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Research Article

Molecular Characterization of Infectious Bronchitis Virus Strains Isolated from Vaccinated Flocks in Serbia and Their Comparison with the Isolated Strains from Neighboring Countries

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Abstract

The aim of this study was to isolate, genetically characterize and determine phylogenetic relationship of strains detected in Serbia in comparison to other strains. A total of 480 samples collected from 13 different commercial layer flocks, obtained from tracheal swabs were included. Samples taken from 2016 to 2017 were molecularly analyzed by real-time RT-PCR, multiplex nested RT-PCR, and by sequencing of the S1 gene. Phylogenetic analyses based on partial S1 sequences revealed that six strains were classified as the D274 genotype, two strains as the QX genotype and two strains as the 4/91 genotype. The difference in nucleotide similarity between the Serbian isolates belonging to the D274 group ranges from 0 to 1.2%. Comparison of the obtained strains and D274 (X15832) showed differences from 0 to 0.9%. The greatest nucleotide similarity of detected QX strains was with Chinese QXIBV (KC795604), ranging from 98.8% to 99.1%. Two Serbian strains belonging to the 4/91 genotype had 99.7% and 98.8% nucleotide similarities with vaccine strain 4/91 (KF377577). This study has shown that viruses belonging to D274, QX, and 4/91 genotypes were circulating in poultry flocks in Serbia during 2016 and 2017.

Keywords: *IBV, Genotyping, QX, D274, 4/91*

Sırbistan'da Aşılı Sürülerden İzole Edilen Enfeksiyöz Bronşitis Virus Suşlarının Moleküler Karakterizasyonu ve Komşu Ülkelerden İzole Edilen Suşlar İle Karşılaştırılması

Öz

Bu çalışmanın amacı, Sırbistan'da Enfeksiyöz bronşitis virus suşlarını izole etmek, genetik karakterizasyonlarını yapmak ve diğer suşlar ile olan filogenetik alakasını belirlemektir. Çalışmada, 13 farklı ticari yumurtacı tavuk sürülerinden trakeal swab yoluyla toplanan toplam 480 örnek kullanıldı. 2016 ile 2017 arasında alınan örneklerin gerçek-zamanlı RT-PCR, multiple nested RT-PCR ve S1 gen sekanslaması ile moleküler analizi gerçekleştirildi. Kısmi S1 sekanslama temelli filogenetik analiz altı suşun D274 genotipi, iki suşun QX genotipi ve iki suşun 4/91 genotipi olduğunu gösterdi. D274 grubuna ait Sırp izolatları arasında nükleotid benzerliğindeki farklılık 0 ile %1.2 arasında değişti. Elde edilen suşlar ile D274 (X15832) karşılaştırıldığında 0 ile %0.9 arasında farklılık tespit edildi. Belirlenen QX suşlarının en yüksek nükleotid benzerliği %98.8 ile %99.1 arasında olmak üzere Çin QXIBV (KC795604) ileydi. 4/91 gentopine ait iki Sırp suşu ile aşı suşu olan 4/91 (KF377577) arasında %99.7 ile %98.8 nükleotid benzerliği gözlemlendi. Bu çalışma D274, QX ve 4/91 genotiplerine ait virusların 2016 ile 2017 yılları arasında Sırbistan'daki kanatlı kümeslerinde bulunduğunu göstermiştir.

Anahtar sözcükler: *IBV, Genotiplendirme, QX, D274, 4/91*

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INTRODUCTION

Infectious bronchitis (IB) is highly contagious viral disease of poultry affecting respiratory tracts, but the disease is also associated with the nephritis, poor weight gain, and reproductive signs as a decline in egg production and quality. The disease is caused by infectious bronchitis virus (IBV), of the family *Coronaviridae*. Its genome consists of positive sense single-stranded RNA, containing genes coding for four structural proteins: spike (S), membrane (M), nucleocapsid (N), and small envelope (E) proteins [1]. S protein is responsible for virus attachment and fusion of the virus with the host cell and it is cleaved into S1 and S2 subunits. S1 is highly variable, including three hypervariable regions (HVRs) [2] and induces neutralizing antibody production in the host [3]. The molecular identification of IBV is based mainly on the analysis of the S1 protein gene [4].

The disease can be managed through an adequate implementation of biosecurity measures and vaccination. Control of vaccination is complicated due to small changes in the amino acid sequences of the S protein that can result in the generation of new antigenic types [5]. For that reason and despite the use of live and inactivated vaccines, there is a continuous emergence of variants responsible for worldwide outbreaks of IB and economic losses of poultry production. It is, therefore, necessary to constantly monitor the field situation and identify circulating IBV genotypes to adequately adjust vaccination program, which will protect poultry flocks. Many different genotypes of IBV have been identified in the world. In Europe, the predominant ones are 793B (4/91), Massachusetts, Italy02, and QX [6].

In Serbia, IB is endemic and is controlled by the use of mainly Massachusetts strains, 4/91 and D274 vaccines. To date, there is no information available on the circulation of variant IBVs in Serbia, which makes this investigation especially important. Therefore, the objective of the present study was to isolate, genetically characterize and determine phylogenetic relationship of strains detected in Serbia in comparison to other strains reported in Europe and around the world.

MATERIAL and METHODS

Sampling

A total of 480 samples were included in this study. Samples were collected from 13 different commercial layer flocks in Central Serbia (Zlatibor, Morava, Raška and Rasina regions) from June 2016 to February 2017. All samples were obtained from tracheal swabs of layer flocks without clinical signs of IB, with the history of sporadic outbreaks previously. The age of the flocks and the vaccinating programs are shown in *Table 1*. Testing was carried out at the Veterinary Specialist Institute, Department for Laboratory Diagnostic in Kraljevo, Serbia. Samples were placed in sterile phosphate-buffered saline (Sigma-Aldrich, Schnelldorf, Germany).

RNA Extraction and Real-time RT-PCR

RNA was extracted directly from tracheal swabs by a QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The extracted RNA was stored at -80°C until the use in the real-time RT-PCR reaction.

For the detection of IBV-specific nucleic acid, a TaqMan-probe based group-specific real-time PCR assay was used as previously described [7]. Forward primer IBV5_GU391 (5'-GCT TTT GAG CCT AGC GTT-3') located at nucleotide positions 391 to 408 of the IBV M41 strain genome, reverse primer IBV5_GL533 (5'-GCC ATG TTG TCA CTG TCT ATT G-3') located at nucleotide positions 533 to 512 of the IBV M41 strain genome, and TaqMan dual-labelled probe IBV5_G (5'-CAC CAG AAC CTG TCA CCT C - 3') located at nucleotide positions 494 to 473 of the IBV M41 strain genome were used to amplify and detect a 143-base-pair fragment of the 5'-untranslated region (UTR). PCR amplification was performed on Stratagene Mx3000P (Stratagene, USA), using SuperScript III Platinum Quantitative One-Step RT-PCR kit (Thermo Fisher Scientific, USA) under following conditions: 50°C for 30 min; 95°C for 2 min; 45 cycles of 95°C for 15 s followed by 60°C for 60 s. A total volume of 25 µL reaction mixture containing 9 µL nuclease-free water, 12.5 µL reaction Mix, 0.2 µL of each primer, 0.5 µL of Taq Mix enzymes and 0.125 µL of TaqMan probe for each tube. The limit of detection for this assay was 100 genome copies per reaction.

Multiplex Nested RT-PCR

Nested PCR were performed according to Worthington et al.[6]. The initial PCR used primers SX1+ (5'-CACCTAG AGGTTTG T/C T A/T GCAT-3') and SX2- (5'-TCCACCT CTATAAACACC C/T TT-3'). The amplicon was further amplified in a second internal PCR that used primers SX3+ (5'-TAATACTGG C/T AATTTTTCAGA-3'), SX4- (5'-AATAC AGATTGCTTACAACCACC-3'). In first round SuperScript III Platinum Quantitative One-Step RT-PCR kit (Thermo Fisher Scientific, USA) was used under following conditions: 50°C for 30 min; 95°C for 2 min; 30 cycles of 95°C for 15 s followed by 48°C for 1,5 min and 72°C for 2 min. Second round was performed using DreamTaq Hot Start Green PCR Master Mix (Thermo Fisher Scientific, USA): 95°C for 10 min; 30 cycles of 95°C for 15 s followed by 48°C for 1,5 min and 72°C for 2 min.

The amplified products were analyzed by electrophoresis on a 1% agarose gel stained with ethidium bromide. The gel purification process was carried out using the GeneJet Gel Extraction Kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. The purified DNA was stored at -20° C until sequencing.

Sequencing

The sequencing of the obtained purified PCR product was carried out using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Woolston, UK), according to the manufacturer's instructions. Sequences were analyzed with 3130 Genetic Analyzer (Applied Biosystems, Woolston, UK). The obtained results were processed using the SeqScape program (Applied Biosystems, Woolston, UK) and corrected in Chromas Lite program (Technelysium Pty, Ltd, Brisbane, Australia).

Phylogenetic Analysis

MEGA 7.0 software [8] was used for phylogenetic analysis and genotyping. Pairwise and multiple sequence alignment was done by Clustal O, a part of Unipro UGENE - a unified bioinformatics toolkit [9]. The evolutionary history was inferred by using the maximum likelihood method based on the general time reversible model [10].

Isolated IBV sequences were pairwise compared with the S1 gene as follows: Genogroup GI-1- GI-11 (Beaudette (M95169), Holte (GU393336), Gray (L14069), Holte (L18988), N1/62 (U29522), VicS (U29519), TP/64 (AY606320), L165 (JQ964061), ARK99 (M99482), B (AF151954), UFMG/G (JX182775)); GI-12 (D3896 (X52084), D274 (X15832); GI-13 - GI-27 (Moroccan-G/83, (EU914938), B1648 (X87238), B4 (FJ807932), IZO 28/86 (KJ941019), CA/Machado/88 (AF419315), JP8127 (AY296744), 58HeN-93II (KC577395), Qu_mv (AF349621), Spain/97/314 (DQ064806), 40GDGZ-97I (KC577382), Variant 2 (AF093796), V13 (KF757447), CA/1737/04 (EU925393), NGA/B401/2006 (FN182243), GA08 (GU301925)); Genogroup GII-1 (D1466 (M21971)); GIII-1 (N1/88 (U29450)); GIV-1 (DE/072/92 (U77298)); GV-1 (N4/02 (DQ059618)) and GVI-1 (TC07-2 (GQ26594)) [11], QXIBV (KC795604), Ark52930 (FJ899688), H120 (M21970), It/497/02 (DQ901377), L-1148 (DQ431199), D207 (M21969 J04329), and vaccine strains (H120 (FJ888351), M41 (DQ834384), MA5 (KU736747), and 4/91 (KF377355)).

GenBank Accession Number

Gene bank accession numbers of the Serbian S1 sequences used in this investigation are presented in *Table 1*.

RESULTS

The detection and quantifications limits were determined using cycle threshold (CT) values obtained for each reaction containing from 10^7 to 10^2 copies of the standard RNA. The assay was negative below 100 template copies. Therefore, the limit of detection and quantification were both determined to be 100 template copies. Results were analysed in terms of CT values. A CT value below 38 cycles was regarded as a positive result, and a negative result was represented by a CT value ≥38.

Among 480 examined samples from tracheal swabs of layer flocks, IBV was detected in 10 cases. Based on their partial S1 gene sequences, IBVs identified in Serbia during this study can be divided into three groups. The first group represents D274 genotype within isolates MH010330, MH010331, MH010332, MH010334, MH010335 and MH010336. The second genotype is 4/91 where isolates MH010333 and MH010339 are positioned. The third genotype belongs to QX with isolates MH010337 and MH010338 *(Fig. 1)*. Isolates MH010330, MH010331 showed 100% mutual nucleotide similarity, the same as isolates MH010332 and MH010336. Isolate MH010335 showed 99.7% similarity to MH010330, MH010331, and MH010334, and 98.8% to MH010332 and MH010336, respectively. Strain MH010334 showed 99.1% nucleotide similarity to MH010332 and MH010336. Strains MH010330 and MH010331 were also closely related (99.4% similarity) to the MH010332 and MH010336 isolates. The differences in nucleotide similarities between the Serbian D274 strains in this study varied from 0 to 0.9% comparing with D274 (X15832) and 2.4 to 2.7% comparing with D3896 (X52084) isolated in Netherland in 1978 *(Fig. 1)*. Our isolates, belonging to the QX genotype, showed 99.7%

Table 1. *Serbian IBV strains used in the study with the epidemiologic data, vaccination program and accession numbers*

Strain	Type of Chicken	Age (weeks)	Vaccination Program	Accession No.
1	Layer	25	1 d/MA5, 35d/4/91	MH010330
2	Layer	25	1 d/MA5, 35d/4/91	MH010331
3	Layer	26	1 d/MA5, 35d/4/91	MH010332
4	Layer	26	1 d/MA5, 35d/4/91	MH010333
5	Layer	26	1 d/MA5, 35d/4/91	MH010334
6	Layer	26	1 d/MA5, 35d/4/91	MH010335
7	Layer	26	1 d/MA5, 35d/4/91	MH010336
8	Layer	29	1 d/MA5, 10 d 4/91, 31d/MA5	MH010337
9	Layer	29	1 d/MA5, 10 d 4/91, 31d/MA5	MH010338
10	Layer	21	1 d/MA5, 10 d MA5, 56d/4/91	MH010339

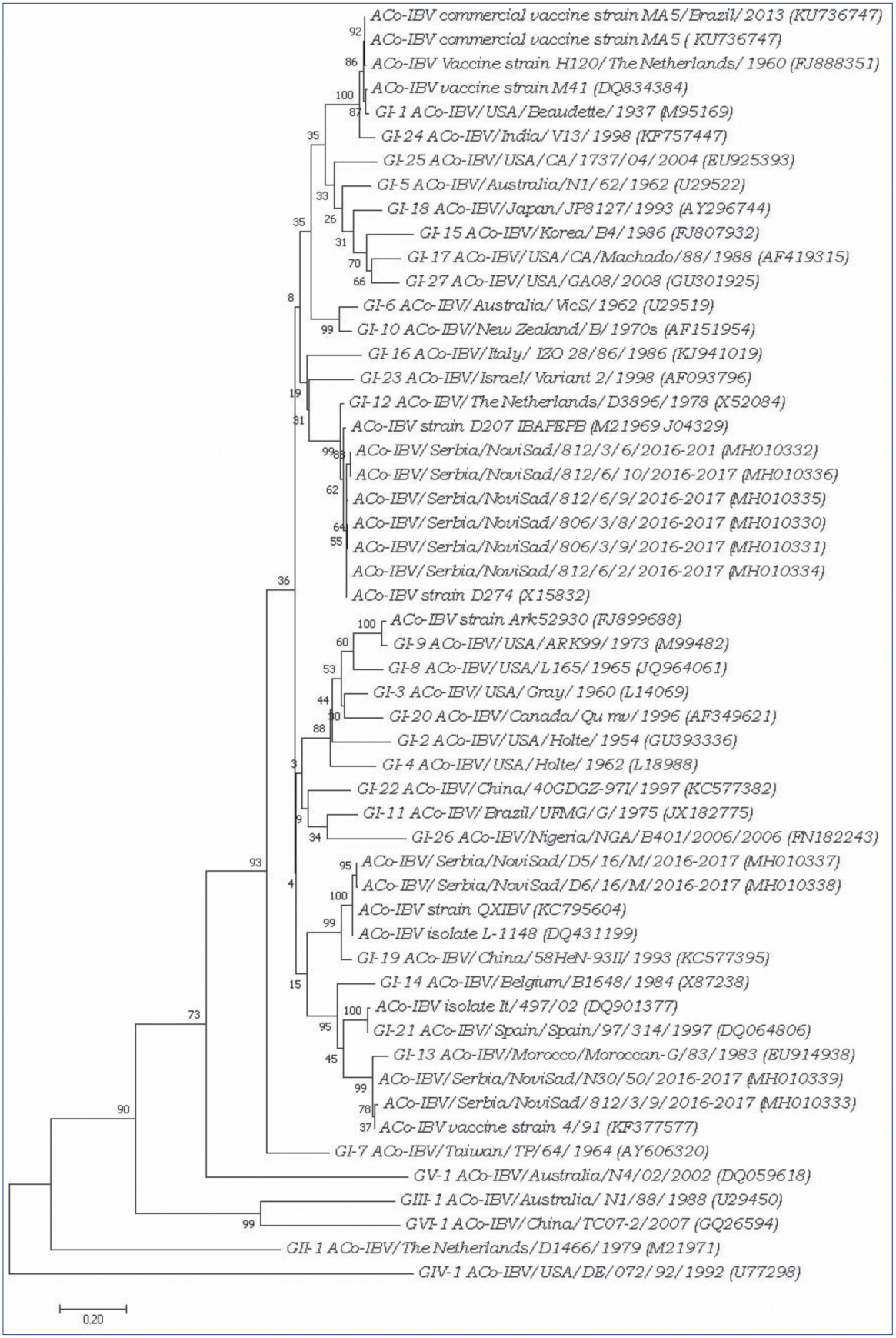

Fig 1. Maximum likelihood tree (1000 bootstop replicates) generated by neighbor-join method, representing the IBV isolates detected in Serbia and selected IBV reference strains

mutual nucleotide similarity. The difference in nucleotide similarity between the isolates belonging to the D274 group ranged from 0 to 1.2%. Serbian strains MH010337 and MH010338, closely related to QXIBV (KC795604) and L-1148 (DQ431199), varied from 0.9 to 1.2%, and QX (KC577395) varied from 5.8 to 6.1% *(Fig. 1)*. Serbian strains MH010333 and MH010339, belonging to 4/91 genotype, showed 98.5% mutual nucleotide similarity, and isolate MH010339 was 99.7% similar to 4/91 vaccine strain (KF377577), while isolate MH010333 was 98.8% similar to the same 4/91 strain *(Fig. 1)*.

DISCUSSION

The dominant genotype detected within this study was D274, where it was detected six out of 10 strains. The difference in nucleotide similarity between the isolates belonging to the D274 group ranged from 0 to 1.2%. Comparing the obtained Serbian strains and D274 (X15832) it can be seen that strain MH010334 showed 100% similarity with D274 (X15832); strains MH010330, MH010331, and MH010335 had 99.7% similar nucleotides and strains MH010332 and MH010336 showed 99.1% nucleotide similarity. This is probably not surprising given that D274 strains are widely used for vaccination. The disadvantage of live vaccines is that they spread easily in the field [12,13], which enables the survival and circulation of the vaccine virus in flocks. This is also supported by the fact that there were no clinical symptoms of the disease in the flocks from which the virus was isolated, but the virus circulated. Based on the data presented in *Table 1*, it can be seen that the flocks from which the virus was isolated were not vaccinated with the D274 vaccine. This vaccine is largely used in Serbia and, together with MA5 and 4/91, recommended for controlling IB in laying flocks. In the past, the D274 vaccine was used on the farms where Serbian strains originated from, and also on farms close to the farms from which Serbian strains belonging to D274 genotype were isolated. Also, strain D3896 (X52084) isolated in the Netherlands in 1978 had a high similarity with our strains ranging from 97.3-97.6% and strain D207 (M211969 J04329) ranging from 97.6% to 98.5%. The most common IBV genotype in some Western European countries in the early and middle 1980s was D274-like, and that was the reason for initiating vaccination [14,15].

Two QX strains (MH010337 and MH010338) were detected in this study. The greatest nucleotide similarity of detected strains was with QXIBV (KC795604) and L-1148(DQ431199), and there was 99.1% similarity for isolate MH010337 and 98.8% for isolate MH010338. Comparing our strains with QX (KC577395) strain MH010337 showed 94.2% similarity and strain MH010338 93.9%, respectively. The QX strain was initially isolated in China [16] during 1990s. This strain at first did not circulate outside Asia, but later was detected in broilers in Italy in 2011 [17]. Also, Chinese QX type was detected in Poland [18] and Hungary [19]. In Europe, beside Chinese strains, QX-like strains characterized as European QX was detected in the United Kingdom [20-22], Finland [23], Russia [24], Slovenia [25], Spain [26], and Sweden [27]. This is the first confirmation of QX in Serbia, so it is hard to say where it comes from, but most likely from neighboring countries. QX viruses have been isolated from a flock where vaccination with MA5 and 4/91 was performed. Since the virus has been isolated from a flock without clinical signs, this indicates that although the virus was present, due to adequate vaccination there were no clinical manifestations. Terregino et al.[28] showed that MA5 and 4/91 vaccines administered at 1 day and 14 day protect chickens from infections and QX-like IB disease, which this vaccinating protocol makes useful in reducing economic losses caused by QX strains.

Since it was first described in the early nineties in the UK [29], 4/91 IBV genotype spread over many other countries and became one of the most predominant in Europe [12,29-32]. The present study showed that 4/91 is also present in Serbia and demonstrated its circulation in our country. It is hard to say how long it has been present since this is the first study in Serbia dealing with the IBV genotyping. Vaccine 4/91 was used in both flocks from which the virus was isolated *(Table 1)*. Serbian strain MH010339 showed 99.7% and strain MH010333 98.8% similarity to vaccine strain 4/91 (KF377577). The differentiation of the vaccine strain from a wild strain is difficult and possible by nucleotide sequencing of the SI gene, where the percentage of nucleotide similarity is important. Worthington et al.[6] have categorized vaccine-related IBVs that have less than 99% part-S1 similarity as field IBV viruses. In our study, where isolate MH010339 showed above 99% similarity to vaccine strain, it was a vaccine virus. Strain MH010333 differed by 1.2% indicating that it can also be a field strain, especially considering the fact that such viruses may have emerged through antigenic drift over time, potentially encouraged by continuous use of homologous vaccine strains, resulting in vaccine pressure [33]. There was also a high similarity of our isolates to Moroccan-G (EU914938), which is 95.9% for isolate MH010333 and 96.2% for isolate MH010339.

In conclusion, this study has shown that viruses from D274, QX, and 4/91 genotypes were circulating in poultry flocks in Serbia during 2016 and 2017, and that this is the first genotyping of IBV in Serbia. Considering the fact that isolated strains originated from a flock without clinical symptoms, it can be said that the vaccination applied in Serbia, using MA5, 4/91, and D274 vaccines, represents a good protection for the present, because it protects flocks from IB and therefore prevents economic losses. Certainly, in the future, the situation with new genotypes in Serbia should be monitored and studies should be carried out involving a large number of farms and a wider area.

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