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VARIANTS OF INFECTIOUS BRONCHITIS VIRUS (IBV) IN CHICKENS IN TURKEY AND PRODUCTION OF RECOMBINANT IBV-N PROTEIN BY USING BACULOVIRUS EXPRESSION SYSTEM

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Summary

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The avian coronavirus, infectious bronchitis virus (AvCoV-IBV) is recognized as an important global pathogen because new variants are a continuous threat to the poultry industry worldwide. Hence, the European Union has started a prevention and control action (COST FA-1207). In line with this action, this project (Number: 113O411) was supported by TUBITAK. The aims of this project were detection of S1 variants of infectious bronchitis virus, phylogenetic analyses, production of recombinant N protein by cloning IBV-N genes and development of an *in-house* ELISA test for diagnostic purposes by using the recombinant N protein produced in our laboratory. For these purposes, between 2014 and 2016, internal organs or tracheal swabs were taken from 108 broiler and 26 layer flocks (4-5 samples for each farm) located in different regions in Turkey and the virus isolation was performed. AvCoV-IBV RNA was detected in 98 (91%) broiler flocks and 16 (61%) of the layer flocks by TaqMan real-time RT-PCR. A phylogenetic tree based on partial S1 sequences of the 73 detected AvCoV-IBVs in broiler flocks revealed that viruses detected in 2 (2.7%) samples were similar to the 793/B genotype, 59 (80.8%) samples to Israeli variant-2 genotype, 10 (13.6%) samples to Ma5, M41, H120 genotypes, 2 (2.7%) samples to Morocco strain genotype and 1 (1.3%) sample to D274 genotype. Phylogentic analyses of partial S1 gene of IBV detected in 8 layer fams showed that viruses detected in 1 (12.5%) sample was similar to Massachuset and 7 (87.5%) 793/B, Israeli variant-1 genotypes. Recombinant IBV-N protein was produced by cloning of N gene of Israeli variant-2 in Baculovirus system and SF9 cells. A band of 52-54 kDa was observed on Western immunobloting with recombinant N protein. Studies were conducted to develop an *in-house* ELISA test using recombinant N protein. The newly developed ELISA test was compared with the commercial IBV-ELISA test kit. Concordance was found in data obtained from both tests. These results indicate that the ELISA test developed by our laboratory can be used to detect IBV antibodies in chicken sera in the field. In conclusion, Israeli variant-2 genotypes of IBV were mostly determined in samples taken from chickens. Epidemiological studies should be performed before vaccination to determine IBV genotypes and variants to dtermine which vaccine to be used to get a better immunity to protect

chickens against circulating genotypes around the region. Also, mutations and recombinations in IBV viruses need to be monitored. ELISA developed in our laboratory can be used to detect IBV antibodies in chickens.

Key words: chickens, infectious bronchitis virus

INTRODUCTION

Infectious bronchitis (IB) has been reported in many countries including Turkey and causes economic losses. Many countries, including neighbouring countries to Turkey, have different IBV variants and cause problems in protection. The important point is that there is no good response to vaccination against mutant or recombinant variant strains. (Dewitt *et al.*, 2010; Cook *et al.*, 2012). It is useful to perform epidemiological studies prior to vaccination and to identify variants of circulating IBV strains. Sequencing and phylogenetic analyses of S1, S2 and N genes are generally used to identify strains. The emergence of nephropathogenic strains such as QX and Israeli variant-2 in recent years and their detection in our neighbors are important issue. In addition, there is no test system that can distinguish the vaccine virus and the field virus. For his purpose, sequence analyses are necessary. Identification of the genotype characteristics of the virus is important for an effective vaccine development, diagnosis, protection and control. (Cook *et al.*, 2012; Jackwood, 2012; Yilmaz *et al.*, 2016).

The presence of variant IBV strains in Turkey has been reported (Kahya *et al.*, 2013; Yilmaz *et al.*, 2016;) and it is thought that existing vaccines may be insufficient to protect. Although variant vaccines of IBV are used in our country and the presence of IBV variant strains was detected, there is no comprehensive study for S1 variant strains. An ELISA kit developed for N protein is not yet available in our country. Fast diagnostic kits are required. This study was supported by TÜBİTAK in line with The European Union's COST Project (COST FA1207). In this study, isolation of IBV, rapid diagnosis, sequence analysis of variable region in S1 gene, determination of variants in Turkey, cloning of IBV-N gene, production of N protein gene and development of *in house* ELISA test using N protein were performed.

MATERIAL AND METHODS

Samples, RNA extraction and reverse transcription

In this study, organs or tracheal swabs were taken from 108 broiler farms (27–42 days) and 26 layer farms. From each farm, 4–5 chicks showing respiratory, digestive, excretory and reproductive system disorders were sampled, located in the Marmara, Aegean, Western Black Sea, Mediterranean and Central Anatolia regions in 2014 and 2016, After RNA extraction with the RNeasy Mini Kit (Qiagen, Kat.No: 74106) from the samples, cDNA was obtained according to the method decribed by others (Yilmaz *et al.*, 2016).

TaqMan real time RT-PCR and phylogenetic analyses

The samples were examined by TaqMan real-time RT-PCR for rapid diagnosis (Callison *et al.*, 2006) and virus isolation was performed in embriyonated SPF chicken eggs from positive samples with low CT values. Sequence analyses were performed to determine the variations in

S1 gene (Worthington *et al.*, 2008), and phylogenetic analyses were performed using S1 gene sequences by the MEGA-6 programme.

Recombinant IBV-N protein production and ın house ELISA test development

Recombinant N-protein was produced in SF9 cells after the expression of N gene from the local isolate of the Israeli variant-2 strain by using Baculovirus expression system (Baulodirect, Invitrogen) as described by the manufacturer. SPF chicks were immunized with adjuvanted and non adjuvanted recombinant N protein. The reaction of vaccinated and IBV positive chicken sera collected from different poultry farms to N perotein was examined by Western immunoblotting. Studies were conducted to develop an *in house* ELISA using recombinant N protein by using conventional methods.

RESULTS

TagMan real time RT-PCR and phylogenetic analyses

Clinical signs like respiratory disorders, depression, feather deformation and growth retardation were observed in chickens. Infectious bronchitis virus was detected in 98 (91%) of the 108 broiler farms and 16 (61%) of the 26 layer farms by TaqMan real time RT-PCR. The CT values of the positive samples and positive control were in the range of in the range of 25 to 39th cycle. No CT value was obtained in negative control and negative samples.

When positive samples from the real-time RT-PCR analysed by RT-PCR for sequencing, a 393 bp product was obtained in agarose gel electrophoresis (Fig. 1). Sequence analysis of these samples was performed by a commercial company (Medsantek).

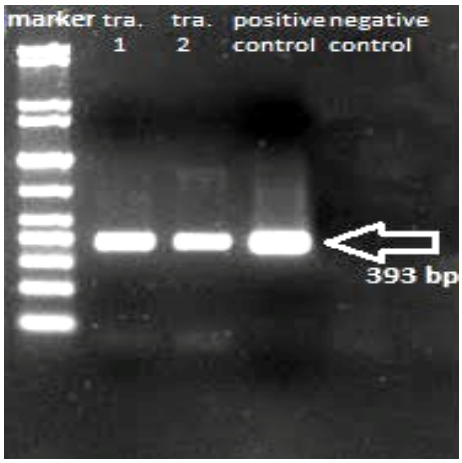

Fig. 1. Agarose gel electrophoresis of 393 bp product obtained by RT-PCR. 100bp Marker; 1 and 2: trachea samples, positive and negative controls.

According to the phylogenetic tree generated as a result of S1 gene sequence and phylogenetic analysis of 73 samples that can be detected and sequenced in broilers , 2 samples (2.7%) similar to the genotype 793/B(GI-13), 59 sapmles (80.8%) similar to Israel variant-2 genotype (GI-23), 10 samples (13.6%) similar to genotypes Ma5, M41, H120 (GI-1), 2 samples(2.7%) similar to Morocco strain genotype (GI-13) and 1 sample (1.3%) similar to genotype D274 were found. In layers, 8 samples which can be sequenced, 1 sample (12.5%) similar to Massachuset strain genotype (GI-1) and 7 samples (87.5%) similar to 793/B, Israel variant-1 (GI-13) were found (Fig. 2).

Production of recombinant IBV-N protein and development of ın house ELISA

As a result of cloning the N gene by using baculoverus expression system and pro-

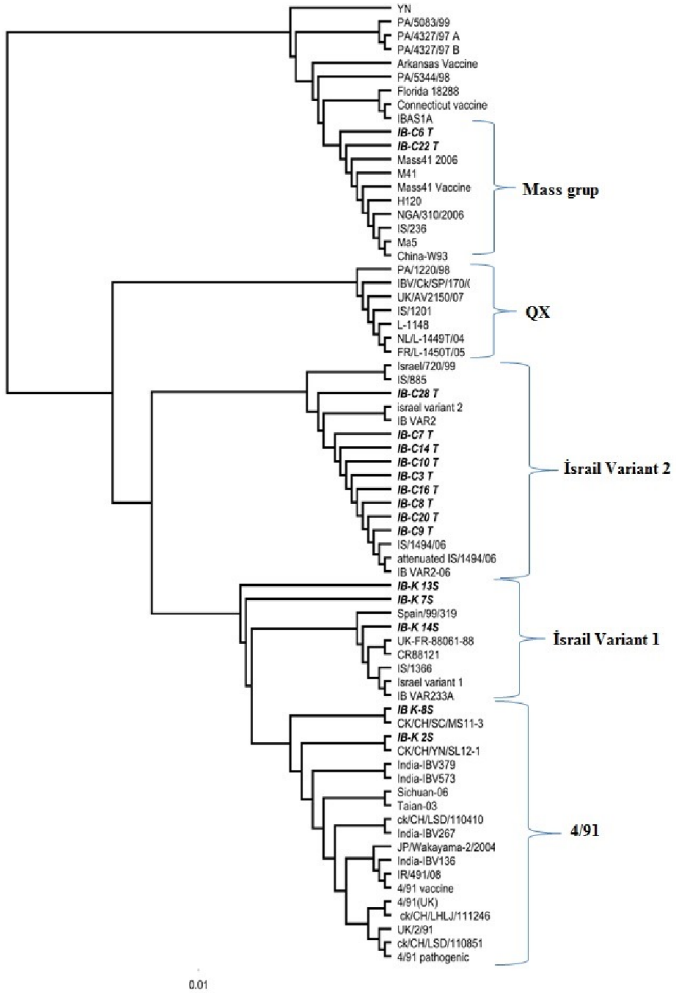

Fig. 2. Phylogenetic tree generated by using the S1 gene sequences obtained in this study and compared to other IBV S1 sequences reported to GenBank from other countries (The dark letters indicate the positives found in this study).

duction of recombinant N protein, a 52-54 kDa molecular weight protein was detected by Western immunoblotting (Fig. 3).

Western immunoblotting studies with non vaccinated but IBV-infected chicken serum from the field resulted in a single band of 52-54 kDa molecular weight (Fig. 4). The *in house* recombinant N protein ELISA was compared with the commercial ELISA kit and it was found that there was concordance between the two tests. It was shown that the in house recombinant N protein ELISA assay could be used to measure IBV antibody titres in chicken sera (Fig. 5).

DISCUSSION

Due to the detection and spread of variant IBV strains in recent years, many coun-

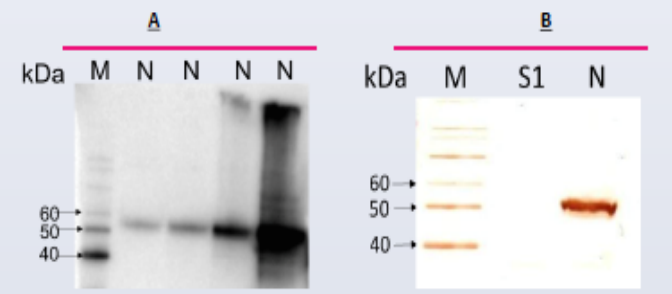

Fig. 3. Western immunoblotting to charactirise recombinant N protein. **A.** The diluted N protein bands with the monoclonal antibody developed against the IBV Massachusetts strain (B819M) N protein. **B.** A single band of recombinant N protein with the Israeli variant-2 strain and the infected chicken serum (about 52-54 kDa molecular weight of N protein) was detected. A reaction with recombinant S1 was not observed. M = marker; MagicMarker XP Western Standard (Invitrogen, Cat No. P/N LC5602).

tries have problems in IB control and diagnosis. For this reason, the European Union initiated the project "FA1207 - Control of Avian Coronaviruses: Diagnosis, Surveillance and Vaccination Strategies" in April 2013 on the basis of COST action. In line with this action, similar studies were carried out in this project (113O411), which was supported by TUBITAK, in order to solve the problems related to IBV infections in our country.

RT-PCR and real time RT-PCR are used for the diagnosis of IBV infections. (De Witt *et al.*, 2010; Jackwood, 2012; Yilmaz *et al.*, 2016). In one study, 323 samples taken from the field were examined and 176 of these samples were found to be positive by real-time RT-PCR, but IBV could be isolated from only 3 samples (Jones *et al.*, 2011). Similar findings were obtained in this project and as a result of the analysis by TaqMan real time RT-PCR, infectious bronchitis virus was detected in 98 (91%) broiler farms and 16 (61%) of 108 layer farms. As a result of the sequence analyses, most of the samples were identified as Israeli variant-2 genotype. In the USA, more strains of Masshachusets, Connecticut, Arkansas,

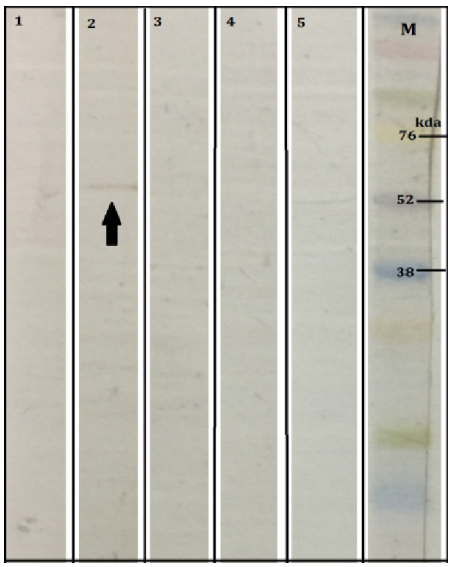

Fig. 4. Western immunoblotting with IBV-N protein. 1: Chicken serum taken on 23rd day from inactivated virus chicken group, 2: Non-vaccinated chicken serum with high OD value in ELISA; 3: Negative control; 4: Chicken serum from the 1st day of the group given the adjuvant IBV-N protein; 5: Chicken serum from the 1st day of the group given the adjuvant IBV-S1 protein; M: Rainbow marker.

DE072, GA98, GA08 were detected. Among these, it was seen that the Mass strain is more localized. In Europe, variants 793 / B, M41, QX, Q1 and IT-02 are frequently identified. However, D274, 624 / I and B1648 were found less frequently. (De Witt *et al.*, 2010; Cook *et al.*, 2012; Toffan *et al.*, 2013). In a multi-country study in Western Europe, 793 / B strains were frequently detected and phylogenetic analysis revealed that up to 50% of these strains were vaccine strains (Worthington *et al.*, 2008). Although Italy 02 and QX strains were detected less frequently, QX strains were found to increase in the Netherlands, Germany, Belgium and France, while Italy 02 strains were found to increase in Italy, Spain and the UK (Worthington *et al.*, 2008).

Geographically, Turkey is a bridge between Asia and Europe, and because has borders with both continents, it is important to control infectious diseases in Turkey. In the Middle East, disease outbreaks, which may occur as a result of animal movements and trade, especially due to the problems experienced at the border, have made the position of our country more critical. When this situation is evaluated in terms of infectious bronchitis virus, the situation of our country should be investigated in terms of the strains present in the Middle Eastern countries and the strains present in European countries. For this reason, in this project, like in the other Middle Eastern countries, Israel (Callison et al 2006; Meir *et al.*, 2004), Jordan (D274 and 4/91) (Roussan *et al.*, 2008), Iran (4/91 and QX) (Seyfi Abad Shapouri *et al.*, 2004) Iraq group I, variant 2 [IS / 1494 -like]; Group II, 793/B-like; Group III, QX-like; Group IV, DY12-2-like (Seger *et al.*, 2016) were investigated as well as the strains in European countries (4/91, 793/B, QX, Italy-02) (Worthington *et al.*, 2008) and the previously reported (Israel variant-2) (Kahya *et al.*, 2013) in our country. In this study, it is seen that more Israeli variant-2 (IS / 1494/06) strains are circulating in Turkey. There are also vaccine strains in Turkey (H120, Ma5 and 4/91). IS/1494/06 strains identified in this study were first reported in Israel (GenBank Accesion number: EU780077) and then reported in Jordan, Egypt, Turkey, Iraq and Iran (Mahmood *et al.*, 2011; Kahya *et al.*, 2013; Najafi *et al.*, 2016; Seger *et al.*,

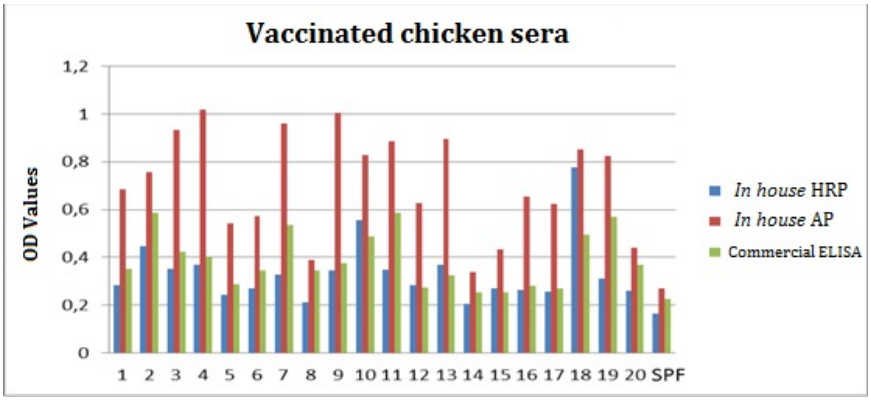

Fig. 5. OD values of *in house* ELISA developed with recombinant N protein by using vaccinated broiler sera with horse radish peroxidase (HRP) and alkaline phosphatase (AP). Blue bars: *in house* ELISA-HRP; red bars: *in house* ELISA-AP and green bars: commercial ELISA.

2016; Yilmaz *et al.*, 2016). These findings indicate that the Israeli variant-2 strain will continue to be and will be a problem in the Middle East countries.

Another aim of this project was to clone S1 gene using recombinant Baculovirus system and to produce recombinant N protein. The recombinant protein produced is around 52-54 kDa examined by Western immunoblotting. The results of our *in house* recombinant N protein ELISA were in concordance with the commercial ELISA results.

In conclusion, the Israeli variant-2 genotype was found to be more frequent than the other IBV genotypes in chickens in Turkey. Epidemiological studies are needed before vaccination to identify IBV genotypes and their variants. It is useful to use potential vaccines that can protect against circulating genotypes. Mutations and recombinations in viruses need to be monitored. The *in house* ELISA assay developed with the recombinant IBV-N protein produced in this study can be used to detect IBV antibodies in chicken.

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