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CLASSICAL AND MOLECULAR CHARACTERIZATION OF PIGEON PARAMYXOVIRUS TYPE 1 (PPMV-1) ISOLATED FROM BACKYARD POULTRY – FIRST REPORT IN MACEDONIA

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ABSTRACT

Aim of this study was to characterize pigeon variant of Newcastle disease virus (NDV) isolated from backyard poultry using classical and molecular methods. In standard hemagglutination inhibition (HI) test both polyclonal NDV antiserum and monoclonal antibodies 161/617 specific for pigeon variants of NDV showed inhibition of heamagglutination of the isolated virus. Intracerebral pathogenicity index (ICPI) has shown that the isolate is mesogenic virus (ICPI = 0.81). One-step RT-qPCR for detection of M gene was performed indicating a presence of NDV and RT-qPCR for discrimination between lentogenic and velogenic strains based on F gene was also performed indicating a presence of virulent NDV. A portion of the F gene was amplified and sequenced for determination of virulence and phylogenetic characterization. The F protein cleavage site sequence of the isolate had multiple basic amino acids at residues 112–116 and a phenyl alanine at residue 117 (112RRQKR*F117) which is typical for velogenic strains. The nucleotide sequence of 374 bp was aligned to begin at nt 47 and finish at 420 immediately after the cleavage site and compared with other reference strains from the region and worldwide. In the phylogenetic tree, the isolate clustered into genotype VIb, typical for PPMV-1. This strain is phylogenetically very similar to other PPMV-1 isolated from pigeons in Macedonia. Poultry infected with PPMV-1 can spread the virus in the absence of clinical signs, thus PPMV-1's are constant threat to domestic poultry. This is the first report of evidenced spillover of PPMV-1 into poultry in Macedonia.

Key words: PPMV-1, backyard poultry, RT-qPCR, nucleotide sequencing

INTRODUCTION

Newcastle disease (ND) virus may be present in natural or experimental hosts in 241 species from 27 to 50 orders of birds and it is very likely that all birds are susceptible to infection, but the severity of the disease would depend on the type of the bird (19). The ND virus (NDV) belongs to order Mononegavirales, family Paramyxoviridae, subfamily Paramyxovirinae, genus *Avulavirus* (22). In this genus there are 11 serotypes of APMV, labeled APMV-1 to APMV-11 (9, 24). Newcastle disease virus is APMV-1, for which there are two different classifications based on genomic structure (2, 7, 12).

Since the first discovery of ND in 1926 (16) to date, four panzootics had occurred worldwide (3). The first isolation of virulent NDV responsible for the panzootics in pigeons is done by Kaleta et al. (18) in the sample from Iraq from 1978. In 1981 virus reaches Italy (8) and afterwards spreads globally to become panzootic by 1984/85 (5). Several authors consider that the reason for fast spread of the virus is developed international trade as well as races and exhibitions of pigeons (5). Pigeon strains most probably are a result of interspecies transmission of the virus which occurred several times in the past. Spread from pigeons to poultry must have happened earlier then 1980's because significant adaptation and evolution of these strains was present during the

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epizootics in pigeons (29). Virus is characterized as an antigenic variant of NDV with use of monoclonal antibodies (mAb's) (6). Because of the antigenic difference and for pragmatic purposes these viruses are termed pigeon paramyxovirus type 1 (PPMV-1) viruses beside the fact being able to infect poultry but with decreased virulence (23). These viruses are also able to infect wild pigeons, doves and ornamental birds (17, 20). The disease in pigeons has an enzootic character with occasional spread to wild pigeons and doves and represents a constant threat to poultry (4).

Amino acid sequence of cleavage site (CS) of fusion (F) protein is a major determinant of virulence (25, 26), although other proteins have a role in virulence depending on the strain of the virus (14). In order for the virus to be virulent a basic amino acid at residue 113, a pair of basic amino acid at residues 115 and 116 and phenylalanine at residue 117 of the F gene is required (10).

For proper diagnosis it is not enough only to detect NDV or prove infection with the virus, but it is also necessary to distinguish whether the virus is virulent or not by recommended classical and molecular methods (30). Nucleotide sequencing can determine virulence and can assess genetic makeup, genotype and phylogenetic characteristics of NDV (11, 21, 27). These techniques allow only 250 nucleotides to be sufficient for reliable phylogenetic analysis (2, 21, 27).

The role of pigeons in epizootiology of NDV in Macedonia has not been studied previously. The aim of this study was to characterize PPMV-1 isolated from backyard poultry by classical and molecular methods and to assess its phylogenetic relationship with other NDV isolates.

MATERIAL AND METHODS

Virus and virus isolation

Pool of internal organs obtained from dead chickens were homogenized in antibiotic medium using sterile sand, checked for sterility and inoculated in to the 9-11 days old embryonated chicken eggs (ECE). All dying embryos and remaining embryos after the incubation period of five days were checked for haemagglutination activity according to recommended protocols (29). Isolated virus was named as follows: serotype/host/country of origin/laboratory identification number (reference number)/year of sampling.

GenBank accession numbers of the viruses used for construction of the phylogenetic tree are shown in parentheses in Fig. 2. Several sequences without accession numbers were obtained from colleagues from the region. Accession number of the Macedonian strain is given in the results section.

Strain under investigation was isolated from backyard chicken on 10.02.2010 from village Rankovce in the northeastern part of Macedonia (N 42o10'03. E 22o07'03.) Isolation was donefrom the pool of visceral organs. Strain was labeled as NDV/chicken/Macedonia/231/2010 according to serotype/host/location/laboratory number/sampling date.

Hemagglutination inhibition test (HI test)

The supernatant of allantoic fluid was collected and used in standard HI test using polyclonal NDV antiserum and monoclonal antibodies (mAb): U85 for detection of classical strains, 161/617 for detection of PPMV-1 strains and 7D4 for detection of F and La Sota vaccine strains.

In vivo pathogenicity test

The pathogenicity of PPMV-1 isolate was assessed by intracerebral pathogenecity index (ICPI) test. One-day-old specific pathogen free (SPF) chickens were inoculated intracerebrally with 0.1 ml of a 1:10 dilution of infective allantoic fluid. Chicks were monitored during an 8-day observation period and scored daily as normal (score 0), sick (score 1), and dead (score 2). Total scores were determined and the mean daily scores were calculated to obtain the ICPI.

Preparation of viral RNA, real-time reverse transcription-polymerase chain reaction (RT-qPCR) and reverse transcription-polymerase chain reaction (RT-PCR)

Viral RNA was extracted from allantoic fluid of ECE using Invisorb Spin Virus RNA Mini Kit (Invitek, Germany) following manufacturers protocol.

One step RT-qPCR for detection of matrix (M) gene was performed with iScript One-Step RT-PCR Kit for Probes (Bio-Rad) with primers and hydrolysis probe as described by Wise et al. (31): forward primer M+4100

5'-AGTGATGTGCTCGGACCTTC-3', reverse primer M-4220 5'-CCTGAGGAGAGGCATTTGCTA-3' and hydrolysis probe M+4169 5'- (FAM) TTCTCTAGCAGTGGGACAGCCTGC(TAMRA) - 3'. Thermal protocol was as follows: 50 ^{0}C 10 min, 95 ^{0}C 5 min and 40 cycles on 95 ^{0}C 10 seconds and 55 ^{0}C 30 seconds.

The procedure for detection of fusion (F) gene is the same as described above except for primers and hydrolysis probe used: forward primer F+4839 5'-TCCGGAGGATACAAGGGTCT-3', reverse primer F-4939 5'-AGCTGTTGCAACCCCAAG-3' and hydrolysis probe F+4894 (VFP-1) 5'-(FAM) AAGCGTTTCTGTCTCCTTCCTCCA(TAM RA)-3' and the temperature of annealing of primers which was 58 ^{0}C instead of 55 ^{0}C, according to Wise et al. (31).

Two-step RT-PCR for the F gene was performed according to methods described by Collins et al., (10) with primers according to Aldous et al., (2) where forward primer was used instead of random primers in the RT step. In the PCR step forward primer MSF1 5'-GACCGCTGACCACGAGGTTA-3' and reverse primer #2 5'-AGTCGGAGGATGTTGGCAGC-3' were used with the thermal protocol of 94 ^{0}C for 3 min, 42 cycles of 94 ^{0}C for 1 min, 50 ^{0}C for 1 min and 72 ^{0}C for 3 min, and final extension at 72^{0}C for10 min. The PCR product of 700 base pairs (bp) was synthesized, subjected to electrophoresis in 1.5% agarose gel and the DNA band was excised from the gel and purified using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). The purified PCR product was used for sequencing.

Nucleotide sequencing and analysis of sequence data

The sequence of the amplified 374 bp region of the F-gene was obtained using Big Dye Terminator v3.1 kit (Applied Biosystems, USA) and F-gene-specific primers, forward primer #7 5'-GACCGCTGACCACGAGGTTA-3' and reverse primer#2 5'-TTAGAAAAAACACGGGTAGAA-3' according to Aldous et al., (2). All sequencing reactions were performed with fluorescent dideoxynucleotide terminators in the ABI 310 automated sequencer (Applied Biosystems Inc., Foster City, CA) and sequencing product was purified with 50 µl of ethanol (96-100%), 2 µl of 3M sodium acetate and 2 µl of 125 mM EDTA. Sequence editing was performed with BioEdit Sequence Alignment Editor version 7.0.9.0 while alignment with Clustal V method was done in MEGA5 software (MEGA, version 5). The same region of the F-gene was used to construct phylogenetic trees and to classify PPMV-1 isolate among other class II genotype reference sequences. Phylogenetic analysis was performed using MEGA5 software (MEGA, version 5) (28). The evolutionary distances were inferred using un-rooted maximum-likelihood method with 1,000 bootstrap replicates to give credibility to the grouping and included the first, second, and third coding and noncoding positions.

RESULTS

Virus was isolated from a holding of 40 chickens, out of which five have died. Clinical signs involved inapetence, stretched wings and dispnoea. Gross lesions were located predominantly in the respiratory system and involved congestion and edema of the lungs. Haemagglutination activity of allantoic fluid was detected after the second passage in inoculated ECE. Infective allantoic fluid has demonstrated inhibition of haemagglutination with polyclonal NDV serum with titer of 8 $\log^2$ while it was negative for H5N1 and H7N1 antisera. When tested with mAb's it demonstrated inhibition of haemagglutination with mAb 617/161 with titre of 5 $\log^2$ while it was negative for mAb U85 and mAb 7D4. Value of ICPI was 0.81 classifying the virus as mesogenic strain regarding pathogenicity. On the basis of RT-qPCR for detection of the M and the F gene, the virus proved to be virulent strain of class II NDV with Ct values of 21.3 and 27.5, respectively. These results were confirmed with RT-PCR for the F gene with visible band on the gel with expected size of 700 bp.

GenBank accession number of the partial F gene nucleotide sequence is KC915211. Nucleotide sequencing of the CS of the F gene revealed amino acid motif characteristic for virulent strains of NDV 112RRQKR*FIG119 (Fig. 1). Based on the position in the phylogenetic tree it can be concluded that this strain belongs to genotype VI, subgenotype VIb. Results obtained from HI test and phylogenetic tree clearly indicate that this strain is PPMV-1 although isolated from chicken.

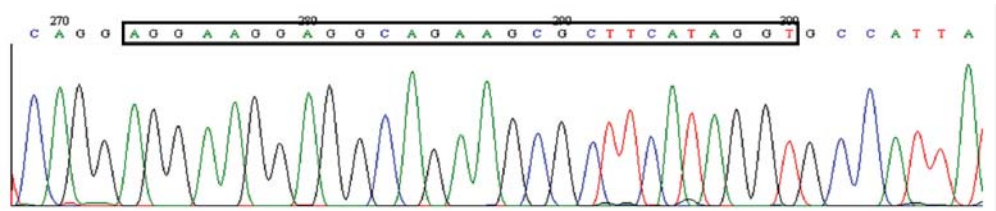

Figure 1. Electropherogram of part of the nucleotide sequence of the F gene of NDV/chicken/Macedonia/231/2010 encompassing the cleavage site (framed)

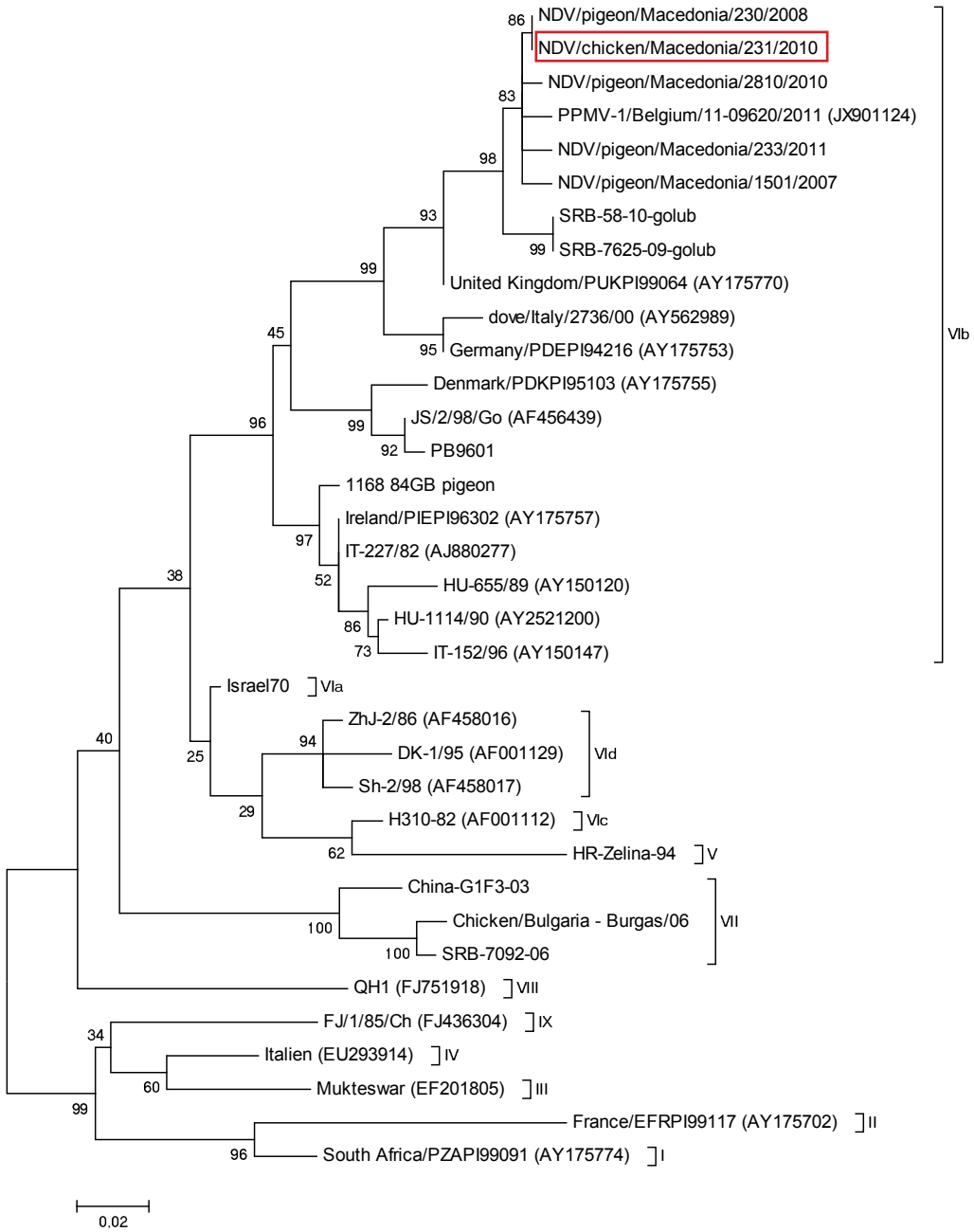

Figure 2. Phylogenetic tree based on the partial nucleotide sequence of the F gene (nt 47 – 420). Classification of the genotypes is marked on the right side of the tree. Tree is constructed with 'maximum-likelihood' method in MEGA 5.1. with "bootstrap" 1000 in order to give credibility to the grouping. Strain investigated in this study is framed. GenBank accession numbers where available are shown in parentheses.

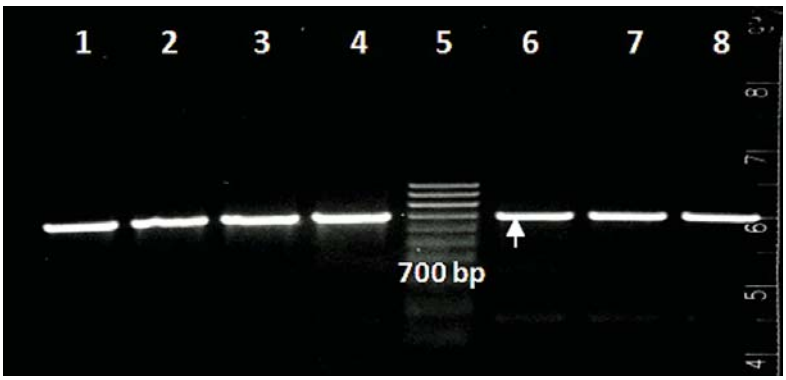

Figure 3. Results of PCR visualization of strain NDV/chicken/Macedonia/231/2010 using primers ' MSF1 и #2 (Aldous et al., 2003), Size 700 bp; (arrow)1-4, 7-8 Different PPMV-1 strains isolated in Macedonia; 5- Marker 100-1000 bp

DISCUSSION

Despite vaccination PPMV-1 is still enzootic in many countries, and birds other than domestic pigeons, such as doves and wild birds and birds in ZOOs, get infected (1, 23). Most PPMV-1 strains have reduced virulence for chickens, but because of the presence of virulent cleavage site motif of the F gene they belong to the group of virulent strains (1, 23, 29). Simultaneous presence of virulent CS of the F gene with lower ICPI values is not uncommon for PPMV-1 and it is previously reported (11, 23). This phenomenon is not associated with the F protein (13) but with replication complex consisting of nucleoprotein, phosphoprotein and polimerase protein (15). It is reported that PPMV-1 isolates from dead racing pigeons with nervous signs possessing amino acid motif of the CS of the F gene 112RRQKR*FIG119 have highly variable but low ICPI values (average 0,69) while PPMV-1 with 112GRQKR*FIG117 have high values of ICPI (average 1,44) (23). These results support recommendation that *in vivo* pathogenecity test should always be accompanied by sequencing (13).

Based on the partial nucleotide sequence of the F gene segment, strain NDV/chicken/Macedonia/231/2010 belongs to genotype VI, subgenotype VIb according to classification by Czegledi et al., (12). This subgenotype is further divided into two groups (28) and according to this classification Macedonian strain belongs to the group VIb/1 of recent European strains (EU/re). This group of strains (VIb/1) probably originated in North-East Africa (29). Strain NDV/chicken/Macedonia/231/2010 possess amino acid CS motif of the F gene 112RRQKR*FIG119 that classifies it in virulent viruses (10) which is characteristic for PPMV-1 isolated from the 1990s onwards (23).

Pigeon variants are very contagious and can spread from infected pigeons to other pigeons or other poultry when inadequate biosecurity measures are present. Poultry infected with PPMV-1 can spread the virus in the absence of clinical signs. Thus they are a constant threat to domestic poultry. In the EU during the period 2000-2009, 14 outbreaks of NDV caused by PPMV-1 in poultry are reported with most of the outbreaks occurring in small backyard and ornamental flocks (4). Based on the nucleotide sequence, strain NDV/chicken/Macedonia/231/2010 even though isolated from chickens belongs to PPMV-1 . This strain is phylogenetically very similar to other PPMV-1 isolated from pigeons in Macedonia in 2007, 2008, 2010 and 2011. Most probably chickens contracted the virus from infected pigeons carrying the virus. It is possible that other introductions of the PPMV-1 into poultry have occurred but have gone unnoticed. If there is a large reservoir of PPMV-1 in domestic and wild pigeons including other wild birds there is a large possibility for spread of the virus to poultry (4).

The phylogenetic tree perceives no clear geographical demarcation between these strains globally. Thus, although geographically distant, PPMV-1 strains are phylogenetically close. This is primarily due to the specificities of this industry where there is intense mixing of pigeons from different geographic locations as a result of international and internal trade, exhibitions of live birds, racing pigeons, etc. This is the first evidenced spillover of PPMV-1 into poultry in Macedonia.

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