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Virology Reports

journal homepage: www.elsevier.com/locate/virep

Phylogenetic analysis and full-length characterization of S1 gene of IS-1494 (Variant 2) like infectious bronchitis virus isolates, Iran, 2015



Hamzeh Nabavi^a, Vahid Karimi^b, Arash Ghalyanchilangeroudi^{c,*}, Saeed Shateri^a, Waleed Seger^{c,d}, Hamideh Najafi^c

^a Department of Avian Medicine, Faculty of Veterinary Medicine, Azad University, Babol Branch, Iran

^b Department of Avian Medicine, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

^c Department of Microbiology and Immunology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

^d Department of Pathology and poultry diseases, Faculty of Veterinary Medicine, University of Basra, Basra, Iraq

ARTICLE INFO

Article history:

Received 9 March 2016

Accepted 20 May 2016

Available online 21 May 2016

Keywords:

Avian infectious bronchitis

IS-1494

Variant 2

Iran

Phylogenetic analysis

Spike

ABSTRACT

Infectious bronchitis virus (IBV), a major pathogen of commercial poultry flocks, circulates in the form of different genotypes. Three IB viruses were isolated from broiler chickens showing respiratory and renal lesions. The isolates were characterized by reverse transcriptase polymerase chain reaction and sequence analysis of the Full-length of the S1. Three isolates were belonged to Variant 2 like (IS/1494 like) IBV genotype. Phylogenetic analysis showed that Variant 2 like isolates formed two clusters and the Iranian and Iraqi isolates were included in the cluster II. Cluster I composed of Israeli, Egyptian and Turkish Variant 2 like IBV Isolates. Three hyper variable regions (HVR) of S1 were determined. The Most variation was seen in HVR2. The findings emphasize the importance of continuous monitoring of IBV, in addition to adjust diagnostic methods, molecular epidemiological studies, development and use of vaccines which are adapted to the changing disease scenario.

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1. Introduction

Infectious bronchitis (IB), caused by infectious bronchitis virus (IBV) is one of the most economically important viral diseases of poultry and can result in respiratory disease, drops in egg production and hatchability, nephritis and enteric problems. The IBV belongs to the genus *Gammacoronavirus* within the *Coronaviridae* family. IBV positive-sense single-stranded RNA genome (27.6 kb) encodes four structural proteins: the spike glycoprotein, the membrane glycoprotein, the envelope protein, and the phosphorylated nucleocapsid protein. The spike (S) proteins are the major structure proteins of IBV proteins, which are responsible for the induction of neutralizing and serotype-specific antibodies. Diversity in S1 probably results from mutation, insertions, deletions, or RNA recombination of the S1 genes. Moreover, three hypervariable regions (HVRs) comprising amino acid residues 38 to 51, 99 to 115 and 274 to 387, respectively, have been located within the S1 subunit and have been associated with haemagglutination-inhibiting and virus-neutralizing epitopes. Numerous genotypes have been detected, some of which have quickly disappeared while others have caused major worldwide disease with economic relevance. Genotyping of IBV strains isolated in Iran showed seven distinct phylogenetic groups (Mass, 793/B like, IS/1494 like, IS/720-like, QX-like, IR-1, and IR-2) based

* Corresponding author at: Department of Microbiology, Faculty of Veterinary Medicine, University of Tehran, Azadi Ave., Tehran, Iran.

E-mail address: arashghalyanchi@gmail.com (A. Ghalyanchilangeroudi).

on analysis of mainly HVRs of the S1 gene (Najafi et al., 2014–2015; Hosseini et al., 2015). In spite of the use of different vaccines (Mass & 793/B type) in poultry farms in Iran, outbreaks have been occurred with high mortality. The presence of Variant 2 viruses (IS/1494/06 like) in Iranian commercial flocks have been demonstrated (Hosseini et al., 2015). The isolation of IS/1494/06, one of the Israeli Variant 2 isolates, and information on its S1 gene sequence in GenBank (Accession number: EU780077) has first been reported by Meir & Maher from Israel (Meir et al., 2004). The IS/1494/06 is still a major IBV variant involved in Israel chickens' farm and in Jordan, Egypt, Turkey and other countries in the Middle East (Hosseini et al., 2015; Kahya et al., 2013; Hussein et al., 2014). IS/1494/06 is known to be nephropathogenic and it also affects the respiratory system (Susan et al., 2010). The aim of this study was to determine the complete nucleotide sequences and phylogenetic analysis of spike gene of Iranian IS-1494 (Variant 2) like IBVs.

2. Materials and methods

2.1. Sample collection & virus isolations

In this study, samples were collected from Iranian broiler chicken farms in 2015. The samples (Trachea and kidney) were taken from chickens showed IB suspected clinical signs (respiratory problems such as gasping, sneezing and bronchial rales, and nephritis lesions such as enlargement, and congestion in kidneys). The samples were collected aseptically and frozen at -70°C . The details of positive samples are available in Table 1. Samples from each bird were homogenized, and a 10% (w/v) suspension was made in PBS. Subsequently, samples were centrifuged at $1500 \times g$ for 20 min at 4°C . The supernatant content was used to inoculate fertile specific pathogen-free (SPF) eggs. Homogenized tissue samples supplemented with 10,000 IU penicillin, 10,000 IU streptomycin, and 250 IU amphotericin B ml^{-1} were used for this isolation. After a period of 1 h at room temperature, 200 μL aliquots of the homogenates were inoculated into the allantoic cavity of 9–11-day-old SPF embryonated chicken eggs. Five eggs were used for each sample. The inoculated eggs were incubated at 37°C and candled daily to check for embryonic viability. After 2–3 days of incubation, the allantoic fluid was harvested and used for subsequent passages. In addition, 3 uninoculated SPF eggs considered as a control in each isolation process.

2.2. RNA extraction

RNA was extracted from samples using Cinna Pure RNA Extraction Kit (Sinaclone, Iran) according to manufacture instructions.

2.3. cDNA synthesis and PCR reaction for S1 amplification

For cDNA synthesis, 1 μL (0.2 μg) of random hexamer primer (SinaClon, Iran) was added to 5 μL of extracted RNA and the mixture was heated at 65°C for 5 min. Fourteen microliters of cDNA master mix containing 7.25 μL DEPC-treated water (SinaClon, Iran), 2 μL dNTP mix (SinaClon, Iran), 0.25 μL RiboLock RNase Inhibitor (Thermo Fisher Scientific, USA), 0.5 μL Revert Aid Reverse Transcriptase (Thermo Fisher Scientific, USA), and 4 μL $5 \times$ RT Reaction Buffer was added to each tube, resulting in a final volume of 20 μL . Then, the mixture was incubated at 25°C for 5 min, 42°C for 60 min, 95°C for 5 min, and 4°C for 1 min, respectively. RT-PCR was carried out using primer sets New oligo 5–5'TGAAACTGAACAAAAGACA 3'- and New oligo 3–5'CCATAAGTAACATAAGGRCRA 3'-targeting the S1 subunit of spike glycoprotein of IBV. The PCR condition for amplification was 94°C for 5 min, 30 cycles of 94°C for 40 s, 60°C for 40 s, and 72°C for 2 min, followed by 72°C for 10 min. The products were analyzed on 1.0% agarose gel. The PCR products were cloned into pTZ57R/T vector (InsTA clone PCR Cloning Kit, Cat No: K1213) for later sequencing.

2.4. Phylogenetic analysis

Sequencing was performed with the universal primers (both directions) (Bioneer Co., Korea). Chromatograms were evaluated with ChromasPro (ChromasPro Version 1.5). Multiple amino acid alignments were performed on S1 genes of representative viruses using Clustal W (MEGA5) and CLC sequence viewer. The phylogenetic tree was constructed by using of the MEGA 5.1 software with neighbor-joining method and each tree was produced using a consensus of 1000 bootstrap replicates (Tamura et al., 2011). The nucleotide sequences of a full length of the S1 gene were compared with several S1 sequences from gene bank. The mentioned sequences of the IBV was submitted to the GenBank database with the following accession numbers: XXXXX-YYYY.

Table 1

The sample data of Iranian IBV-IS-1494 like strains used for S1 gene characterization.

Strain name	Flock type	Year	Vaccines used in flock	Organ of isolation	Accession numbers
GKHWNF-16	Broiler	2015	H120, 4/91	Kidney	
GKHWNF-4	Broiler	2015	H120, 4/91	Trachea	
GKHWNF-5	Broiler	2015	Ma5, 4/91	Kidney	

3. Results

Phylogenetic trees were constructed based on the complete and partial nucleotide sequences of S1 gene (Figs. 1 and 2). Variant 2 IBV strains formed two different clusters, Cluster 1 composed of IS/1494/06, Eg/CLEVB-2/IBV/012, and TR8 (Fig. 1), while Iraqi and Iranian IS/1494-like viruses were included in cluster II (Fig. 2). Based on complete nucleotide sequences of S1 gene, GKHWNF-4, GKHWNF-5 and GKHWNF-16 IBVs which isolated from clinically suspected broilers between 2015 and 2016 belonged to cluster II of the Variant 2 genotype. Sequence similarities varied between 99.20 and 100% among the three isolates of this work. Comparing the complete nucleotide sequences of S1 gene of these three IBV isolates with published sequences reference strains in GenBank, three strains shared 95.12% nucleotide identity with Attenuated IBVAR2-06, 95.22% homology with both IS/1494/06, Eg/CLEVB-2/IBV/012 and 95.42% nucleotide similarity with TR8 strains. When the Iranian three field isolates and reference strains phylogenetically analyzed based on deduced amino acid sequences of the HVR, these field isolates were grouped with Variant 2 IBV strains (Figs. 1 and 2). The amino acids similarity varied between 99.19% and 100% among three strains. GKHWNF-4 and GKHWNF-5 shared amino acid sequence similarities of 92.16% with Attenuated IBVAR2-06 and 92.36% with both IS/1494/06, and Eg/CLEVB/2_/IBV_012. GKHWNF-16 had the amino acid identities of 92.57%, 92.77%, 92.77% and 92.57% with IS-1494 like IBV strains Attenuated IBVAR2-06, IS/1494/06, Eg/CLEVB/2_/IBV_012 and TR8 consecutively (Table 2). Three hyper variable regions (HVR) of S1 were determined. The Most variation was seen in HVR2 (Fig. 3). (See Table 3.)

4. Discussion

Infectious bronchitis (IB) is one of currently major diseases in poultry production. The disease has occurred frequently in vaccinated and non-vaccinated flocks and caused severe economic losses in recent years in Iran. The first isolation of IBV in Iranian flocks was reported in 1994. Currently, Ma5, H120, and attenuated 4/91 IBV-based vaccination strategies have been applied for IB control in poultry farms in Iran (Seyfi Abad Shapouri et al., 2004; Hashemzadeh et al., 2013). However, there have been frequent reports of IB-suspected cases from all over the country because of the immune failure. The reason for immune failure is the poor cross-protection between the field virus and vaccine strain and the continual emergence of new variant [10]. Variant 2 like IBV (IS/223/96), was described by Callison et al. in 2001. Later other viruses, which were highly related to Variant 2 (IS/378/97, IS/572/98, IS/585/98, IS/589/98), recovered from the respiratory or renal disease in Israel (Callison et al., 2001). The isolation of IS/1494/06 IBV, one of the Israeli Var2 isolates (Callison et al., 2001), and information on its S1 gene sequence in GenBank (EU350551) was first reported by Meir & Maharat in Israel (unpublished data, Direct submission;2006) (Najafi et al., 2015). This isolate was known as a nephropathogenic IBV strain. Nephropathogenic IBV usually damages kidneys and reproductive tract of chicken, causing a high mortality rate in young flocks.

In this study, we isolated and characterized three Variant 2 like IBV strains from chicken flocks in Iran in 2015–2016. To provide deeper insight into the comprehensive epidemiological situation and evolutionary process of IBV in Iran, we analyzed the S1 genes of all these IBV isolates with sequence information available in GenBank. Our phylogenetic analysis of S1 glycoprotein genes

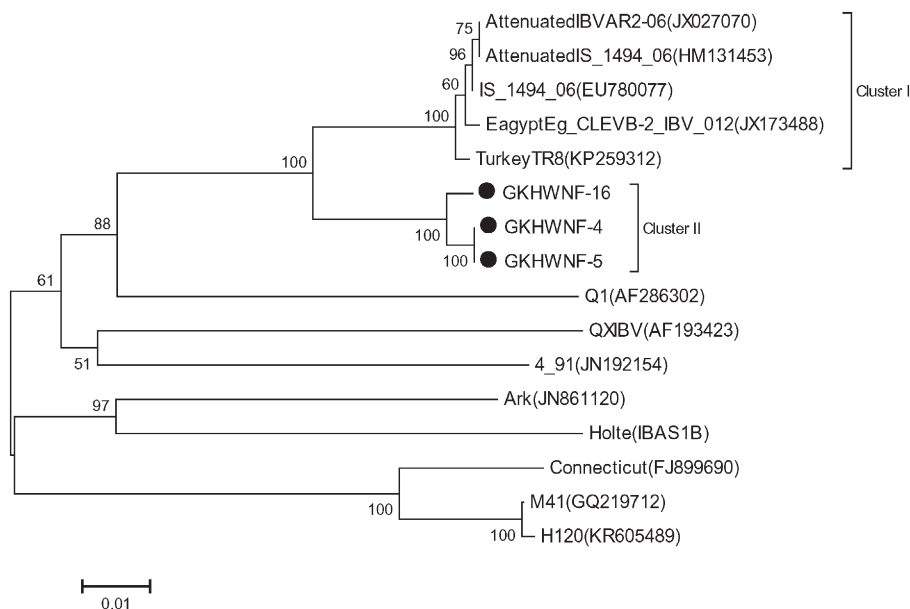


Fig. 1. Phylogenetic tree based on a full-length sequence of the S1 gene, showing the relationship between the Iranian IS-1494 like IBVs and other IBV strains. The neighbor joining method with the Kimura-2 parameter substitution model and 1000 bootstrap replicates to assign confidence level to branches constructed phylogenetic tree. Some of the viruses isolated in the current study highlighted with a black circle. The vertical lines are for spacing branches and labels. The scale bar represents the distance unit between sequence pairs. The sequences obtained from GeneBank.

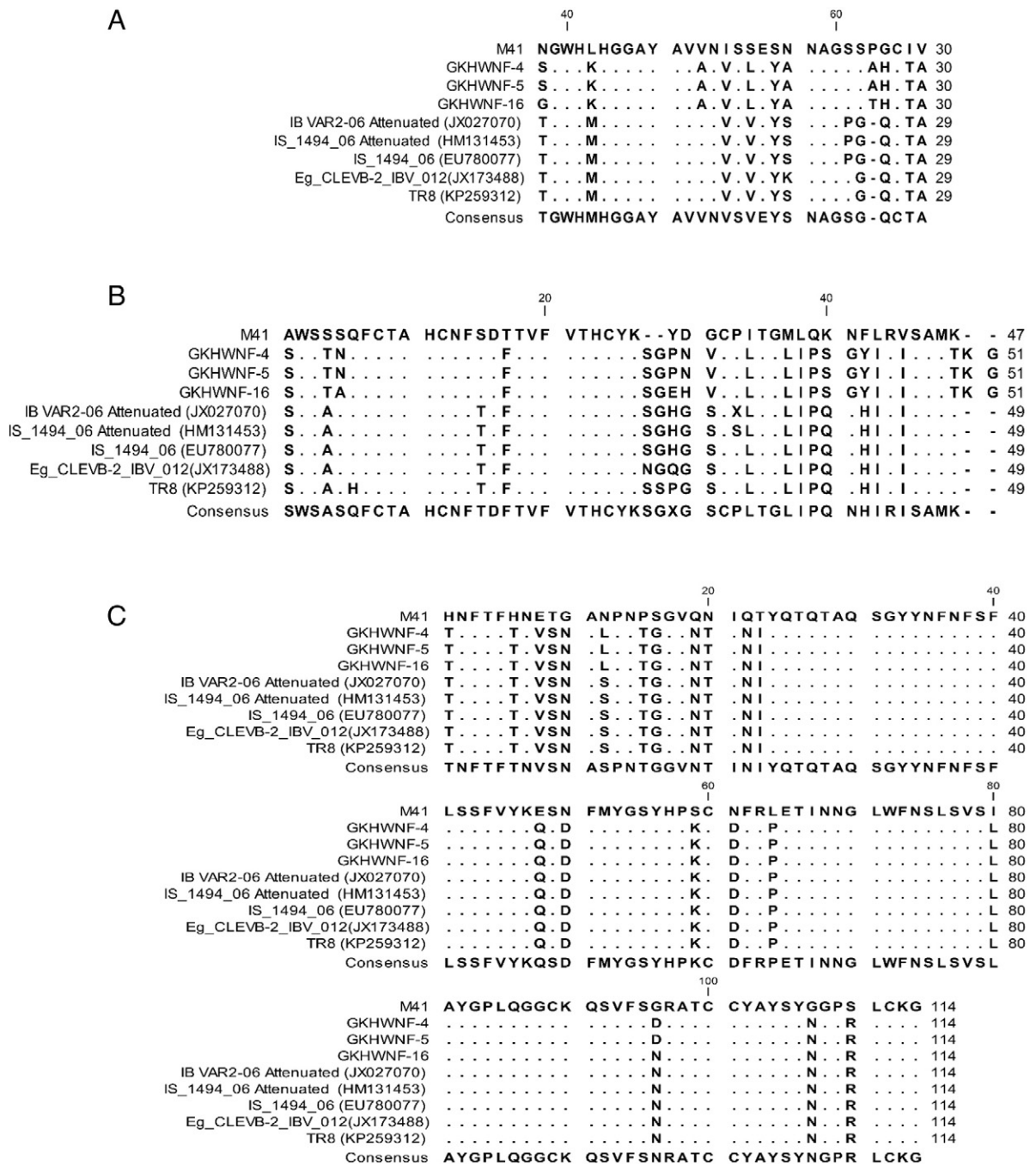


Fig. 3. A,B,C shows Hyper variable regions 1 (aa 38–67), 2 (aa 91–141), 3 (aa 274–387) of S1 protein of Infectious Bronchitis Viruses (IBV) respectively (base on M41 AA numbering system). GKHWNF-4, 5, 16 are Iranian IS-1494 like IBV isolates.

In this study, the most clinical findings in postmortem examination included serious kidney damages, accumulation of uric acid in ureters, followed with visceral gout that might cause mortality in layer birds (Hosseini et al., 2015). In the study done by Najafi et al. (2015), Variant 2 like viruses with the total prevalence of 34% were the most predominant IBV type in Iranian chicken flocks. They shared the highest identity of 99.22% with IS/1494/06, Turkey/TR8, and Eg/CLEVB-2/IBV/012.

The Variant 2 like IBV is still a major IBV Variant involved in Jordan, Egypt, Turkey, Israel and other countries in the Middle East (Hosseini et al., 2015; Kahya et al., 2013; Hussein et al., 2014; Najafi et al., 2015). Seger et al. (2016) determined genotypes of IBVs from broiler farms in Iraq during 2014–2015. Phylogenetic analysis revealed that isolates belong to four groups (Group I: Variant 2 (IS/1494-like), Group II: 793/B-like, Group III: QX-like, and Group IV: DY12-2-like). Their study has discovered that group I (Variant 2) is the dominant IBV genotype (46.87%) (in published data). In 2001, the Egypt/Beni-Suef/01 isolate was

Table 3

Aminoacide matrix showing the three Iranian IS-1494 like IBV isolates and their relation to the reference IBV strains.

	Name of strains	1	2	3	4	5	6	7	8	9	10
1	GKHWNF-4		100	99.19	92.16	92.36	92.36	82.32	79.11	79.11	77.30
2	GKHWNF-5			99.19	92.16	92.36	92.36	82.32	79.11	79.11	77.305
3	GKHWNF-16				92.57	92.77	92.77	82.73	79.31	79.51	77.51
4	AttenuatedIBVAR2-06(JX027070)					99.79	99.19	81.32	79.71	80.52	76.90
5	IS_1494_06(EU780077)						99.39	81.52	79.91	80.72	77.10
6	EgyptEg_CLEVB-2_IBV_012(JX173488)							81.32	79.51	80.32	77.10
7	Q1(AF286302)								77.30	77.51	75.90
8	QXIBV(AF193423)									80.32	78.11
9	4_91(JN192154)										80.32
10	H120(KR605489)										

detected in Egypt with 99% similarity with IS/1494/06 (Abdel-Moneim et al., 2012), and Variant 2 like IBV was detected in Jordan in 2009 (Ababneh et al., 2012). In Egypt, the isolate Eg/CLEVB-2/IBV/012 was reported in 2012, and it shared 99.22% homology with our Variant 2 like viruses. In May 2012, the virus designated IBV/chicken/Egypt/VRLCU154/2012, isolated from tracheal swabs from a vaccinated broiler flock and it was in close relationship with the IBV isolate IS/1494/06 (Hussein et al., 2014). Another study of IBVs isolated from broiler and layer chicken farms in Egypt revealed that 11 out of 13 isolates had a close relationship with Israeli variants (IS/885 and IS/1494/06), with nucleotide homology reaching up to 89.9% and 82.3%, respectively (Selim et al., 2013). In Libya, 12 IBV strains obtained from broiler flocks with respiratory disease and high mortality were differentiated by sequencing of the S1 gene. Sequences from one farm formed a cluster, with 100% relatedness to Eg/CLEVB-2/IBV/012 and IS/1494/06 (Awad et al., 2014). Kahya et al. detected eight Turkish IBVs from samples of broiler and breeder chicken flocks in 2011; all eight IBV isolates were related to that of EU780077 (IS/1494/06 IBV strain) with 99% identity. S1 sequences of TR8 Turkish isolates, found by Kahya et al., shared 99.22% identity with Var2-like viruses in our survey (Kahya et al., 2013). In Oman, Al-Shekaili et al. examined the oropharyngeal swabs taken from 243 different backyard flocks using RT-PCR in 2012; 2.56% of the samples showed the high homology to IS/1494/06. The isolate designated Oman/Ibri/4/12 (KJ206465) showed 99% relatedness to Variant 2-like viruses isolated in this survey (Al-Shekaili et al., 2015), and the latter was related to IBV/Chicken/Kurdistan-Sulaymania/12VIR10065-5 reported in 2012 in Iraq. Currently, the major control measures of IB are vaccination of live attenuated IBV vaccine (mass serotypes) such as H120, and Ma5 and 793/B strains in Iran (4/91, IB 88 & iBird); however, the chicken flocks vaccinated with the live vaccines usually fail to present full protection to field virulent IBV challenge, and the vaccinated chicken flocks were often subjected to the attack of nephropathogenic IBV. Challenge studies revealed that the H120 vaccine provides poor protection (25%) against Variant 2 (Gelb et al., 2005). Awad et al. (2015) showed H120 at day-old followed by CR88 at 14 days old, the tracheal ciliary protection conferred were 60% and 80% from IS/885/00-like and IS/1494/06-like, respectively (Awad et al., 2015). Our experiences showed H120 (1-day-old) & 793/B type (14-day-old) provide 68–74% protection against Iranian IS-1494 like IBV isolate (Unpublished data). As the results indicate, IS-1494 (Var2) IBV based vaccination strategies should be applied for the control of IB in Iran chickens. This data complete the puzzle of molecular epidemiology of IBV in Iran.

Acknowledgment

This study was conducted under grant of research council, Azad University (No. ##). The authors gratefully acknowledge Mr.Behrooz Asadi for his extensive technical supports.

References

- Najafi, H., et al., 2014–2015. Molecular characterization of infectious bronchitis viruses isolated from broiler chicken farms in Iran. *Arch. Virol.* 1–10.
- Hosseini, H., et al., 2015. Epidemiology of Avian Infectious Bronchitis Virus Genotypes in Iran (2010–2014). *Avian Dis.*
- Meir, R., et al., 2004. Identification of a novel nephropathogenic infectious bronchitis virus in Israel. *Avian Dis.* 48 (3), 635–641.
- Kahya, S., et al., 2013. Presence of IS/1494/06 genotype-related infectious bronchitis virus in breeder and broiler flocks in Turkey. *Ank. Üniv. Vet. Fak. Derg.* 60, 27–31.
- Hussein, A.H., et al., 2014. Sequence Analysis of Infectious Bronchitis Virus IS/1494 like Strain Isolated from Broiler Chicken Co-Infected with Newcastle Disease Virus in Egypt During 2012. *Int. J. Poult. Sci.* 13 (9), 530–536.
- Susan, S., El-Hady, M., Soliman, Y., 2010. Isolation and characterization of nephropathogenic strain of infectious bronchitis virus in Egypt. *J. Am. Sci.* 6, 669–675.
- Tamura, K., et al., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28 (10), 2731–2739.
- Seyfi Abad Shapouri, M., et al., 2004. A survey of the prevalence of infectious bronchitis virus type 4/91 in Iran. *Acta Vet. Hung.* 52 (2), 163–166.
- Hashemzadeh, M., et al., 2013. Phylogenetic study of Iranian infectious bronchitis virus isolates during 2010–2011 using glycoprotein S1 gene. *J. Vet. Res.* 68 (2), 135–141.
- Najafi, H., et al., 2015. Molecular characterization of infectious bronchitis viruses isolated from broiler chicken farms in Iran, 2014–2015. *Arch. Virol.* 1–10.
- Callison, S.A., Jackwood, M.W., Hilt, D.A., 2001. Molecular characterization of infectious bronchitis virus isolates foreign to the United States and comparison with United States isolates. *Avian Dis.* 45 (2), 492–499.
- Abdel-Moneim, A.S., Affi, M.A., El-Kady, M.F., 2012. Emergence of a novel genotype of avian infectious bronchitis virus in Egypt. *Arch. Virol.* 157 (12), 2453–2457.
- Ababneh, M., et al., 2012. Presence of infectious bronchitis virus strain CK/CH/LDL/971 in the Middle East. *ISRN Vet. Sci.*
- Seger, Waleed, et al., 2016. Genotyping of infectious bronchitis viruses from broiler farms in Iraq during 2014–2015. *Arch. Virol.* 161, 1229–1237.
- Selim, K., et al., 2013. Molecular characterization of infectious bronchitis viruses isolated from broiler and layer chicken farms in Egypt during 2012. *Int. J. Vet. Sci. Med.* 1 (2), 102–108.
- Awad, F., Baylis, M., Ganapathy, K., 2014. Detection of variant infectious bronchitis viruses in broiler flocks in Libya. *Int. J. Vet. Sci. Med.* 2 (1), 78–82.

- Al-Shekaili, T., Baylis, M., Ganapathy, K., 2015. Molecular detection of infectious bronchitis and avian metapneumoviruses in Oman backyard poultry. *Res. Vet. Sci.* 99, 46–52.
- Gelb Jr., J., et al., 2005. S1 gene characteristics and efficacy of vaccination against infectious bronchitis virus field isolates from the United States and Israel (1996 to 2000). *Avian Pathol.* 34 (3), 194–203.
- Awad, F., et al., 2015. Protection conferred by live infectious bronchitis vaccine viruses against variant Middle East IS/885/00-like and IS/1494/06-like isolates in commercial broiler chicks. *Vet. Rec. Open* 2 (2), e000111.