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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.

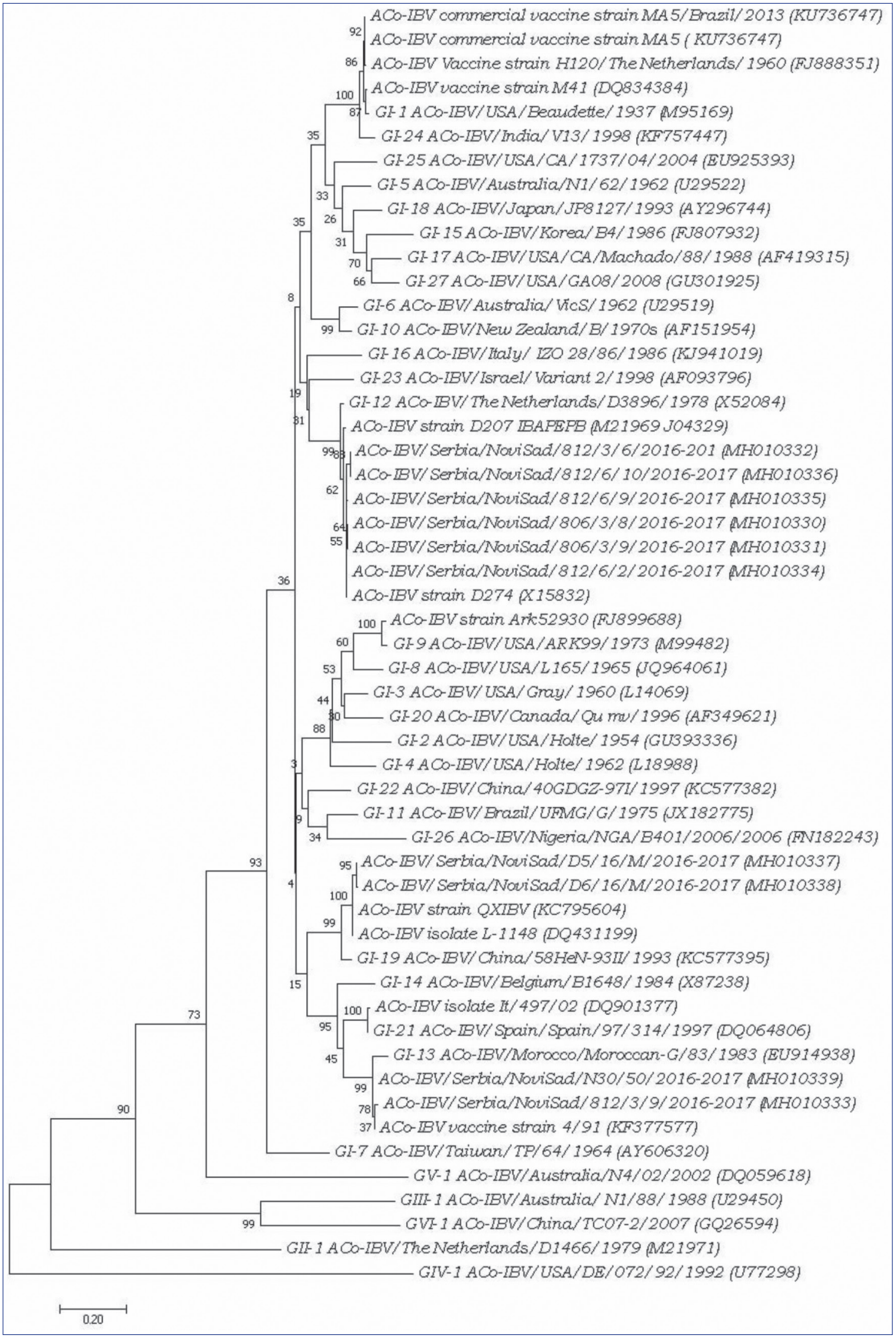

Fig 1. Maximum likelihood tree (1000 bootstop replicates) generated by neighbor-join method, representing the IBV isolates detected in Serbia and selected IBV reference strains

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