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


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 TTT GCA 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 nuclelease-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. [8]. The initial PCR primers SX1+ (5’-CACCTAG AGGGTTG T/C T A/T GCAT-3’) and SX2- (5’-TCCACCT CTAACACCC C/T TT-3’). The amplicon was further amplified in a second internal PCR that used primers SX3+ (5’-TAAACTGGC/T AATTTTTCAGA-3’), SX4- (5’-AATAC AGATTGCTTAAACCACC-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 \cite{11} 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 \cite{9}. The evolutionary history was inferred by using the maximum likelihood method based on the general time reversible model \cite{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 (D274 (X15832)); GI-13 - GI-27 (Moroccan-G/83, (EU914938), B1648 (X87238), B4 (JF807932), IZO 28/86 (KJ941019), CA/Machado/88 (AF419315), JP8127 (AY296744), 58HeN-93II (K577395), Qu_mv (AF349621), Spain/97/314 (DQ064806), 40GDGZ-97I (K577382), Variant 2 (AF093796), V13 (KF757447), CA/1737/04 (EU925393), NGA/B401/2006 (FN182243), GA08 (GU301925)); Genogroup GI-1 (D1466 (M21971)); GII-1 (N1/88 (U29450)); GIV-1 (N4-02 (DQ509618)) and GVI-1 (TC07-2 (GQ26594)) \cite{11}; QXIBV (KC795604), Ark52930 (FJ899688), H120 (M21970), It/497/02 (DQ01377), L-1148 (DQ431199), D207 (M21969 J04329), and vaccine strains (H120 (FJ888351), M41 (DQ834384), MAS (KU736747), and 4/91 (KF377355)).

Gene 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 $10^2$ to $10^5$ 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% similarity to the QX genotype.

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Type of Chicken</th>
<th>Age (weeks)</th>
<th>Vaccination Program</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Layer</td>
<td>25</td>
<td>1 d/MA5, 35d/4/91</td>
<td>MH010330</td>
</tr>
<tr>
<td>2</td>
<td>Layer</td>
<td>25</td>
<td>1 d/MA5, 35d/4/91</td>
<td>MH010331</td>
</tr>
<tr>
<td>3</td>
<td>Layer</td>
<td>26</td>
<td>1 d/MA5, 35d/4/91</td>
<td>MH010332</td>
</tr>
<tr>
<td>4</td>
<td>Layer</td>
<td>26</td>
<td>1 d/MA5, 35d/4/91</td>
<td>MH010333</td>
</tr>
<tr>
<td>5</td>
<td>Layer</td>
<td>26</td>
<td>1 d/MA5, 35d/4/91</td>
<td>MH010334</td>
</tr>
<tr>
<td>6</td>
<td>Layer</td>
<td>26</td>
<td>1 d/MA5, 35d/4/91</td>
<td>MH010335</td>
</tr>
<tr>
<td>7</td>
<td>Layer</td>
<td>26</td>
<td>1 d/MA5, 35d/4/91</td>
<td>MH010336</td>
</tr>
<tr>
<td>8</td>
<td>Layer</td>
<td>29</td>
<td>1 d/MA5, 10 d 4/91, 31d/MA5</td>
<td>MH010337</td>
</tr>
<tr>
<td>9</td>
<td>Layer</td>
<td>29</td>
<td>1 d/MA5, 10 d 4/91, 31d/MA5</td>
<td>MH010338</td>
</tr>
<tr>
<td>10</td>
<td>Layer</td>
<td>21</td>
<td>1 d/MA5, 10 d MA5, 56d/4/91</td>
<td>MH010339</td>
</tr>
</tbody>
</table>
Fig 1. Maximum likelihood tree (1000 bootstrap replicates) generated by neighbor-join method, representing the IBV isolates detected in Serbia and selected IBV reference strains.
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%. Serbian strains MH010337 and MH010338, closely related to QXIVB (KC795604) and L-1148 (DQ431199), varied from 0.9 to 1.2%, and QX (KC777395) 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).

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 strain. 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 [13]. 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 infections and QX-like IB disease, which this vaccinating protocol makes useful in reducing economic losses caused by QX strains.

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Infectious Bronchitis Virus in Serbia

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