Acta Veterinaria (Beograd), Vol. 62, No. 4, 365-374, 2012.

DOI: 10.2298/AVB1204365M UDK 578.831.11:636.5:577.21

MOLECULAR CHARACTERIZATION OF SOME STRAINS OF NEWCASTLE DISEASE VIRUS ISOLATED IN PROVINCE OF VOJVODINA, REPUBLIC OF SERBIA

MILIĆ N*, LAZIĆ S**, VIDANOVIĆ D***, ŠEKLER M***, NIŠAVIĆ J*, RESANOVIĆ RADMILA* and PETROVIĆ T**

**University of Belgrade, Faculty of Veterinary Medicine, Serbia*
***Scientific Veterinary Institute "Novi Sad", Novi Sad, Serbia*
****Veterinary Specialized Institute "Kraljevo", Kraljevo, Serbia*

(Received 12th December 2011)

Five strains of Newcastle disease virus (NDV) were obtained from poultry in Vojvodina, Serbia during the outbreaks in 2006 and 2007. These isolates were confirmed and genotypically characterized by reverse transcription polymerase chain reaction (RT-PCR) with primer specific to the viral fusion (F) protein (572bp), and by sequencing of partial F gene for phylogenetic analysis. Phylogenetic analysis showed that all five isolated strains of Newcastle disease virus belong to genotype VII. At the same time, all five isolates were clustered in NDV subtype VIId. The examined NDV isolates express high similarity to each other (99.7-100%) and group together with the strains of Newcastle disease virus isolated previously from wild birds in Serbia during the same 2006 - 2007 outbreak. The analysis of the isolates F gene cleavage sites has shown that all five isolated strains of Newcastle disease virus had a cleavage site motif 112R–R–Q–K–R–F-117 characteristic for highly virulent, velogenic strains.

Key words: Newcastle disease virus, phylogenetic analysis, RT-PCR

INTRODUCTION

Newcastle disease (ND) is one of the most devastating diseases in the poultry industry. It is caused by virulent strains of Newcastle disease virus (NDV), a member of *Paramyxoviridae* family, designated as an avian paramyxovirus 1 (Wehmann *et al*., 2003). The enveloped virus has a negative-sense single stranded genome of approximately 15.2 kb containing six genes 3'NP-P-M-F-HN-L-5' which encodes at least six proteins: the nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein (HN) and the large (L) protein (Millar *et al*., 1988). Newcastle disease is a highly contagious and widespread disease which causes severe economic losses in domestic poultry, especially among chickens. The virus has a wide host range. It has been reported that birds belonging to 27 orders

have been affected by the disease, the mortality rate being the highest in chickens. The infectious virus may be ingested or inhalated. NDV isolated strains are categorized into three main pathotypes depending on the severity of the disease produced by the isolates in chickens. Lentogenic isolates do not usually affect adult birds and are considered avirulent. Viruses of intermediate virulence that cause this respiratory disease are termed mesogenic, while virulent viruses accompanied by high mortality rate are termed velogenic. Neurotropic and viscerotropic forms of velogenic viruses have been reported worldwide, causing major economic losses in poultry. The molecular basis of NDV pathogenicity is dependent on the fusion protein cleavage site amino acid sequence and the ability of specific cellular proteases to cleave the fusion proteins of differnt pathotypes. The F protein is synthesized as a biologically non-active precursor (F0). Previous studies comparing the precursor F0 amino acid sequences of NDVs differing in virulence for chickens have shown that viruses that are virulent for chickens have the amino acid sequence ^{112}R/K-R-Q-K/R-R^{116} at the C terminus of the F2 protein and phenylalanine at residue 117, the N terminus of the F1 protein, while low virulence viruses have the sequence ^{112G}/E-K/R-Q-G/E-R^{116} at the C terminus of the F2 protein and leucine at residue 117 (Collins *et al*., 1993).

In the territory of the former Yugoslavia Newcastle disease was first reported in Croatia (Hupbauer and Topolnik, 1944). The severe epizootic that followed was partly brought under control in the mid '50s by using mesogenic vaccines. A significant reduction of Newcastle disease in the former Yugoslavia was achieved by the second half of the '60s and the incidence diminished to 200-300 cases per year only a decade later. Wehmann *et al., (*2003) subjected 68 NDV strains to genetic analysis which were isolated during the period from 1979 to 2002 on the territories of the former Yugoslavia (Serbia, Croatia and Bosnia and Hercegovina). All the 68 strains were classified in genotype V.

In order to reveal what genotypes of Newcastle disease virus were responsible for the outbreaks during 2006 and 2007 in the Province of Vojvodina, Republic of Serbia, we have performed the molecular characterization of five Newcastle disease virus strains, isolated during 2007 in Vojvodina, Serbia.

MATERIAL AND METHODS

Isolates

Five isolates were obtained from Newcastle disease outbreaks in poultry in the Republic of Serbia (Province of Vojvodina) during 2007 (Table 1, Figure 1). These isolates were recovered from the samples taken from diseased or dead poultry. The viruses were grown in the allantoic cavities of 9 -11 day old chicken embryonated eggs using standard procedures, and identified with the standard hemagglutination (HA test) and hemagglutination inhibition tests (HI test).

Table 1. Representative data for five NDV isolates from Vojvodina Province, Serbia used for sequencing and molecular characterization

No.	Date	Location	Isolates name	Number of poultry cases (dead/destroyed)
1	8.05.2007	Bačka Palanka, Southern – Bačka county	SRB-4497-07	1862
2	15.05.2007	Novi Kozarci, Kikinda, Northern – Bačka county	SRB-4652-07	37
3	15.05.2007	Pećinci, Srem county	SRB4-537-07	45
4	18.05.2007	Bogaraš, Senta, Northern – Bačka county	SRB-4755-07	76
5	30.05.2007	Sirig, Temerin	SRB-5111-07	40

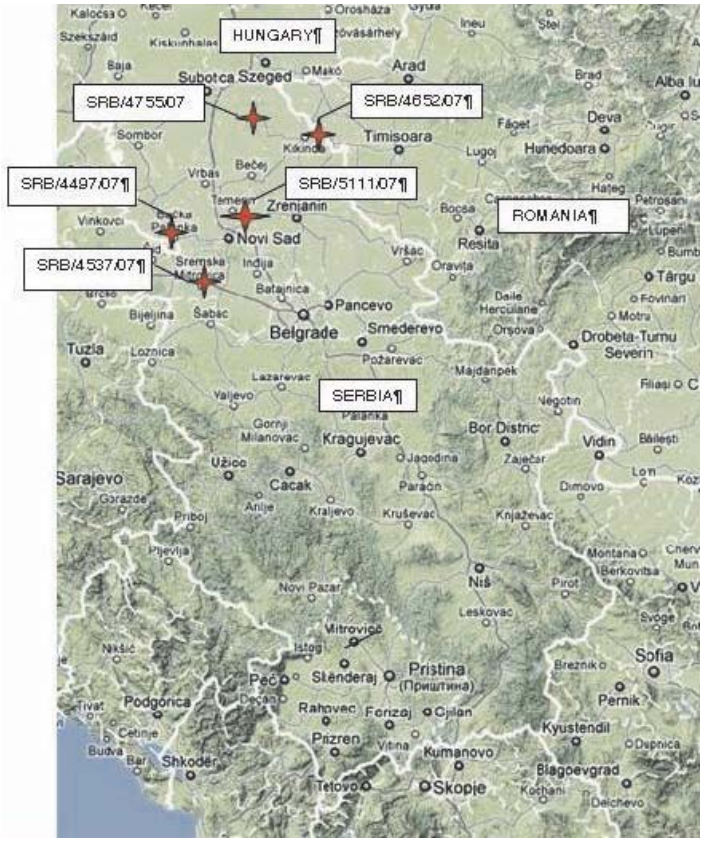

Figure 1. Geographical locations of five sequenced NDV poultry isolates from Serbia

Molecular methods used in characterization of NDV isolates

RNA extraction and RT-PCR

For the molecular characterization of Newcastle disease virus, total RNA was extracted from 250 μL of alantoic fluids from chicken embryos individually infected with five NDV poultry isolates from 2007. The RNA extraction was done by using the TRIreagent (Adiagen) according to the manufacturer recommendations. A partial sequence of 572 bp within F gene of the viral RNA, including the F gene cleavage site was amplified by the forward and reverse primers MV1 and B2 described by Lomniczi *et al*. (1998) (Table 2). Amplification was performed by one-step RT-PCR using Access RT-PCR system (Promega, USA) according to the manufacturer recommendations. Briefly, in the reaction volumes of 50 μL, 10 μL of RT/PCR buffer, 1 of 10 mM dNTP mix, 2 μL of 50 mM $MgSO_4$, 1 μL 5U AMV reverse transcriptase, 1 μL of 5U DNA polymerase, 0.25 μL of both primers with a concentration of 100 pmol/μL, and 28.5 μL of PCR grade water were included in RT-PCR mix. The amount of template was 6 μL. The temperature condition was: reverse transcription 45 min at 48^{o}C, reverse transcriptase inactivation and DNA polymerase activation 2 min at 94^{o}C, 40 cycles of denaturation 30 second at 94^{o}C, annealing 1 minute at 50^{o}C and elongation 2 min at 68^{o}C. The last elongation was 10 min at 68^{o}C. The obtained RT-PCR products were visualized by UV light on 2% agarose gel stained with ethidium bromide.

Table 2. Primers used for RT-PCR amplification and nucleotide sequencing of the F gene of NDV isolates from Serbia

Primer	Sequence (5'-3')	Target gene	Genome position[a]	Expected products size (bp)
MV1[b]	CCYRAATCAYYRYGRYRCYRGATAA	M	4424-4448	572
B2[b]	KCRGCRTTYTGKKTGGCTKGTAT	F	4973-4995	

[a] Based on GenBank accession number DQ839397 (isolate KBNP-4152)
[b] described by Lomniczi *et al.*, (1998)

Sequencing of partial F gene and phylogenetic analysis

The PCR products of 572 nucleotides of NDV isolates F genome part were used as templates in cycle sequencing reactions primed with the same MV1 and B2 primers used in RT-PCR reaction. The amplified RT-PCR fragments were commercially sequenced by Macrogen Inc., Seoul, Korea. Phylogenetic analyses of the NDV isolates were conducted using MEGA version 5 (Tamura *et al.,* 2011). The individual sequence homology search was conducted by the National Center for Biotechnology Information (NCBI) using BLAST network service (http://www.ncbi.nlm.nih.gov). The 374 bp long F gene nucleotide sequences of NDV isolates were aligned by Clustal W program, together with published

sequences in NCBI GenBank. The phylogenetic tree was constructed using neighbour – joining method based on bootstrap of 1000 replicates.

In addition to the F gene 374 nucleotides long sequences of NDV isolates, obtained from poultry in Serbia, a corresponding nucleotide sequence of 23 NDV sequences, representatives of all class II NDV genotypes I – IX and genotype VII subtypes, were included in the analysis for comparison. The nucleotide sequence GenBank accession numbers of these NDV genotype representatives were: AY562991 chicken/N.Ireland/Ulster/67 (I); AF077761 LaSota (II); EF201805 Mukteswar (III); AY741404 Herts/33 (IV); AF001107.1 H-10/72 (V); AF001111.1 Israel 70 (VIa); AF109885.1 GB 1168/84 (VIb); AF083961.1 TW/94P (VIIA); AF109876.1 ZA360/95 (VIIb); AY865652 Sterna/Astr/2755/2001 (VIIb); AF109883.1 CZ3898/96 (VIIc); FJ434391.1 KAZ342/03 (VIId), AY390299.1 G1F3/03 (VIId); AF456442.1 JS/5/01 (VIId); FJ436302.1 F48/E; FJ872531 Muscovy duck/China(Fujian)/FP1/02 (VIId). Two previously isolated strains from this region (former Yugoslavia) namely, AY117008.1 YU(Vo)-1-94 and AY117010.1 HR-Zelina-94 as representatives of NDV genotype V, as well as 5 strains of NDV previously isolated from wild birds in Serbia during 2007 (Vidanović *et al.*, 2011), were also included. Genotyping was performed according to the system suggested by Ballagi-Pordany *et al*. (1996) and Kim *et al*. (2007).

RESULTS

Five Newcastle disease strains, isolated on the territory of Vojvodina, Serbia, were subjected to genetic analyses. The analysis of the isolates F gene cleavage sites have shown that all five isolated strains of Newcastle disease virus had a cleavage site motif ^{112}R-R-Q-K-R-F^{117} characteristic for highly virulent, velogenic strains. The results of the phylogenetic analysis revealed that all five sequenced NDV isolates, isolated from poultry in Serbia during 2007, belong to NDV genotype VII. At the same time, all five isolates were clustered in NDV subtype VIId.. Isolates 4652-07, 4755-07 and 5111-07 had a 100% identical sequences while isolates 4497-05 and 4537-05 showed 0.1 and 0.2% distance from first three isolates and 0.3% distance between themselves. The overall mean distance between all five isolates was 0.2%. The examined NDV isolates express high similarity to each other and group together with the NDV isolates obtained previously from wild birds in Serbia during the same 2006 - 2007 outbreak with overall mean distance of 0.2%. When compared to the published sequences in GenBank, this sequences of five Newcastle disease strains showed the highest homology (99%) with Newcastle disease virus isolate GX/4/05/Ch isolated in China in 2005.

The phylogenetic tree was constructed using Neighbor Joining method with 1000 bootstrap replicates (using MEGA version 5). The percentage of replicate trees is shown next to the branches. The length of the horizontal lines is proportional to the genetic distance among the isolates. The scale bar indicates the branch length based on the number of nucleotide substitutions per site. All the isolates from Serbia, characterized in this study, are indicated with a big black point, while the isolates with an asterix (*) represent the NDV strains previously

isolated from Serbian wild birds (Vidanović *et al.,* 2011). The genotype grupings and subgroupings are indicated on the right side of the phylogenetic tree. GenBank accession numbers and other background information on the virus sequences used for the phylogenetic analysis are given in Materials and Methods section.

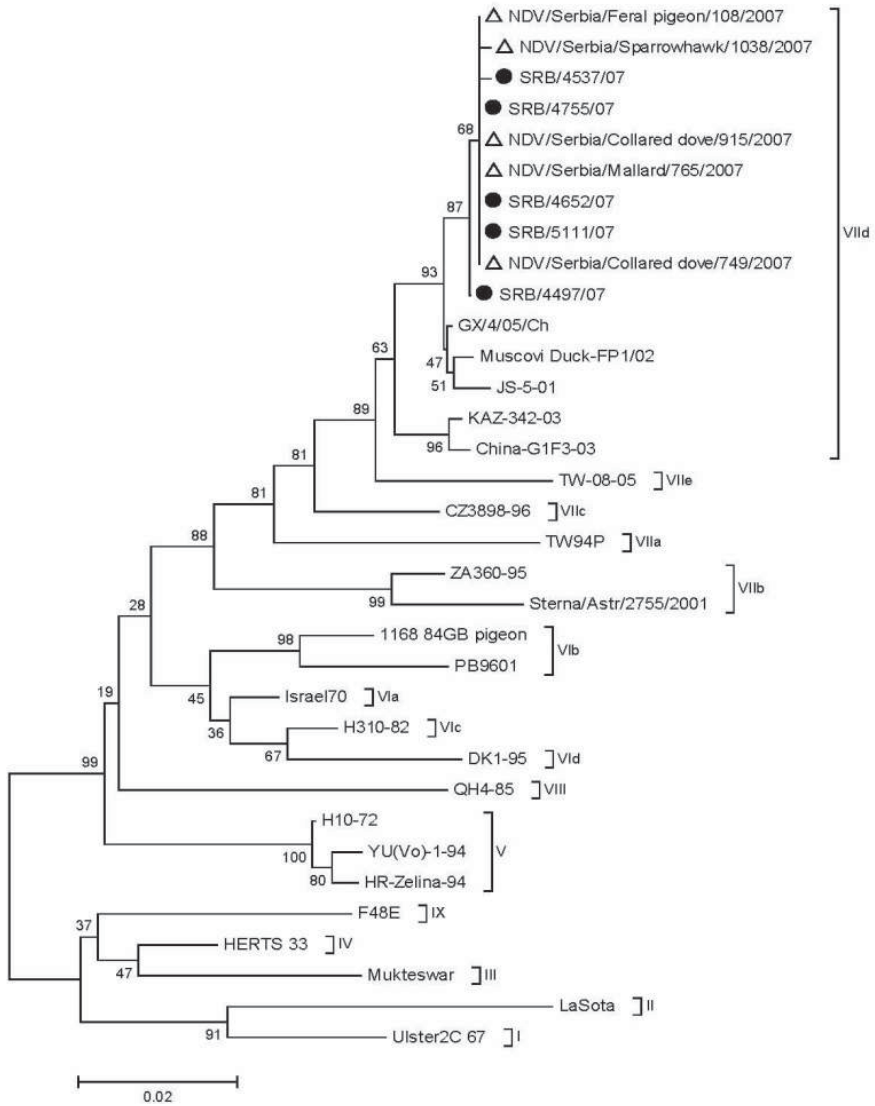

Figure 2. Phylogenetic tree of the nucleotide sequences of published NDV strains and five isolates from domestic poultry from Serbia in 2007 based on a variable portion (nt 47-420) of F gene

DISCUSSION

All the five strains of the Newcastle disease virus were previously isolated by using the standard virological method of virus isolation in embyonated chicken eggs and hemagglutination and hemagglutination – inhibition tests. Alexander (2000) believes that the virus isolation in embryonated chicken eggs is one of the most reliable methods for Newcastle disease diagnosis. In 2008 the International Organization for Epizooties recommended that for the isolation of Newcastle disease virus on chicken embryos it is necessary to make two passages of the virus in the case the hemagglutination activity of the virus has not been established after the first passage (OIE Mannual, 2008). The virus isolation in embryonated chicken eggs, in addition to its advantages, has some disadvantages. It is considered to be slow, demanding and requiring *in vivo* testing, with no information concerning the origin and distribution of the virus (Aldous and Alexander, 2001).

In order to examine the possibilities of the molecular methods for a fast and reliable characterization of the new NDV isolates, we compared a part of the F gene sequences (374 nucleotides), received from the poultry NDV isolates, obtained in Serbia in 2007, with the remaining NDV representatives of different virus genotypes and subtypes. In addition to the F gene 374 nucleotides long sequences of NDV isolates obtained from poultry in Serbia, a corresponding nucleotide sequence of 23 NDV representatives of all NDV class II genotypes I – IX and genotype VII subtypes was included in the comparative analysis. Among those 23 NDV sequences representatives were also two previously isolated strains from this region (former Yugoslavia), namely YU(Vo)-1-94 and HR-Zelina-94 as representatives of NDV genotype V, as well as five strains of NDV previously isolated from wild birds in Serbia during 2007, (Vidanović *et al.,* 2011). The results of the phylogenetic analysis revealed that all five sequenced Serbian NDV isolates from poultry, isolated during 2007, belong to the genotype VII. Also, all 5 isolates were clustered in the subtype VIId and express high similarity to each other. The same high similarity of the characterized NDV isolates in this study was also observed within the NDV isolated previously from wild birds in Serbia during the same 2006 – 2007 outbreak (Vidanović *et al.*, 2011), with an overall mean distance of 0.2%. This result was expected, as it was in conformance with the previously published results in Serbia and other countries.

The genetic studies, previously carried out on NDV strains from Serbia, Bosnia-Herzegovina, Croatia, and Slovenia, identified only the presence of the NDV genotype V strains, endemic in the region between 1979 and 2002, Wehmann *et al.* (2003). Lomniczi *et al*. (1998) compared the strains of Newcastle disease virus, isolated during the epizootic outbreaks between 1992 and 1996 in the Western European countries, using the restriction enzyme cleavage site mapping of the fusion (F) protein gene between nucleotides 334 and 1682 and by sequence analysis between nucleotides 47 and 435. Both methods revealed that NDV strains responsible for the epizootics belong to two distinct genotypes. The strains derived from the sporadic cases in Denmark, Sweden, Switzerland and Austria were classified as genotype VI, the same group which caused the

outbreaks in the Middle East and Greece in the late '60s and in Hungary in the early '80s. In contrast, the viruses that caused the epizootics in Germany, Belgium, The Netherlands, Spain and Italy could be classified as a novel genotype termed VII, that was at that time undetected in Europe. It is possible that the genotype VII viruses originated in the Far East, because they showed a high genetic similarity (97%) to NDV strains isolated from Indonesia in the late 1980's.

Vidanović *et al*. (2011) in their recent study analysed 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 and typed them in the VII genotype. They also claimed that the Serbian isolates from 2006 - 2007 outbreaks among wild birds and poultry belonged to the group most closely related to the concurrent isolates from Bulgaria and Ukraine, the poultry isolates from these three counties being most likely a part of the NDV epizootic in the Balkans and Eastern Europe from 2006 to 2008.

In addition, many other authors have indicated that the genotype VIId viruses are responsible for the majority of the outbreaks of ND since late '90s (Liu *et al*., 2008; Bogoyavlenskiy *et al*., 2009). Our results of the phylogenetic analysis of five Newcastle disease viruses isolated from poultry in the northern part of Serbia in 2007 fully confirm this hypothesis. It was also observed that nucleotide sequences of the NDV isolates from wild birds and domestic poultry in Serbia were genetically highly similar, indicating interspecies transmission of the virus. In addition to the clinical observations from the field, the velogenic character of all five analyzed NDV isolates was also confirmed by the presence of the typical cleavage sites of the fusion protein for the velogenic viruses ^{112}R-R-Q-K-R-F^{117}.

On the basis of the performed analysis we could conclude that the methods used for the molecular characterization are very reliable for a fast and precise characterization of the new isolated NDV strains and could be easily used for the molecular epidemiology of Newcastle disease virus.

ACKNOWLEDGMENT

This work is part of the research within the framework of the project No. 31008 and partly of the project No. 31084 funded by the Ministry of Education and Science of Republic of Serbia.

Address for correspondence
Nenad Milic, PhD, full professor
Department of Microbiology
Faculty of Veterinary Medicine
University in Belgrade
Bulevar oslobodjenja 18
11000 Belgrade, Serbia
nenadmilic@vet.bg.ac.rs

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MOLEKULARNA KARAKTERIZACIJA NEKIH SOJEVA VIRUSA NEWCASTLE BOLESTI IZOLOVANIH U POKRAJINI VOJVODINI REPUBLIKE SRBIJE

MILIĆ N, LAZIĆ S, VIDANOVIĆ D, ŠEKLER M, NIŠAVIĆ J, RESANOVIĆ RADMILA i PETROVIĆ T

SADRŽAJ

Pet sojeva virusa Newcastle bolesti (NDV) je izolovano iz uzoraka suspektnog materijala poreklom od živine 2006. i 2007.godine tokom epizootije

atipične kuge živine na teritoriji Vojvodine, Srbija. Ovi izolati su potvrđeni i genotipski tipizirani primenom metoda RT-PCR uz korišćenje prajmera specifičnih za deo genoma virusa koji kodira sintezu fuzionog F proteina (572bp) i sekvenciranjem dela F gena sa filogenetskom analizom. Filogenetska analiza je ukazala da je svih pet izolovanih sojeva virusa Newcastle bolesti pripadalo genotipu VII. Istovremeno, svih pet sojeva je grupisano u podtip VIId navedenog virusa. Izolovani sojevi virusa Newcastle bolesti su međusobno bili veoma slični (99,7-100%) i grupisali su se sa sojevima virusa prethodno izolovanih iz divljih ptica u Srbiji tokom izbijanja bolesti 2006. i 2007. godine. Molekularnom karakterizacijom gena na mestu deobe fuzionog F proteina ustanovljeno je da svih pet izolovanih sojeva virusa pripada visoko virulentnim velogenim sojevima.