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Original article

S1 gene based phylogeny of Israel variant-2 infectious bronchitis virus isolated in Turkey in a five year period

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Abstract

Infectious bronchitis (IB) is an important disease that causes severe economic loses in the poultry industry worldwide. Furthermore, the spread of new variants poses a challenge for diagnosis and control of the disease. This study investigated the situation of infectious bronchitis virus (IBV), specifically the Israel variant-2 (IS var-2) also known as GI-23 genotype, in Turkey. Between 2014 and 2019, 214 flocks vaccinated against H120 from Marmara, Western Black Sea, and Inner Anatolia were examined, with 127 (59.3%) flocks testing positive for IBV, of which 92 (72.4%) were positive for IS var-2. Of the latter samples, 60 were randomly selected and subjected to full S1 gene sequencing. The analysis indicated that the field strain in Turkey was located on the same branch as the GI-23 genotype, which is one of the most frequently observed wild-type cluster found in the Middle East. The DNA similarities between the GI-23 isolates from 2014 to 2019 were 99%. In conclusion, the IS var-2 genotype has been circulating in broiler flocks in Turkey. It is recommended that establishing the vaccine strategy it should be considered the current circulating strains for the prevention and control of the disease among poultry.

Key words: broilers, genotyping, infectious bronchitis virus, molecular epidemiology, Israel variant 2

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Introduction

Infectious bronchitis (IB) is an important and highly contagious disease that causes serious economic loses in the poultry industry worldwide (Cavanagh 2007, He et al. 2012). Since it was first identified in chickens in North Dakota, USA, IB has become a common disease, especially in countries with large commercial poultry populations (Bande et al. 2016). Infectious bronchitis virus (IBV) causes respiratory, reproductive, digestive, and renal infections in chickens (Jackwood 2012). Morbidity can reach 100% while mortality can also be very high, especially in conjunction with other secondary viral or bacterial infections (Bande et al. 2016).

IBV belongs to the *Coronoviridae* family in the *Gammacoronavirus* genus. It is a positive-sense, single-stranded, enveloped RNA virus of about 27 kb length (Lai and Cavanagh 1997). The genome has about 10 open reading frames (ORFs) (Liu et al. 2009). The major part of the genome comprises two ORFs, 1a and 1b, while the main structural proteins, spike (S), envelope (E), membrane (M), and nucleocapsid (N), are encoded in the remaining part (Mo et al. 2012). The spike protein has S1 and S2 subunits. The S1 subunit is responsible for cell attachment, serotype specificity, and virus neutralizing while the S2 subunit anchors S1 to the viral membrane (Cavanagh et al. 1986, Ignjatovic and Galli 1994, Belouzard et al. 2012). Small mutations in spike region sequences can result in the emergence of new genotypic variants that may differ from current vaccine types (Adzhar et al. 1996). The spread of new IBV variants has become a major concern for preventing and controlling IB (Jackwood 2012). More than 50 different antigenic and genetic IBV types have been identified worldwide (Sjaak et al. 2011, Jackwood 2012).

The most used method for detecting the agent is genotyping because it is fast and correlates with the serotype. In particular, S1 gene sequencing has been used to differentiate IBV variants (Bochkov et al. 2006, Ladman et al. 2006). Similarities between the nucleotide and amino acid sequences of the S1 subunit with phylogenetic analysis have been used to distinguish viral genotypes. Each IB serotype may differ by 20-25% at the genomic scale and 50% at the protein scale (Cavanagh et al. 2005). Valastro et al. (2016) used S1 whole-gene phylogeny to define 32 IBV lineages, which they then genetically classified into 6 divergent groups, or 'genotypes (GI)'. All the genotype lineages have an IBV serotype identified by a different name from different world regions. For example, the Massachusetts (also known as Mass or M41), H120, and Connecticut types fall into the GI-1 lineage, the 793B type (also known as 4/91 and CR88) into the GI-13 lineage, the Israel Variant-2 (IS var-2) into the GI-23 lineage, and D274 into the GI-12 lineage (Valastro et al. 2016).

In Turkey, both broilers and layers are vaccinated against IBV using both live and inactivated vaccines, including Ma5, 4/91, H120, IS var-2, M41, and a combination of H120-D274 strains. The predominant genotype circulating in Turkey appears to be IS var-2 (Kahya et al. 2013, Yilmaz et al. 2016). In these studies, samples were taken from both vaccinated (including with IS var-2 vaccine) and unvaccinated flocks from different regions of Turkey (Kahya et al. 2013, Yilmaz et al. 2016). However, these findings are problematic because live vaccine strains may spread to unvaccinated flocks while the persistence of both live and inactivated vaccine strains complicates IBV diagnosis since field and vaccine strains are closely related. In addition, there is no method to distinguish field and vaccine strains of IBV (Jackwood and Lee 2017).

Therefore, to better understand the current situation of IBV and establish if IS var-2 is the predominant genotype circulating in Turkey, we specifically collected samples over five years from broiler chickens with IB symptoms that were not vaccinated against the IS var-2 genotype.

Materials and Methods

Samples

Between 2014 and 2019, 2,140 tracheal swab samples (10 swabs/flock) were analyzed, taken from 214 broiler flocks showing IB clinical symptoms, such as gasping, sneezing and nephritis. The flocks were selected from the regions of Marmara, Western Black Sea, and Inner Anatolia, which house Turkey's largest poultry population. All the sampled chickens had been vaccinated against the H120 strain.

RT-qPCR assay

Total RNA was extracted from the pooled tracheal swab samples by using RINA-M14 Robotic Nucleic Acid Isolation System (Bio-Speedy, Turkey) according to the manufacturer's recommendations. To detect IBV and specific IBV genotypes (IS var-2, H120, 793B, D274), IBV RT-qPCR detection kit (Bio-Speedy, Turkey) and IBV RT-qPCR genotyping kit (Bio-Speedy, Turkey) were used, respectively.

Full S1 gene sequencing

Ten IS var-2 genotype-positive samples were selected from each year, randomly. A total 60 samples were subjected to full S1 gene sequencing. The sequence analysis was performed with BigDye Direct Cycle

Table 1. Infectious bronchitis virus genotyping results by sampling year.

Genotype	Number of positive flocks (%)						Total
	2014	2015	2016	2017	2018	2019	
IS var-2	19 (82.6)	14 (70)	7 (77.7)	21 (80.7)	17 (62.9)	14 (63.6)	92 (72.4)
H120	2 (8.6)	2 (10)	1 (11.1)	4 (15.3)	3 (11.1)	7 (31.8)	19 (14.9)
793B	2 (8.6)	4 (20)	1 (11.1)	1 (3.8)	6 (22.2)	1 (4.5)	15 (11.8)
D274	-	-	-		1 (3.7)	-	1 (0.7)
Total	23/36 (63.8)	20/43 (46)	9/29 (31)	26/40 (65)	27/34 (79.4)	22/32 (68.7)	127/214 (59.3)

Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions. Sephadex G-50 (Sigma-Aldrich) was used to purify the amplicons while the sequence analysis was performed on an Applied Biosystems 3500 Genetic Analyzer (Applied Biosystems). The sequences were analyzed using CLC Main Workbench software, Version 7. The primers for S1 gene amplification and sequencing were taken from Franzo et al. (2019) and Cavanagh et al. (1999).

The obtained nucleotide sequences were submitted to GenBank (Acc. Numbers MN685714– MN685773).

Phylogenetic analysis

Alignment of all S1 gene sequences and the reference strains obtained from the NCBI database (GenBank), and the phylogenetic trees were mapped using MEGA7 software and the Kimura 2-parameter nucleotide substitution model (Tamjura et al. 2013). To determine the genotypes, all sequences were compared with the reference sequences provided by Valastro et al. (2016) in the constructed phylogenetic tree.

The best model for evolutionary analysis of nucleotide substitution in S1 gene region was determined using MEGA7 (Kumar et al. 2016). The analysis involved 93 nucleotide sequences with reference genotypes from GenBank and field isolates from Turkey. The final dataset included 1,693 nucleotides. The models with the lowest BIC scores (Bayesian Information Criterion) were considered to best describe the substitution pattern. For each model, the AICc value (Akaike Information Criterion, corrected), Maximum Likelihood value (lnL), and the number of parameters (including branch lengths) were also calculated (Nei and Kumar 2000).

Results

IBV genotyping results

Between 2014 and 2019, 127 flocks (59.3%) tested positive for IBV from 214 broiler poultry flocks suspected of IB infection. Table 1 shows the number of IBV-positive samples and the distribution of IBV genotypes by year. The predominant genotype was IS var-2 (72.4%), followed by H120 (14.8%), 793B (11.8%), and D274 (0.7%).

Phylogenetic analysis of the full S1 gene sequences

The following three-part model was used to infer the S1 gene sequence's evolutionary history: General Time Reversible (GTR); discrete Gamma distribution (G = parameter = 0.6063); and a certain fraction of evolutionarily invariable (+I) sites (Kumar et al. 2016). The model was assessed with MEGA7 based on the maximum likelihood method while the bootstrap consensus tree was inferred from 1,000 replicates. There were 1693 nucleotide positions per sequence in the final dataset. The analysis indicated that these Turkish IS var-2 IBV field isolates from 2014-2019 are located on the same branch as the GI-23 genotype (Fig. 1). The DNA similarities between these isolates were very high (99%). The isolates from 2019 were also 97% similar to the strain TR08 (KP259312.1), first isolated in Turkey in 2011. These findings suggest that IS var-2 in Turkey mutated by up to 3% between 2014 and 2019.

Discussion

Infectious bronchitis disease is one of the most important problem for the poultry industry worldwide. Although various vaccines (W93, 28/86, H52, Ma5, H120, D274, 793B, IS1494) are used to prevent IBV infection, they are not completely effective (Liu and Kong 2004, Leghari et al. 2016). Given the extreme diversity of vaccination plans, full protection cannot be guaranteed. Therefore, it is crucial to monitor circulating variants in different countries and vaccinate against these specific variants. It would also be hugely beneficial if vaccination plans were made consistent to facilitate the understanding of IBV epidemiology.

This study reported on the IS var-2, located on the same branch as the GI-23 genotype. This was the major

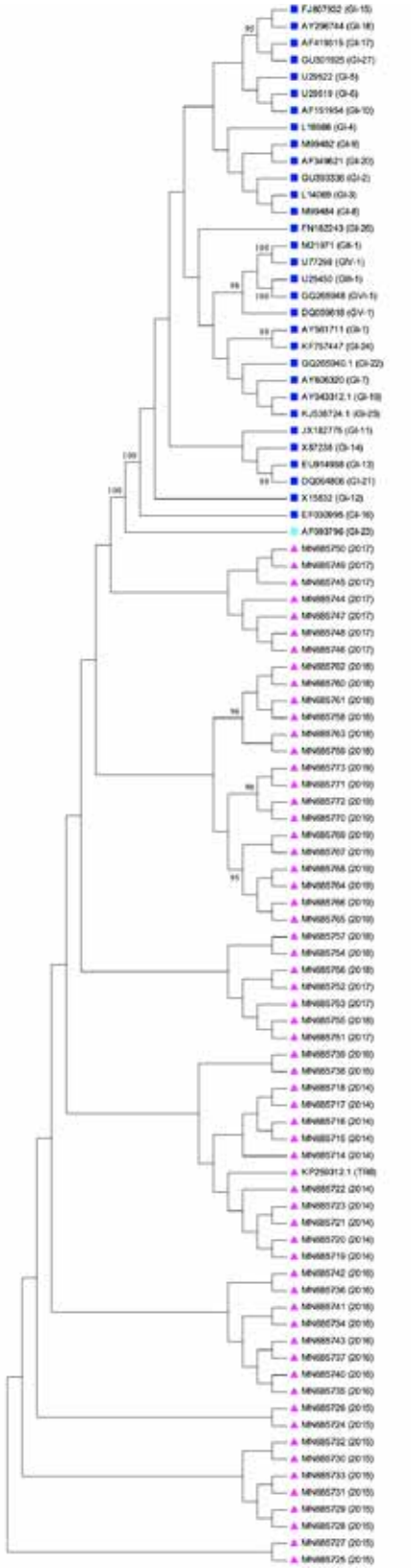

Fig. 1. Molecular phylogenetic analysis of IB viruses by maximum likelihood method.
The evolutionary history was inferred by using the maximum likelihood method based on the General Time Reversible model. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed and less then 95 values were hidden. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. Evolutionary analyses were conducted in MEGA7.

genotype detected in commercial broiler flocks in Turkey between 2014 and 2019. This confirms previous studies indicating that vaccinating against this variant (GI-23) could enable better protection from IBV infection (Houta et al. 2021).

There is no available in-vitro method to clearly distinguish field and vaccine strains (Jackwood and Lee 2017, Klimcik and Currie 2018). Yilmaz et al. (2016) showed that 48.95% of samples with IS var-2 were detected in both vaccinated and non-vaccinated flocks from January 2013 to June 2015 in Turkey. Their phylogenetic comparison of partial S1 gene sequences showed that 24 sequences obtained from the broiler flocks were related to IS var-2 isolates. Since the IS var-2 genotype was detected in flocks vaccinated against H120, IS var-2, and Ma-5, their findings could not clearly indicate the prevalence of the IS var-2 field strain. Therefore, we excluded flocks vaccinated with the IS var-2 from our study. Our results indicate that the most frequently detected IBV genotype in non-vaccinated flocks was IS var-2 (73%). In addition, our sequence analysis of 60 samples was related to IS var-2 isolates (GI-23) comparing with the reference sequence set provided by Valastro et al. (2016). This proves that the rate of IS var-2 detected in this study really belongs to the field strains.

One previous study, conducted in August 2011, showed that isolates obtained from 40–50-week-old breeders that had been regularly vaccinated with IBV H120, were identical with the IS var-2 genotype (Kahya et al. 2013). Based on comparison of 400 bp of S1 gene sequencing, they also concluded that these isolates were distantly related to other vaccine strains (H120, Ma5, and 4/91). Similarly, Ongor et al. (2021) found the IS var-2 genotype in four layer chicken flocks vaccinated with H120 and 4/91. They also sequenced 345 bp of the S1 gene to identify the genotype. However, although sequencing of the 400 bp product of the S1 gene is widely used to identify IBV, complete S1 gene sequencing is more reliable for detecting the type of genotype and the strain's location in IBV's phylogenetic tree (Valastro et al. 2016). Therefore, in this study, we included all strains from Turkey previously submitted to GenBank and performed full S1 gene sequencing.

In a study by Habibi et al. (2017) on white specific pathogen-free (SPF) leghorn chickens, it was reported that Mass (H120, day 0) + 793/B (1/96 strain, day 14) vaccines combination and challenge with IS/1494/06 (variant-2 genotype) make a broader range of protection (69.2%) against variant-2 like viruses in cilliostasis test. Sultan et al. (2019) reported that the best protection in their study was observed in groups primed with IBV var-2 (80-100% protection). They also emphasize the importance of the homology of the IBV vaccine to the predominant field strains to provide the highest protection. On the other hand, earlier studies have been conducted by Kahya et al. (2013) and Ongor et al. (2021) obtaining almost the same results as we have, in which the IS var-2 genotype was determined in flocks vaccinated against H120. Overall, our results suggest that vaccination with different genotypes may not provide sufficient protection against IS var-2 IBV infection.

In practical terms, molecular techniques are a more convenient method to identify IBV. Ongor et al. (2021) reported that commercial rRT-PCR assays (Kylt IB-aCo Kit, AniCon Labor, Hoeltinghausen, Germany) failed to correctly identify the IBV genotype responsible for infection. However, our results from the IBV RT-qPCR Genotyping Kit (Bio-Speedy, Turkey) assays and S1 gene sequencing were compatible.

Our findings indicate that the IS var-2 genotype is highly prevalent in commercial broiler flocks in Turkey. Between 2014 and 2019, this genotype was apparently circulating in 72.4% of flocks not vaccinated against this genotype.

This study has important practical implications in underlining the necessity of conducting IBV surveys in broiler flocks to vaccinate the chickens with the appropriate vaccines. This would reduce uncontrolled circulation of vaccine-derived strains, which can hinder IBV diagnosis and evolution. This study also provides updated information about the strains actually circulating in Turkey.

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