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Detection of Israel variant 2 (IS/1494/06) genotype of Infectious Bronchitis Virus in a layer chicken flock

Hasan ÖNGÖR[1,a,✉], Necati TİMURKAAN[2,b], Fethiye ÇÖVEN[3,c], Burak KARABULUT[2,d], Hatice ERÖKSÜZ[2,e], Burhan ÇETİNKAYA[1,f], Kamil Tayfun ÇARLI[4,g]

[1]Fırat University, Faculty of Veterinary Medicine, Department of Microbiology, Elazığ; [2]Fırat University, Faculty of Veterinary Medicine, Department of Pathology, Elazığ; [3]Bornova Veterinary Control Institute, İzmir; [4]Uludağ University, Faculty of Veterinary Medicine, Department of Microbiology, Bursa, Turkey.
[a]ORCID: 0000-0003-4978-9578; [b]ORCID: 0000-0001-5567-991X; [c]ORCID: 0000-0003-1928-7630;
[d]ORCID: 0000-0002-4907-6159; [e]ORCID:0000-0002-8407-5792; [f]ORCID: 0000-0001-6347-1684;
[g]ORCID: 0000-0001-6045-8644

✉Corresponding author: hongor@firat.edu.tr
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Abstract: The aim of this study was to determine an infectious bronchitis (IB) infection, caused by an Israel variant 2 (IS/1494/06)-like IBV, in a layer chicken flock regularly vaccinated with vaccines containing IBV H120 and 4/91 strains. Mild respiratory symptoms, drop in egg production and soft-shelled eggs and eventually death were observed in a layer chicken flock. Clinical samples from four diseased chickens were examined for the detection and genotyping of IBV by virus isolation, a commercial real time reverse transcription polymerase chain reaction (rRT-PCR) and nucleotide sequencing. Both Israel variant 2 (IS-Var2) and 793/B serotypes were detected from samples by rRT-PCR, but sequencing results of a 345 bp part of S1 gene revealed that our IBV isolate, HFT-IBV, was IS/1494/06 (IS-Var2)-like with the 97.7% genetic similarity. These results suggested that immunity against vaccination with a combination of different genotypes (H120 and 4/91) could not be protective for IS-Var2 IBV field infection. In addition, identification of genotypes from the clinical samples, such as swabs and organ samples by commercial rRT-PCR assays failed to find correct IBV genotype responsible for the IB infection, whereas analysis of nucleotide sequencing of S1 gene of IBV as a gold standard test undoubtedly detected causative genotype of the infection. Also, the findings indicated that there is an urgent need for consider genotype- or protectotype-match vaccination strategies in the field to prevent vaccine- and IB-dependent economic losses of the poultry sector and logically protect chickens from IBV infection.

Keywords: Genotype, Infectious Bronchitis Virus, Israel variant 2, Layer chicken, vaccination.

Yumurtacı bir tavuk sürüsünde İnfeksiyöz Bronşit Virüsünün İsrail varyant 2 (IS/1494/06) genotipinin tespiti

Özet: Bu çalışmanın amacı, IBV H120 ve 4/91 suşları içeren aşılar ile düzenli olarak aşılanan bir yumurtacı tavuk sürüsünde, İsrail varyantı 2 (IS / 1494/06) benzeri IBV'nin neden olduğu infeksiyöz bronşit (IB) enfeksiyonunu belirlemektir. Yumurtacı tavuk sürüsünde hafif solunum yolu semptomları, yumurta veriminde düşüş, kabuk bozuklukları ve bir süre sonra ölüm gözlendi. Hastalıklı tavuklardan alınan klinik örnekler virüs izolasyonu, ticari gerçek zamanlı RT-PCR (rRT-PCR) ve nükleotid dizileme yöntemleri IBV'nin belirlenmesi ve genotiplendirilmesi açısından incelendi. Örneklerin rRT-PCR ile analizi neticesinde hem İsrail varyant 2 (IS-Var2 hem de 793/B serotipleri tespit edilmesine karşın, S1 geninin 345 bp'lik kısmının dizileme sonuçları IBV izolatının, HFT-IBV, % 97,7 oranında genetik benzerlik ile IS/1494/06 (IS-Var2) benzeri olduğunu ortaya çıkardı. Bu sonuçlar farklı genotiplerin (H120 ve 4/91) kombinasyonu ile aşılamaya karşı oluşan bağışıklığın, IS-Var2 enfeksiyonu için koruyucu olamayacağını göstermektedir. Ayrıca, svab ve organ örnekleri gibi klinik örneklerden ticari rRT-PCR testi ile genotiplerin tanımlama işleminin IB enfeksiyonundan sorumlu olan genotipin bulunmasında yetersiz kaldığı, buna karşılık altın standart test olarak kabul edilen IBV'nin S1 geninin nükleotid dizileme analizinin enfeksiyona neden olan genotipi doğru olarak tespit ettiği belirlendi. Buna ilaveten, çalışmanın bulguları kanatlı sektörünün aşı ve IB'ye bağlı ekonomik kayıplarını önlemek ve dolayısıyla tavukları rasyonel bir şekilde IBV enfeksiyonundan korumak için, sahada genotip veya protektotip uyumlu aşılama stratejilerinin dikkate alınmasının gerekliliğine işaret etmektedir.

Anahtar sözcükler: Aşılama, genotip, İnfeksiyöz Bronşit Virüs, İsrail varyant 2, yumurtacı tavuk.

Introduction

Avian infectious bronchitis (IB) is a highly contagious disease and, causes respiratory tract disorders, nephritis and reduction in egg production, and egg quality of chickens. IB is caused by Avian Coronavirus Infectious Bronchitis Virus (AvCoV-IBV) found in Gamma coronavirus genus belonging to *Coronaviridae* family. Genome of IBV is a single strand of positive-sense RNA. IBVs are made up of spike (S), envelope (E), membrane (M), and nucleocapsid (N) structural proteins (10, 11). The S1 subunit of S glycoprotein located outside of the virus plays a role for the fusion between the virus envelope and the host cell membrane (13). The epitopes in this subunit are protective antigens which are responsible for the production of neutralizing antibodies. In the S1 subunit, there are some hypervariable regions (HVR) determining the genotypes and serotypes, which are identified by molecular analyses of these regions (8). Genetic variation caused by mutations in the IBV S1 gene sequence is responsible for the worldwide diversity of IBV isolates (13).

Vaccination is the most important and indispensable way to control IB, but novel S1 gene mutants of IBV continue to emerge due to point or recombination-related variations in the field. Therefore, vaccination with current vaccine strains in the market may be insufficient to produce the protective neutralizing immunity to these novel serotypes and variants. Thus, continuous monitoring of the IBV genotypes from IB cases in the field and designing vaccination strategies with genotype-matched IB strains according to the monitoring results is necessary to improve protection (4-7).

The aim of this study was to determine an IB infection, caused by an Israel variant 2 (IS/1494/06)-like IBV, in a layer chicken flock regularly vaccinated with vaccines containing IBV H120 and 4/91 strains.

Materials and Methods

Samples: In November 2019, four dead chickens from a layer flock were submitted to the Department of Pathology at Fırat University in Elazığ province located in eastern Turkey. The history of respiratory problems such as gasping, sneezing and bronchial rales in addition to production losses was noted in this flock which had the capacity of 4000 Isa Tinted breed type of chickens at the average age of 52 weeks. According to the records, all the chickens in the flock were regularly vaccinated with Mass. (H120, Ma5 and Ma41) and 4/91 (793/B) variant strains, and the mortality rate was about 5-6%. Trachea, lung, kidney and cecal tonsils from those IBV suspected chickens were tested for IBV and variants (IS-Var2, Mass, 793/B, QX and D274) in Poultry Diseases Diagnosis Laboratory at Bornova Veterinary Control Institute, İzmir.

Necropsy: All the dead chickens were subjected to post-mortem examination. At necropsy, two chickens were detected to have thin-walled and fluctuant cysts containing 4-5 ml of clear liquid, attached to isthmus serosa of the oviduct. In only one of the chickens, kidneys showed mild pale appearance. No significant gross lesions were observed in the respiratory organs. Tissue samples for histopathology were taken from sinus, larynx, trachea, lungs, air sacs, kidneys, ovary, and infundibulum, magnum and isthmus sections of the oviduct, then fixed in 10 % neutral formalin solution. After processing routine procedures, the prepared paraffin blocks were cut into 5 µm thick, stained with haematoxylin and eosin (H&E) and, were evaluated by light microscopy.

Virus isolation: Each chicken was evaluated separately for virus isolation. Pooled organs were mixed with Phosphate Buffer Saline (PBS) (Sigma-Aldrich) containing penicillin (2000 units/ml), streptomycin (2 mg/ml) and gentamicin (50 µg/ml) antibiotics and, Mycostatin (1000 units/ml). The organs were homogenized using a MagNA Lyser (Roche) according to the manufacturer's instructions, followed by centrifugation at 3000 rpm for 10 min. The supernatants were filtered through a 0.45µm filter membrane and used for virus isolation and screened by real time reverse transcription polymerase chain reaction (rRT-PCR).

0.2 ml of the supernatants were inoculated onto the chorioallantoic cavity of ten 9-11 day-old specific pathogen free (SPF) eggs and incubated at 37°C. Inoculated eggs were checked twice daily. Those that died within 24h after inoculation were discarded. Deaths between 2 and 7 days post inoculation (PI) were considered to be virus specific. The chorioallantoic fluid was harvested aseptically from embryos that died between 48 and 72h PI, providing that the fluid showed no Hemagglutination (HA) activity. Dead embryos were examined for the presence of embryo stunting, curling, urate in the mesonephros, or focal necrosis in the liver. On day 3 PI, five live embryos were also removed from the incubator and were placed at 4°C for 24h and the chorioallantoic fluid of the embryos was collected for the next passage.

RNA extraction and cDNA synthesis: The chorioallantoic fluids collected on day 4 PI were serially diluted and used in Reverse Transcription-Polymerase Chain Reaction (RT-PCR). High Pure Viral Nucleic Acid Kit (Roche) was employed to extract total RNA from 200 µL of the chorioallantoic fluids according to the manufacturer's instruction. The extracted RNA was stored at -40 °C until PCR was performed. Viral RNA was

reverse transcribed using Transcriptor First Strand cDNA Synthesis Kit (Roche) and the obtained cDNAs were stored at −20°C until use.

Real-Time Reverse Transcription Polymerase Chain Reaction (rRT-PCR): The Real-Time Reverse Transcription-Polymerase Chain Reaction (rRT-PCR) was performed on LightCycler480 (Roche, Mannheim, Germany) by using the Kylt IB-aCo Kit for the detection of Avian Coronaviruses, and the Kylt IBV-Variant O2 Kit for the detection of IBV Middle-East GI-23 lineage (Var2-like), Kylt IBV-Variant IB-aCoV, Kylt IBV-Variant Massachusetts, Kylt IBV-Variant 4/91 (793B), Kylt IBV-Variant D274, Kylt IBV-Variant QX (all were purchased from AniCon Labor, Hoeltinghausen, Germany). rRT PCR tests were performed according to the manufacturer's instruction.

Partial sequencing of S1 gene: The cDNA samples which were detected as positive for IBV by rRT-PCR were subjected to partial sequencing of S1 gene by using two pairs of primers SX1: CACCTAGAGGTTTGY TWGCATG and SX2: TCCACCTCTATAAACACCY TTAC; SX3: TAATACTGGYAATTTTTCAGATGG and SX4: AATACAGATTGCTTACAACCACC (1). The first pair of primers (SX1 and SX2) were selected for use in the initial PCR and the other pair (SX3 and SX4) for nested PCR. First round amplification was performed in a final volume of 20 µL (2 µL D.W, 13 µL Norgenbiotek 2X PCR master mix (Canada), 2 µL of SX1 and SX2 primers and 3 µL of cDNA) with a thermal profile of one step denaturation at 94 °C for 2 min, followed by 35 cycles at 94 °C for 15 sec, 58 °C for 30 sec, 72 °C for 30 sec, and a final step of synthesis at 72 °C for 10 min. Amplifications were performed in an Eppendorf master cycler gradient thermocycler (Eppendorf, Hamburg, Germany). Nested-PCR reactions (total volume: 20 uL) were performed using 1 µL of the first PCR product. The reaction mixture was the same as the abovementioned PCR with the addition of nested primers (SX3 and SX4). The amplification products were analyzed by electrophoresis in 1.5% agarose gels in Tris-Acetate-EDTA (TAE) buffer, stained with GelRed™ (Biotium, USA) and visualized under UV light.

Nucleotide sequencing, alignment analysis and phylogenetic tree: rRT-PCR products were purified and sequenced by ABI Prism BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, USA) in a forward direction using primer SX3 and in a reverse direction using primer SX4. The sequences obtained (345 bp) were compared with the IBV sequences in GenBank database and similarities were analyzed by BLAST. Multiple sequence alignments were carried out with Clustal W and phylogenetic tree was constructed with MEGA software (version 7; Biodesign Institute, Tempe, USA) using the Maximum Likelihood tree method with 1000 bootstrap.

Results

Histopathological findings: Mild epithelial degeneration and desquamation were seen in the magnum of the oviduct of the four chickens. A mild to moderate salpingitis characterized by lymphocytes, macrophages and a few heterophils was present in the propria mucosa of the magnum and isthmus. Also, degenerated ova were seen in the lumen of the magnum. Tubular degeneration of individual cells and mononuclear cell infiltration were multifocally present in the interstitium of the kidneys. In the lungs, there was moderate congestion and oedema, and mild peribronchial lymphoid hyperplasia. No significant microscopic lesion was seen in the other organs.

Egg passage: After the seven passage in SPF embryonated eggs, no specific lesions for IBV such as stunting, curling and uric acid deposition in the kidneys and ureter were observed. The chorioallantoic fluid of inoculated eggs were found to be negative for Newcastle disease virus and Avian Influenza Virus by Hemagglutination (HA) Test.

PCR and rRT-PCR: PCR analysis of the infected chorioallantoic fluid samples could not yield any positive results. rRT-PCR analyses showed positive results, with a threshold cycle value of 29.97 for Avian Coronaviruses, 32.58 for IBV Middle-East GI-23 lineage (Var 02), and 34.33 for IBV 4/91 (793/B). Although the animals were vaccinated with IB 4/91 vaccine against 793/B three times (14, 42 and 84th days), the detection of this serotype put forward that the efficacy of the vaccine against this serotype was questionable or that the field strain obtained here was different from the vaccine strain.

Partial sequencing and alignment analysis of the IBV S1 gene: In the partial sequencing of IBV S1 gene, we obtained 345 nucleotide base in length and the similarity level of our isolate with the other strains deposited in the GenBank of NIH was compared (National Institute of Health, USA). A 345 bp length nucleotide sequence of S1 gene segment of our isolate (HFT) showed great similarity (approximately 99%) with those of IBV Eg/CLEVB-2/IBV/012 (Accession No: JX173488.1), IS/1494/06 (Accession No: EU780077.2) and TR8 (Accession No: KP259312.1). A few nucleotide deletions at the position of 1050, 1088, 1089, 1098, 1099 and 1107 of S1 gene were detected in our isolate (Figure 1). These deletions observed, except in the position of 1050, are probably due to reading errors that occurred in sequencer because of low level of fluorescence signal. The other

selected sequences of IBV strains from the GenBank were significantly different from our isolate HFT IBV.

Phylogenetic analysis of S1 sequence of our HFT IBV isolate also revealed a close genetic relation with IS/1494/06 isolate (GenBank Access No: EU780077.2), Eg/CLEVB-2/IBV/012 (GenBank Access No: JX173488.1) and with our previous laboratory isolate TR8 (GenBank Accession No: KP259312.1) (Figure 2).

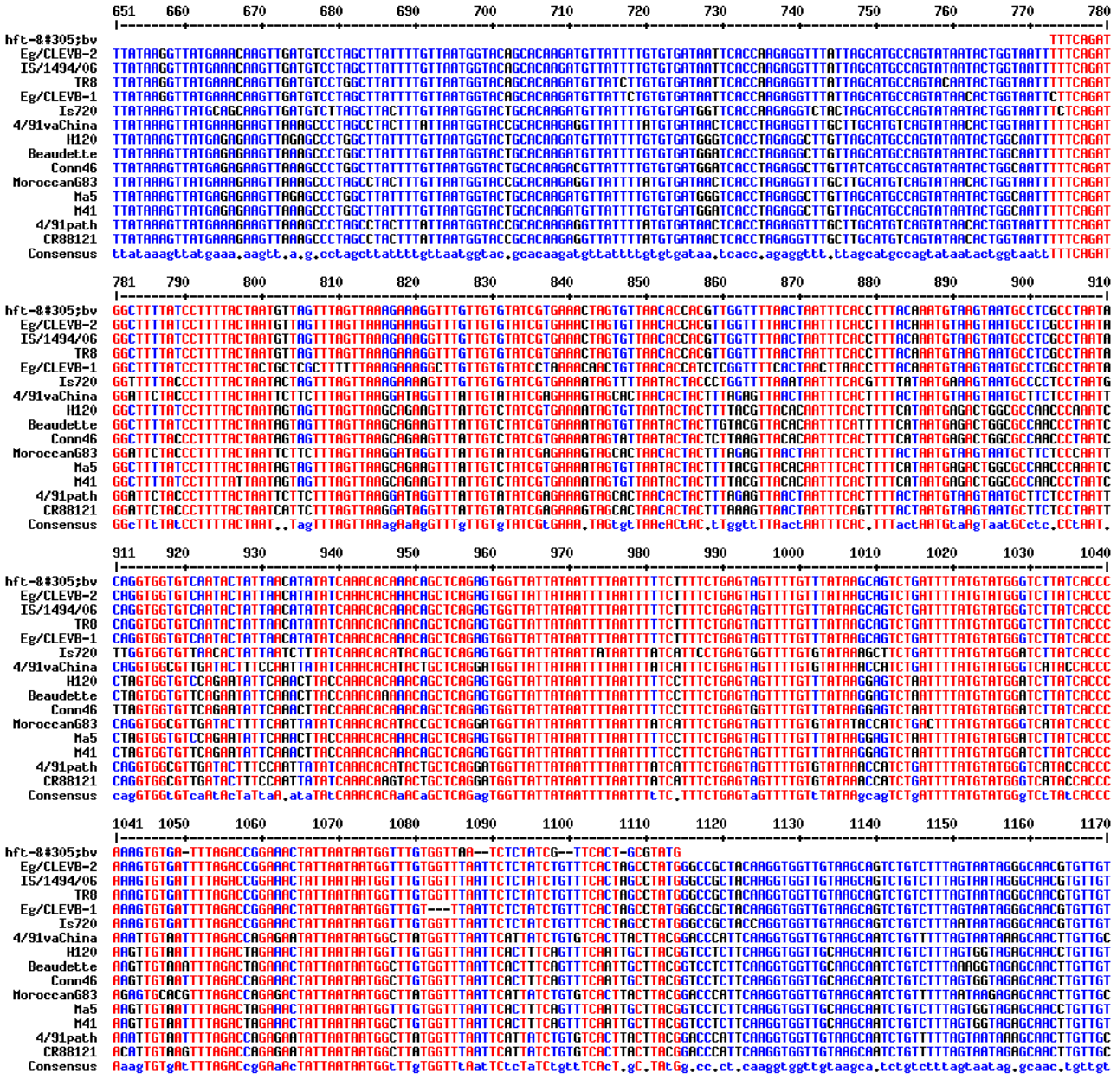

Figure 1. Alignment analysis of a selected 345 bp length S1 gene part of our HFT-IBV with some important IBV isolates from GeneBank, NIH.

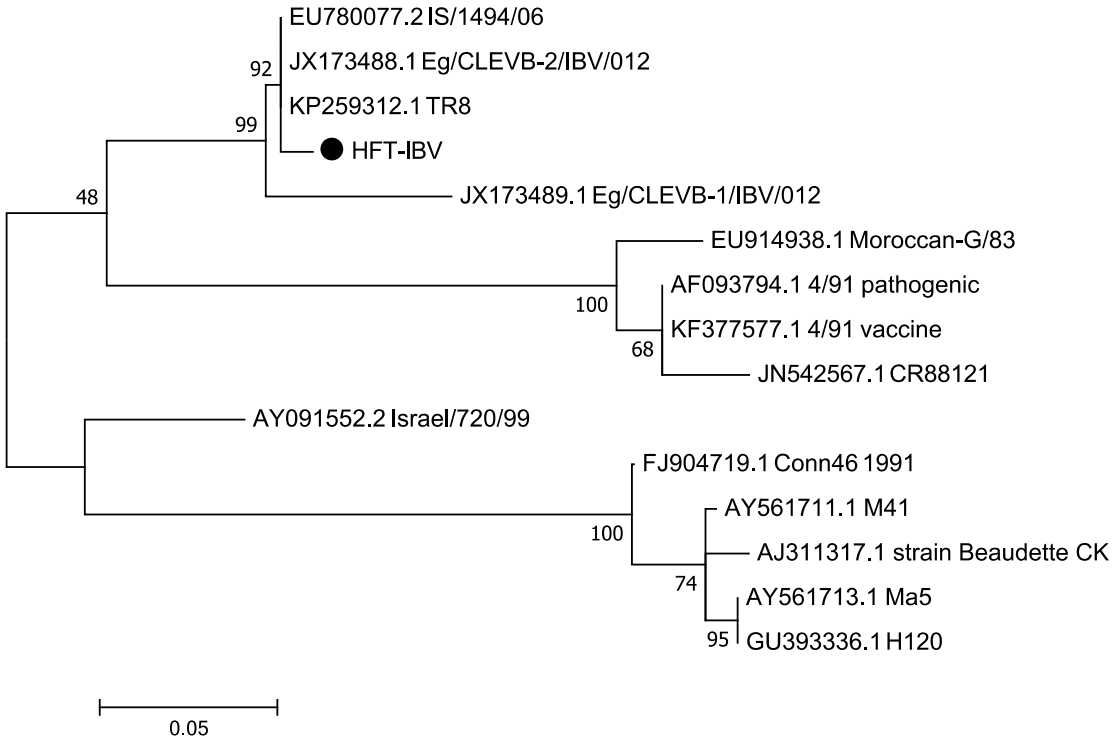

Figure 2. Phylogenetic tree showing genetic relations of S1 sequence of HFT-IBV with the other selected IBV strains from GenBank, NIH.

Discussion and Conclusion

In this study we isolated a IS/1494/06-like IBV (GI-23 lineage) named as 'HFT-IBV' from pooled organs (trachea, lung, kidney and cecal tonsils) of diseased layer chickens which had previously been vaccinated with H120 and 4/91 vaccine strains. Although information about protective antibody levels of this flock after vaccination is missing and some cross protections between H120, 4/91 and IS-Var2 IBV strains were previously reported, it is well known that protective immunity acquired from immunization with H120 and/or 4/91 vaccine strains could not yield sufficient protection against IS-Var2 IBV infections (2, 3, 12). This report can, therefore, be considered as an additional data to strengthen the knowledge that vaccinations with H120 and/or 4/91 IBV strains may not provide sufficient protection against IS/1494/06-like field virus infections. This finding was logically supported by the alignment and phylogenetic analysis of our HFT-IBV isolate. In the BLAST analysis, we found about 97.7% nucleotide sequence similarity with IS- Var2-like isolates, while this similarity with M41-related (H120, Ma5, M41) and 4/91 (793/B, Moroccan G/83 and CR88) strains was below 90% and reason for classifying the genotypes into the different genetic lineages. Because of this large difference in S1 gene, IS-Var2-like genotypes have been classified into GI-23 lineage, while M41 and 4/91-like viruses have been put into GI-1 and GI-13 lineages, respectively (13).

The findings of this study revealed that there was a huge genetic difference in the S1 gene between our HFT IBV isolate and the vaccine strains used in the layer flock examined. The genetic difference in S1 gene with above 10 % generally implied that immunity developed against IBV vaccine strains used in the layer flock was almost totally ineffective against clinical infection caused by IS/1494/06-like field strains such as our isolate HFT IBV. The clinical and pathological findings indicating the presence of IB infection within this flock supported this.

Recent studies carried out in Turkey have shown that IS-Var2 was circulating in the national chicken population and had great economic importance and losses particularly in layer flocks. The presence of this variant has been proved by Kahya et al. (9) who isolated and genotyped it in broiler and breeder chicken flocks. Yilmaz et al. (15) conducted a field study in broiler and layer flocks located in different regions of Turkey to investigate the presence of IBV and phylogenetic analysis of S1 gene, and reported that they detected IS-Var2 in addition to serotypes/variants similar to vaccine strains (Ma5, H120 and M41). The authors also noted that the IS-Var2 showed high similarity with those reported in the Middle East countries. In another study carried out by the same researchers in 2017 (14), a positivity of approximately 81% was reported in the real time RT-PCR analysis of the samples taken from broilers and layer hens, and sequencing of S1 gene and phylogenetic analyses revealed

high similarity with IS-Var2. Although 4/91-like viruses were recently detected from layer flocks at almost the same proportions with the IS-Var2 IBVs, the latter was still considered as the major cause of IB infections in broiler flocks in Turkey. In the present study, IS-Var2 was found to be responsible for IB infection in the layer chickens, and the findings indicated that it was not possible to make the genotype prediction without sequencing the isolated strain, due to the fact that nephropathogenicity and egg production problems could be caused by any of the IBV genotypes like 4/91, IS/1494/06 or M41-like viruses circulating in the country.

Detection and genotyping are crucial steps to control IB infections in chicken flocks by vaccination. This study employed virus isolation on embryonated SPF eggs and a commercial real time RT-PCR for detection of the virus, and S1 gene sequencing for genotyping of the isolate. When the sequencing results were considered, it was observed that commercial real-time RT-PCR test yielded false result by identifying our isolate as 793B (4/91) genotype. Whereas, nucleotide sequencing which is regarded as gold standard for genotyping revealed that our isolate was actually an IS-Var2-like IBV. The results of this report and our previous experience indicated that although commercial RT-PCR tests used for genotype detection can be regarded as quick and cheaper, they may fail in detecting the correct genotype in the samples.

We therefore suggest that nucleotide sequencing should be employed to correctly determine IBV isolates originated from diseased chickens in the field. Otherwise, mistyping results might lead to improper selection of vaccine strains in the field in order to protect the flocks which will consequently result in great economical losses.

Heterogeneity between vaccine strains and field strains is a significant problem in the field as observed in this report. Keeping in mind that no complete cross-protection is available among the IBV serotypes, novel vaccines containing variant strains are needed to combat this disease. We therefore recommend that continuous monitoring of IBV genotypes by virus detection and S1 gene sequencing, followed by selecting genotype or protectotype-match vaccine strains for immunizing is the most plausible way to control IB infection in chicken flocks.

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Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors declared that there is no conflict of interest.

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