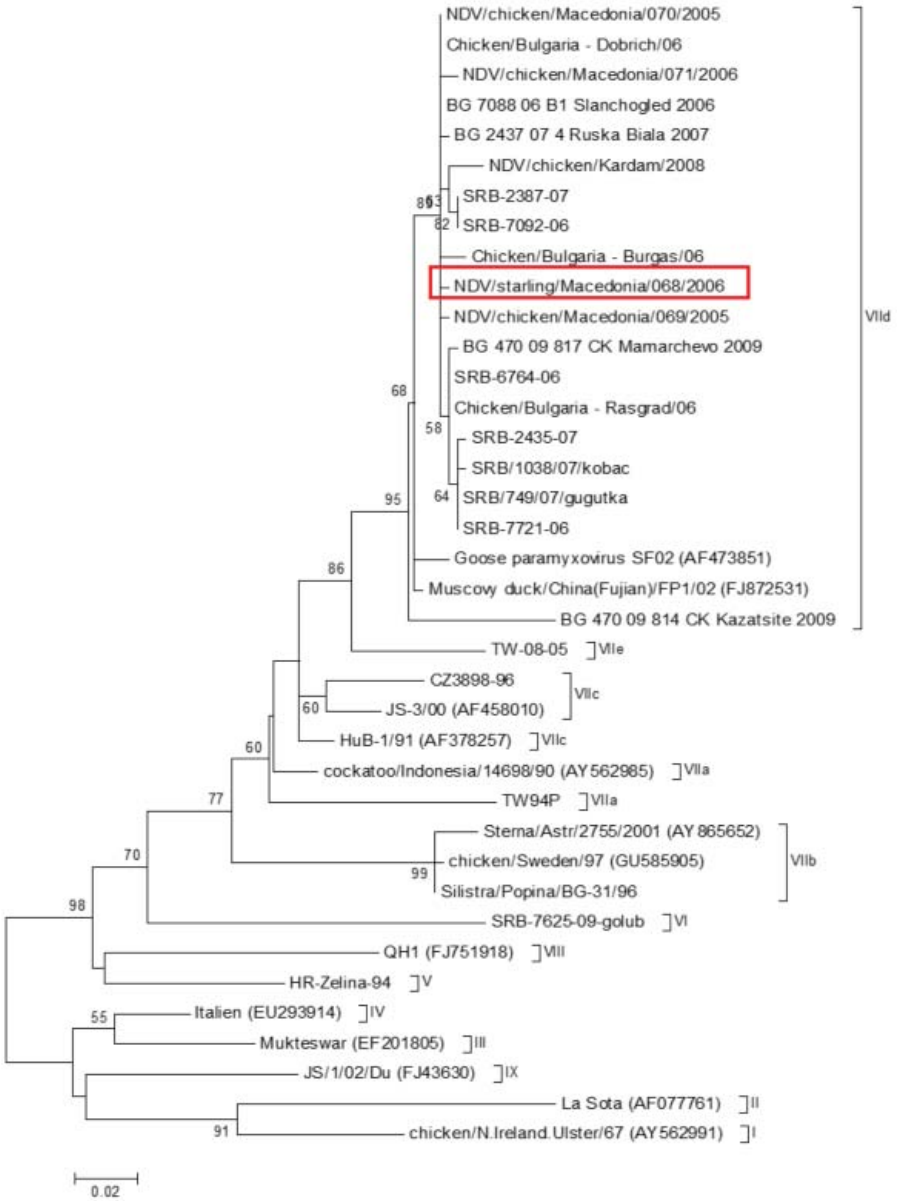

Fig. 3. Phylogenetic tree based on the partial nucleotide sequence of the F gene (nt 47 - 420) of the Newcastle disease virus. The evolutionary history was inferred by using the Maximum

Likelihood method, based on the Kimura 2-parameter model. The tree with the highest log likelihood (-2291.0159) is shown. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. A discrete Gamma distribution was used to model evolutionary rate differences among sites (2 categories (+*G*, parameter = 0.4625)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 38 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 374 positions in the final dataset. Evolutionary analyses were conducted in MEGA5. GenBank accession numbers where available are shown in parentheses.

Discussion

We could not isolate NDV from starlings in ECE, although the pooled sample of visceral organs was positive by RT-qPCR for detection of the M gene and F gene. This is possibly due to the presence of inactivated viral RNA, or very low concentrations of the virus in the sampled material. The latter corresponds to the high Ct values obtained by both RT-qPCR's. Based on the experience of Norwegian colleagues, Brown (BROWN, 2011) reports the impossibility of isolation of virus because of its low concentration, although oropharyngeal and cloacal swabs were positive on RT-qPCR.

Phylogeny results revealed that the detected virus clustered in the genotype VII subgenotype VIId, and it was similar to virulent viruses detected in backyard poultry in Macedonia in 2005 and 2006, and to viruses from poultry and wild birds detected in Serbia and Bulgaria in 2006 and 2007.

Evidence of the virulent virus in starlings (NDV/starling/Macedonia/068/2006) indicates the presence of the virus in the population of wild birds, and this is not uncommon because the virulent virus of the same genotype VIId was isolated from starlings in Romania, with ICPI value of 1.7 (ALEXANDER, 2011). Detection of NDV from starlings with severe nervous signs but lentogenic for chickens has been reported (LIPKIND et al., 1987). In general, lentogenic viruses are more frequently isolated from wild birds, and it is thought that they are reservoirs of these viruses (KIM et al., 2007). Lentogenic strains, highly similar to vaccine strains, can escape from poultry into the environment and be detected in free-living and captive wild birds or waterfowl, and they may thus be involved in their dissemination (GARCIA et al., 2013; SNOECK et al., 2013). There are three exemptions from the absence of endemic virulent NDV in wild birds: panzootics in pigeons, cormorants in North America and occasional evidence of the spread of NDV by wild birds in Europe (ALEXANDER, 2011). The causative agents of the last are viruses belonging to genotype VIIb (ALEXANDER et al., 1999; JORGENSEN et al., 1999). The majority of isolations of virulent viruses are the result of sampling of dead birds found in the proximity of infected poultry holdings (ALEXANDER, 2001). Isolation of genotype VIId virulent virus from sparrowhawks, doves, pigeons and mallards, as a

result of the presence of the virus in poultry, was reported during the widespread country epizootic in Serbia in 2007 (VIDANOVIĆ et al., 2011). In Mexico, virulent strains detected in wild birds belonged to the same subgenotype Vb, together with strains circulating in poultry (GARCIA et al., 2013). In our case, common starlings were probably infected as a result of the ongoing epizootic in domestic poultry, and they did not have any role in the primary introduction and spread of the virus in domestic poultry. Interspecies transmission of virulent NDV has already been reported in Macedonia (DODOVSKI et al., 2013). The finding of 30 dead birds on the site points to acute onset of death due to the high virulence of the virus for starlings. There are 3 large poultry farms in the area where the birds were found, which at that time had not reported any clinical signs or increased mortality, but in the period from autumn 2005 to spring 2006, ND was present in backyard poultry in many parts of the country (DODOVSKI et al., 2007). It is evident that in an epidemiological situation characterized by numerous outbreaks over a prolonged period of time in domestic poultry, the possibility of detection of the same virus in wild birds is increased. Additional epidemiological surveillance is required in wild birds in the proximity of poultry farms in order to determine their role in the spread of NDV (MILLER et al., 2007).

This study represents the first report of the detection and molecular and phylogenetic characterization of virulent NDV from wild birds in Macedonia.

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SAŽETAK

Cilj ovog istraživanja bio je molekularnim metodama odrediti virulenciju i filogenetska obilježja virusa newcastleske bolesti (NB) dokazanog u čvorka (*Sturnus vulgaris*) u Makedoniji. Pokazalo se da nukleotidni slijed na mjestu cijepanja gena F daje aminokiselinski sastav specifičan za virulentne sojeve virusa NB s fenilalaninom na poziciji 117 i uzastopnim bazičnim aminokiselinama između pozicija 112 i 116, tj. ^{112}*RRQKR*FIG*^{119}. Filogenetska analiza područja od 374 bp fuzijskog (F) gena pokazala je da izdvojeni soj pripada podtipu VIId skupine II virusa NB, a srodan je virulentnim sojevima dokazanima u domaće peradi u Makedoniji 2005. i 2006. kao i s virusima izdvojenima iz peradi i divljih ptica u Srbiji i Bugarskoj 2006. i 2007. Epizootiološki podatci upućuju na zaključak da su čvorci vjerojatno bili zaraženi kao posljedica pojave NB u domaće peradi te nisu imali nikakvu ulogu na pojavu i širenje virusa na domaću perad. Ovo je prvo izvješće o dokazu, molekularnim i filogenetskim obilježjima virulentnog virusa NB u divljih ptica u Makedoniji.

Ključne riječi: čvorak, virus newcastleske bolesti, molekularna karakterizacija