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APPLICATION OF STANDARD AND MOLECULAR METHODS FOR THE DIAGNOSIS OF NEWCASTLE DISEASE

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Abstract - Four pooled samples of whole poultry carcasses with their internal organs were used to determine the presence of Newcastle disease (ND) virus. Samples were collected from one epizootiological area in the Republic of Serbia during January 2007. Newcastle disease virus strains were isolated from four samples. The identification of isolated strains was done by using the hemagglutination and hemagglutination-inhibition tests. The nucleic acid of the ND virus was identified in all the four samples It was confirmed that all the isolated strains were velogenic strains. Analysis of the nucleotide sequences of the gene encoding the F cleavage site of the fusion F protein showed the presence of motifs ^{112}RRQKRFIG^{119}, characteristic for the velogenic strains of the ND virus. Phylogenetic analysis of the F gene sequences revealed that all isolated strains of the virus belong to class II and genotype VIId.

Key words: Newcastle disease virus, RT-PCR, Real Time RT-PCR

INTRODUCTION

The Newcastle disease virus (NDV), or avian paramyxovirus type 1 (AMPV1), causes Newcastle disease in poultry and wild birds which is accompanied by high morbidity and mortality rates in the infected animals (Alexander, 2000). It belongs to the genus *Avulavirus*, subfamily *Paramyxovirinae*, family *Paramyxoviridae* and order *Mononegavirales*. The Newcastle disease virus genome consists of a non-segmented, single-stranded negative-sense molecule RNA, with a length of 15,186 to 15,198 nucleotides (Czegledi et al., 2006). The virus has an outer layer/peplos in which there are hemagglutinin-neuraminidase (HN) and fusion (F) antigens that are essential for the process of viral infection of cells. The fusion (F) protein is synthesized as an inactive precursor labeled F0. To become biologically active, the precursor protein must be subjected to proteolytic cleavage by the host cell proteases; this is how the two subunits F1 and F2 that remain connected by disulphide bonds arise. Proteolytic cleavage of the fusion protein is critical for the process of fusion and hemolysis (Peeples et al., 1988). The pathogenicity of Newcastle disease virus strains depends primarily on the presence or absence of specific basic amino acids in the position of F0 protein cleavage. Sequence analysis of genes encoding the fusion F protein of Newcastle disease virus enabled the comparison of the strains of Newcastle disease with low virulence with velogenic and mesogenic strains. All the velogenic and mesogenic strains of Newcastle disease virus have at least three basic amino-acids arginine (R) or lysine (K) in positions 113 to 116 and amino-acid phenylalanine (F) at position 117. Lentogenic strains have less than three basic amino acids in positions 113 to 116 and amino-acid leucine (L) at position 117 (Collins et al., 1993). Natural or artificial infection of

birds with Newcastle disease virus is described in at least 241 species from 27 of 50 orders of birds (Kaleta et al., 1988). Newcastle disease spreads among poultry populations and other birds by direct and indirect contacts. The incubation period lasts from 2 to 15 days, on average from 5 to 6 days. Depending on the degree of virulence, velogenic, mesogenic and lentogenic strains can be differentiated. The velogenic strains of Newcastle disease virus cause a peracute disease that is accompanied by a high mortality rate. The infection of poultry caused by neurotrophic velogenic strains of the Newcastle disease virus leads to the development of nervous symptoms. The mesogenic strains generally cause respiratory symptoms of Newcastle disease and decreased egg production, accompanied with a low mortality rate, except in very young birds. The lentogenic strains of Newcastle disease virus do not usually cause the disease in adult birds. The pathomorphological changes in the internal organs of infected poultry and wild birds depend primarily on the degree of virulence of the Newcastle disease virus strain that cause the infection, the type of infected poultry, their age, immune status and other non-specific factors that influence the course of infection. There are no pathognomonic symptoms to indicate Newcastle disease. Changes in the internal organs include bleeding in the digestive and respiratory systems, the presence of focal necrotic fields accompanied by bleeding, lesions in the cerebellum, extended cord, spinal cord, and altered egg follicles with hemorrhage in laying hens and turkeys (Alexander, 2000). For the laboratory diagnosis of Newcastle disease standard methods of virological diagnosis are used that involve the isolation of the virus from chicken embryos aged 9-11 days, virus isolation in tissue culture, the hemagglutination test and hemagglutination-inhibition test and ELISA. The pathogenicity of the isolated virus is assessed by determining the intracerebral pathogenicity index (ICPI), intravenous pathogenicity index (IPVI) and mean death time of embryos (MDT). In addition to these methods, polymerase chain reaction (PCR)-based molecular methods such as RT-PCR, Real-Time RT-PCR and nucleotide sequencing methods are used (Aldous and Alexander, 2000). The NDV strains show a great genetic diversity and can be divided into two classes with at least nine genotypes each (Czegledi et al., 2006). Because of this, and the fact that the lentogenic strains are very often used as live vaccines, it is important to detect all the genetic groups of NDV and to differentiate between the vaccine strains and velogenic strains. The aim of this study was to isolate and identify the Newcastle disease virus from suspect samples originating from poultry by using the standard virological methods, and to perform the identification, pathotyping, genotyping and phylogenetic analyses of all the isolates using molecular methods.

MATERIALS AND METHODS

Four pooled samples of whole poultry carcasses with their internal organs were used to determine the presence of the Newcastle disease virus. The poultry samples were collected from one epizootiological area in the Republic of Serbia during January 2007. Standard methods for NDV isolation were performed on embryonated chicken eggs aged 9 to 11 days, and for the identification of the isolated strains the hemagglutination test and hemagglutination-inhibition test were used (with the reference polyclonal sera and monoclonal antibodies). In order to determine the presence of NDV nucleic acids in the suspect poultry material, three different protocols were used: RT-PCR described by Seal et al. (1995), Real Time RT-PCR with the use of LUX primers described by Antal et al. (2007), and Real Time RT-PCR with the use of M gene specific primers and probes designed for both the virulent and avirulent strains of NDV described by Wise et al. (2004.). To determine the pathogenicity of the isolated NDV strains the following protocols were used: RT-PCR as described by Creelan et al. (2002), Real Time RT-PCR with the use of F gene specific primers and probes for virulent strains described by Wise et al. (2004), as well as the methods of F gene sequencing.

Viral RNA was extracted using a QIAamp Viral RNA Mini Kit (Qiagen, Germany) according to manufacturer's recommendations. Reverse transcription and polymerase chain reaction for the above-mentioned protocols was performed with a commercial

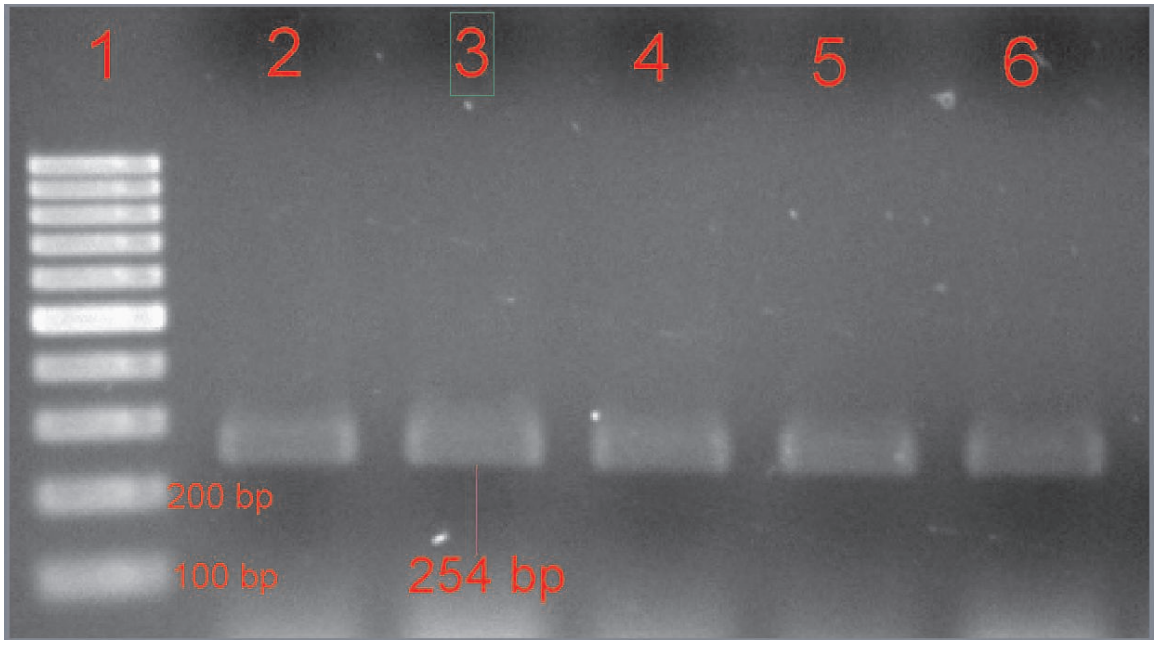

Fig. 1. Agar gel electrophoresis of RT-PCR products. Column 1 - molecular marker O'GeneRuler™ 100 bp DNA Ladder, (Fermentas); columns 2 through 6 - RT-PCR products of strains La Sota, SRB/22/07, SRB/181/07, SRB/182/07, and SRB/183/07, respectively.

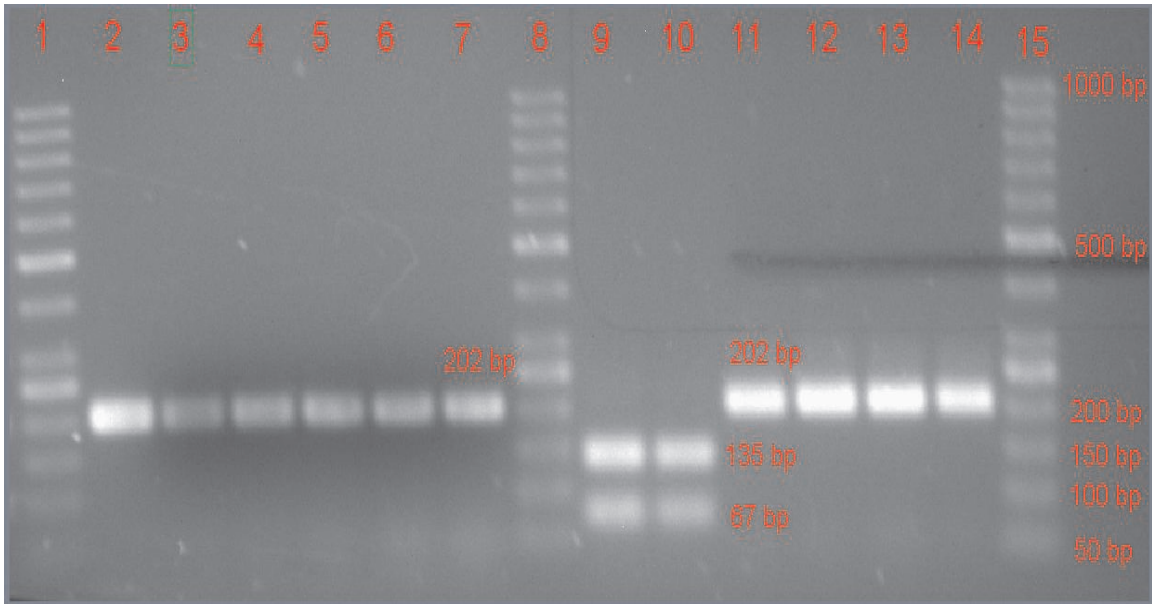

Fig. 2. Agar gel electrophoresis of RT-PCR products. Columns 1, 8 and 15 - the molecular marker O'GeneRuler™ 50 bp DNA Ladder, (Fermentas); columns 2 through 7 - RT-PCR products of strains La Sota, Clone 30, SRB/22/07, SRB/181/07, SRB/182/07 and SRB/183/07, respectively; columns 9 to 14 - RT-PCR products of the above-mentioned strains after digestion with restriction endonuclease BgiI. In lentogenic strains after treatment of PCR products by restrictive enzyme, cleavage occurred and two products of 67 bp and 135 bp were formed. After the treatment of PCR products from velogenic strains with enzyme there was no cleavage of the product.

kit, Superscript III Platinum One-Step Quantitative RT-PCR System (Invitrogen, USA). Real Time RT-PCR amplifications were carried out in a Real Time PCR instrument MX3000P (Stratagene, USA), while RT-PCR amplification and sequencing reaction were done in a 2720 Thermal Cycler (Applied Biosystems, USA). The sequencing of the viral genome was carried out by the Sanger method. The primers for sequencing, reagents used and thermocycling conditions have already been described (Vidanović et al., 2011). For the phylogenetic analysis and genotyping of strains isolated from poultry samples originating from the territory covered by this examination, a part of the nucleotide sequences of the F genes whose length is 375 nucleotides (from position 47 to 421 bp), was used.

RESULTS

The presence of Newcastle disease virus was determined in embryonated chicken eggs aged 9 to 11 days from four pooled suspect poultry samples. The hemagglutination titer values of the isolated strains of Newcastle disease virus were 1:32 for the SRB/181/07 strain, 1:64 for SRB/182/07 and SRB/183/07 strains and 1:256 for the SRB/22/07 strain. The identification of the isolated strains of Newcastle disease virus was done using the hemagglutination-inhibition test (HI test) using reference polyclonal sera and monoclonal antibodies. By applying three different protocols according to Seal et al. (1995) (Fig. 1), Antal et al. (2007) and Wise et al. (2004), the presence of nucleic acids of Newcastle disease virus was observed in all the samples. The results of two different protocols for determining the pathogenicity of the isolated strains of Newcastle disease virus, i.e. the RT-PCR method (Creelan et al., 2002) (Fig. 2.) and Real Time RT-PCR method (Wise et al., 2004) using primers and probes specific for virulent strains of Newcastle disease virus, confirmed that all isolated strains were highly virulent or velogenic. The analysis of the amino-acid sequences of the F0 cleavage site showed the presence of motif ^{112}RRQKRFIG^{119}, a characteristic of velogenic strains. The phylogenetic analysis of the F gene sequences revealed that the isolated strains of Newcastle disease virus belonged to the class II and VIId genotype in the phylogenetic tree with a high similarity to the NDV isolates from wild birds isolated in Serbia in 2007 (data not shown).

DISCUSSION

The presence of Newcastle disease virus was confirmed using the standard virological methods applied in our study in the 4 pooled samples of suspect materials originating from dead poultry. Alexander (2000) believes that the virus isolation in embryonated chicken eggs is one of the most reliable methods for diagnosis of Newcastle disease and all the known strains of Newcastle disease virus replicate in chicken embryos. However, virus isolation is considered to be slow, laborious and requiring undesirable *in vivo* testing with no information on the virus origin and its spread (Aldous and Alexander, 2001). The detection of the viral nucleic acid of the NDV using molecular methods directly from a wide range of specimens, i.e. blood, feces, tissues, and from different avian species, speeds up the diagnostic process significantly. In our study, the presence of the nucleic acids of Newcastle disease virus in the poultry samples was detected by three different protocols in accordance with Seal et al. (1995), Antal et al. (2007) and Wise et al. (2004). By applying these protocols, the presence of the nucleic acids of Newcastle disease virus was confirmed in four samples. Jestin (1991) first applied the RT-PCR method to detect the presence of nucleic acids of Newcastle disease virus in samples of chicken embryo allantoic fluids. The reaction was highly specific and without false-positive results. Wise et al. (2004) established the procedures for carrying out Real Time RT-PCR in order to detect the differentiations of some of the strains of the Newcastle disease virus. The results of these tests confirmed that the application of the first pair of primers and probes targeting the M gene was successful in the detection of different strains of Newcastle disease virus, with no cross-reactions with other avian paramyxoviruses. The pair of primers and probes targeting the F gene was specific to the virulent strains of NDV, including pigeon strains. The results of determining the pathogenicity of the isolated strains of Newcastle disease

virus, using the RT-PCR method of Creelan et al. (2002) and Real Time RT-PCR, using primers and probes specific for virulent strains of Newcastle disease according to Wise et al. (2004), confirmed that all the isolated strains belonged to highly virulent velogenic strains. Collins et al. (1993) sequenced the viral genome of 17 strains of Newcastle disease in order determine their pathogenicity. The obtained results confirmed that all 17 strains belonged to velogenic strains of the Newcastle disease virus. The phylogenetic analysis of the F gene sequences of Newcastle disease virus strains isolated in the Republic of Serbia showed that they belonged to the class II genotype and VIId viruses on the phylogenetic tree. Phylogenetic analysis based on part of the F gene has been widely used in the epidemiological assessment of the origins and spread of the viruses responsible for ND outbreaks (Kim, 2007). Aldous et al. (2003) proposed that the genotyping of NDV isolates should become part of the diagnostic virus characterization for reference laboratories. Such an analysis should guarantee a rapid epidemiological assessment of the origins and spread of the viruses responsible for ND outbreaks.

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