Iran J Virol 2016;10(4): 18-25
©2016, Iranian Society of Virology. All Rights Reserved.

Original Article

Full-length Characterization of S1 Gene of Iranian QX Avian Infectious Bronchitis Virus Isolates, 2015

Davam H[1] , Ghalyanchi langeroudi A[1], Hashemzadeh M[2], Karimi V[3], Zabihi T[3] , Seger W[1,4] , Najafi H[1]

1. Department of Microbiology and Immunology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.
2. Department of Research and Production of Poultry Viral Vaccine, Razi Vaccine and Serum Research Institute, Karaj, Iran .
3. Department of Avian Medicine, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.
4. Department of Pathology and poultry diseases, Faculty of Veterinary Medicine, University of Basra, Basra, Iraq.

Abstract

Background and Aims: Avian infectious bronchitis (IB) has prevalent in the most chicken farms during recent years, in spite of the IB vaccination program which has been widely performed in Iran. To better understand the molecular epidemiology of IBV in Iran, the full length sequences of S1 gene of Iranian QX IBVs were determined and phylogenetic analysis was done using some sequences of IBV.

Materials and Methods: To better understand the molecular epidemiology of IBV in Iran, the full length sequences of S1 gene of Iranian QX IBVs were determined and phylogenetic analysis was done using some sequences of IBV.

Results: Iranian QX IBVs were located in LX4 (Cluster 2). The nucleotides homologies were 99.52% - 100% between the isolates. Phylogenetic analysis revealed that all the IBV isolates were very similar and probably had a common origin. The hyperactive variable regions of S1 were determined.

Conclusions: The results from this study and other published results in the GenBank database showed that the isolates circulating in Iran in recent years were mainly LX4 (Cluster 2) genotype, which is the predominant genotype circulating in Iran in recent years (After first report of QX IBV in Iran, 2011). This finding provides important information on IBV evolution in Iran.

Keywords: Avian Infectious Bronchitis, QX, Iran, Phylogenetic analysis, Spike.

Introduction

Infectious bronchitis (IB) is one of the most contagious diseases that affect poultry worldwide and is responsible for severe economic losses. Infectious bronchitis virus (IBV) is a member of the species of avian coronavirus, genus Gamma-coronavirus (Nidovirales: Coronaviridae -Coronavirinae). IBV has a linear, single-stranded RNA genome of positive polarity of approximately 27 kb in length and produces enveloped virions [1]. The genome encodes four major structural proteins, the spike (S) glycoprotein, the membrane (M) glycoprotein, the nucleocapsid (N) protein and the envelope or small membrane (E) protein[2, 3]. IBV is not a single homogenous type but occurs in different serotypes. More than 50 different serotypes of IBV have been identified, and new variants continue to emerge [4]. To date, different genotypes of IBV have been identified,

* **Corresponding author:** Arash Ghalyanchi Langeroudi, Department of Microbiology, Faculty of Veterinary Medicine, University of Tehran, Azadi Ave , Tehran - Iran, PO.Cod:1419963111
arashghalyanchi@gmail.com , Tel: 021-61117154, Fax: 021-66933222

[Downloaded from journal.isv.org.ir on 2024-03-29]
[DOI: 10.21859/isv.10.4.18]

including Massachusetts, Beaudette, Arkansas, Connecticut, California, Australian native B and C, Holland and QX . Variant serotypes continue to be associated with outbreaks of disease in many countries [5]. S antigen, which is known as the main immunogenic antigen of IBV, has two parts called S1 and S2. The S1, which is the most ever-changing part, is responsible for viral variations and recombination. There are hundreds of IBV serotypes and the majority of them differ from each other by 20 to 25% of S1 amino acids [6, 7]. The precursor S protein is posttranslationally cleaved into S1 and S2 subunits. In the S1 subunit, three Hyper Variable Regions (HVRs) are located within amino acids 38–67, 91–141, and 274–387. Neutralizing epitopes are associated with the defined HVR, and variation in this region is thought to correlate with serotype. A recent study typed IBV based on HVR 1 sequence and demonstrated that genotyping correlated with serotyping by the VN test. The virus is characterized by a great genetic and pathogenic variability. The first isolation of IBV in Iranian chicken flocks was reported by Aghakhan et al (1994) [8] which was Massachusetts serotype. Later, several Iranian researchers identified the 793/B serotype [9]. The 793/B serotype is regarded as one of the dominant genotypes of IBVs circulating in Iran [10]. Bozorgmehri - Fard et al (2014) have demonstrated the presences of QX viruses in Iranian commercial flocks (2011). Genotyping of IBV strains isolated in Iran were classified into seven distinct phylogenetic groups (Mass, /B like, IS/1494 like , IS/720-like, QX-like, IR-1, and IR-2) based on analysis of mainly HVRs of the S1 gene [11, 12]. In spite of the use of different vaccines (Mass & 793/B type) in poultry farms in Iran, outbreaks have been detected with high mortality in farms. Since outbreaks of IBV still occur in vaccinated flocks and the virus strains isolated are frequently different from serotypes of the vaccine strains used, continuous identification of the genotype and production of new generations of vaccines are crucial [13]. The aim of this study was to sequence full-length characterization and phylogenetic analysis of spike gene of circulating Iranian QX strain during 2015.

Methods

Sample collection & Virus Isolations. Three positive QX IBV ioslates (Passages 2) based on partial S1 have been selected. Details of positive samples are available in Table 1.

RNA extraction and cDNA synthesis. RNA was extracted from tissue samples using Cinna Pure RNA Extraction Kit (Sinaclone, Iran). For cDNA synthesis, 1 µL (0.2 ug) of random hexamer primer (SinaClon, Iran) was added to 5 µL of extracted RNA and the mixture was heated at 65°C for 5 minutes. Fourteen µL 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 5X RT Reaction Buffer was added to each tube, resulting in a final volume of 20 µL . The mixture was incubated at 25°C for 5 min at 42°C for 60 min, 95°C for 5 min, and 4°C for 1 min, respectively.

PCR Reaction for S1 Amplification. RT-PCR was carried out using primer sets New oligo 5'TGAAACTGAACAAAAGACA 3' and New oligo 3 5' CCATAAGTAACATAAGGRCRA 3' targeting the spike glycoprotein of S1 subunit of IBV.. The PCR conditions for amplification were 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 (Ins TA clone PCR Cloning Kit , Cat No: K1213) for later sequencing.

Phylogenetic analysis. Sequencing was performed with the primers (both directions) used in the PCR (Bioneer Co., Korea). Chromatograms were evaluated with CromasPro (CromasPro Version 1.5). The multiple alignment of S1 nucleotide was achieved using Clustal X software. The phylogenetic tree was constructed using the MEGA 5.1 software with neighbor-joining

[Downloaded from journal.isv.org.ir on 2024-03-29]

[DOI: 10.21859/isv.10.4.18]

Table 1: The sample data of Iranian IBV – QX strains used for S1 gene charechterization.

Strain Name	Flock type	Year	Vaccines used in flock	Organ of isolation	Location
QXIRGW4	Broiler	2014	H120 , 4/91	Trachea	Qazvin
QXIRGW7	Broiler	2015	H120 , 4/91	Kidney	Azarbayjan
QXIRGW8	Layer	2015	Ma5 , 4/91	Kidney	Sistan-o-Balochestan

method and each tree was produced using a consensus of 1000 bootstrap replicates [14] .The nucleotide sequences of a full length of the S1 gene was compared with several S1 sequences from gene bank.

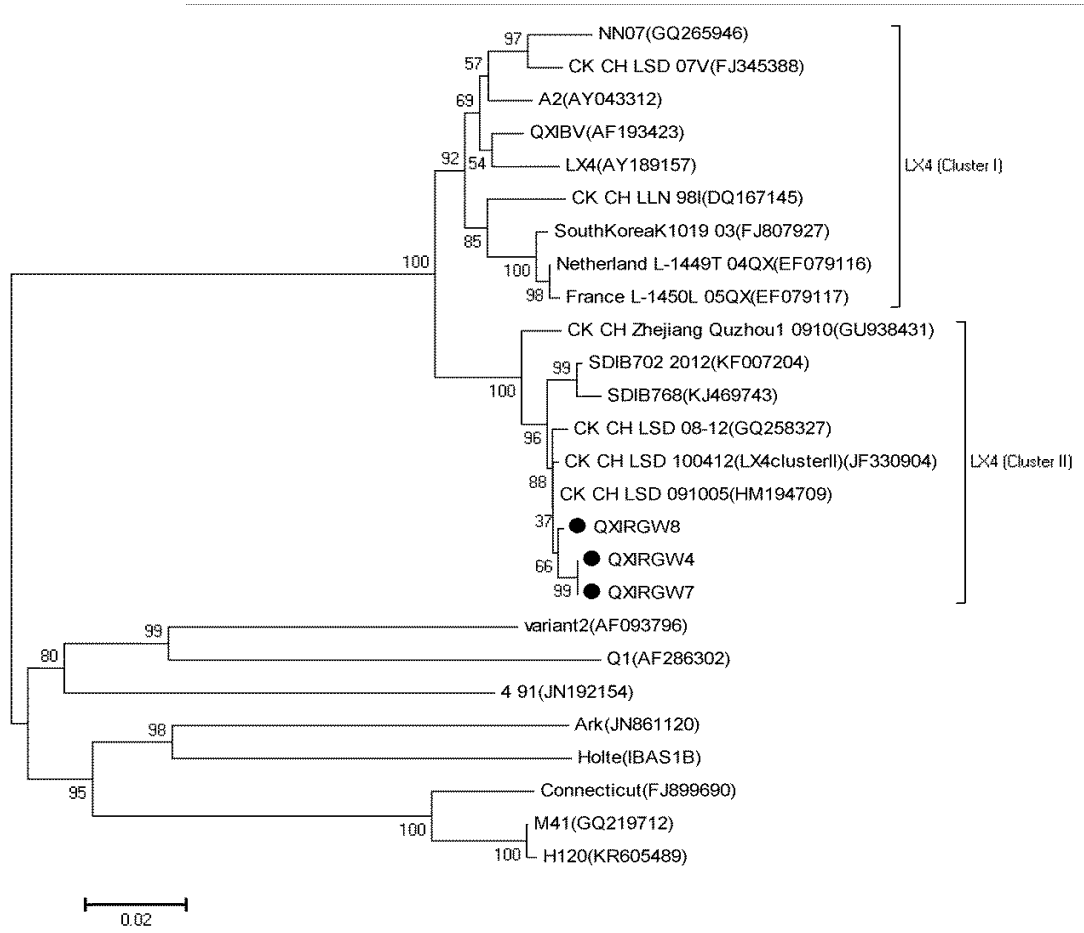


Fig. 1. Phylogenetic tree based on a full-length sequence of the S1 gene, showing the relationship between the Iranian and other IBV strains. The neighbor joining method with the Kimura-2 parameter substitution model and1000 bootstrap replicates to assign confidence level to branches constructed phylogenetic 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 Gene Bank.

[Downloaded from journal.isv.org.ir on 2024-03-29]

[DOI: 10.21859/isv.10.4.18]

Table 2: Nucleotide similarity matrix showing the three Iranian QXIBV isolates and their relation to the reference IBV strains.

No	Names of strains	1	2	3	4	5	6	7	8	9	10	11	12
1	**QXIRGW4**		100	99.52	99.52	98.36	95.08	95.47	94.59	94.79	95.37	78.59	79.30
2	**QXIRGW7**			99.52	99.52	98.36	95.08	95.47	94.59	94.79	95.37	78.59	79.30
3	**QXIRGW8**				99.81	98.65	95.37	95.76	94.78	95.08	95.56	78.78	79.40
4	**CK/CH/LSD 091005(HM194709)**					98.84	95.56	95.95	94.98	95.27	95.76	78.98	79.59
5	**SDIB768 (KJ469743)**						94.60	95.18	94.30	94.31	94.99	78.50	79.21
6	**South Korea(K101903)**							97.30	96.43	99.32	96.53	78.98	79.40
7	**QXIB (AF193423)**								98.07	96.82	97.97	79.65	79.98
8	**LX4 (AY189157)**									96.23	97.78	79.90	80.23
9	**France L-1450L05 (EF079117)**										96.43	78.88	79.50
10	**A2(AY043312)**											79.36	79.79
11	**M41(GQ219712)**												80.08
12	**4/91(JN192154)**												

Results

The S1 gene with full-length sequence was obtained for the three IBV isolates. MEGA5 programs were used to determine the sequence homology of the S1 gene from the three Iranian QXIBV isolates (QXIRGW4, QXIRGW7 and QXIRGW8). Phylogenetic analysis, based on the nucleotide sequences of S1 full length gene of the three QXIBV isolates and other published results in the Gene Bank database (Also. other Iranian QX IBV data; Partial S1), showed that the Iranian QXIBV isolates were located in LX4 (Cluster 2) genotype as shown in figure 1. The nucleotide homology of these isolates was 99.52% -100%. Whereas the nucleotide homology between QX IBV isolates and CK/CH/LSD (091005) , SDIB768 (KJ469743) ,South Korea (K101903), QXIB (AF193423) were about 99.52% -99.52% , 98.36%-98.84% , 94.60%- 95.37% , and 95.18% - 95.95% , respectively (As explained in table 1).The three hypervarileble regions (HVRs) of spike gene protein were determined (Figure2). The most and least changes among HVRs of QX like IBV strains were seen in HVR-1 & HVR-2. Amino acids 38 (T) and 341 (N) were specific for Iranian QX IBVs isolates in HVRs.

Discussion

Avian infectious bronchitis (IB) is one of the most common poultry disease in Iran which is difficult to control and causes outbreaks in commercial chicken farms. One of the major problems of IBV is the frequent emergence of new variants. Different serotypes have been reported worldwide and new variant serotypes continue to be recognized [15]. In September 1997, an outbreak of the disease, characterized mainly by swelling of the stomach

[Downloaded from journal.isv.org.ir on 2024-03-29]

[DOI: 10.21859/isv.10.4.18]

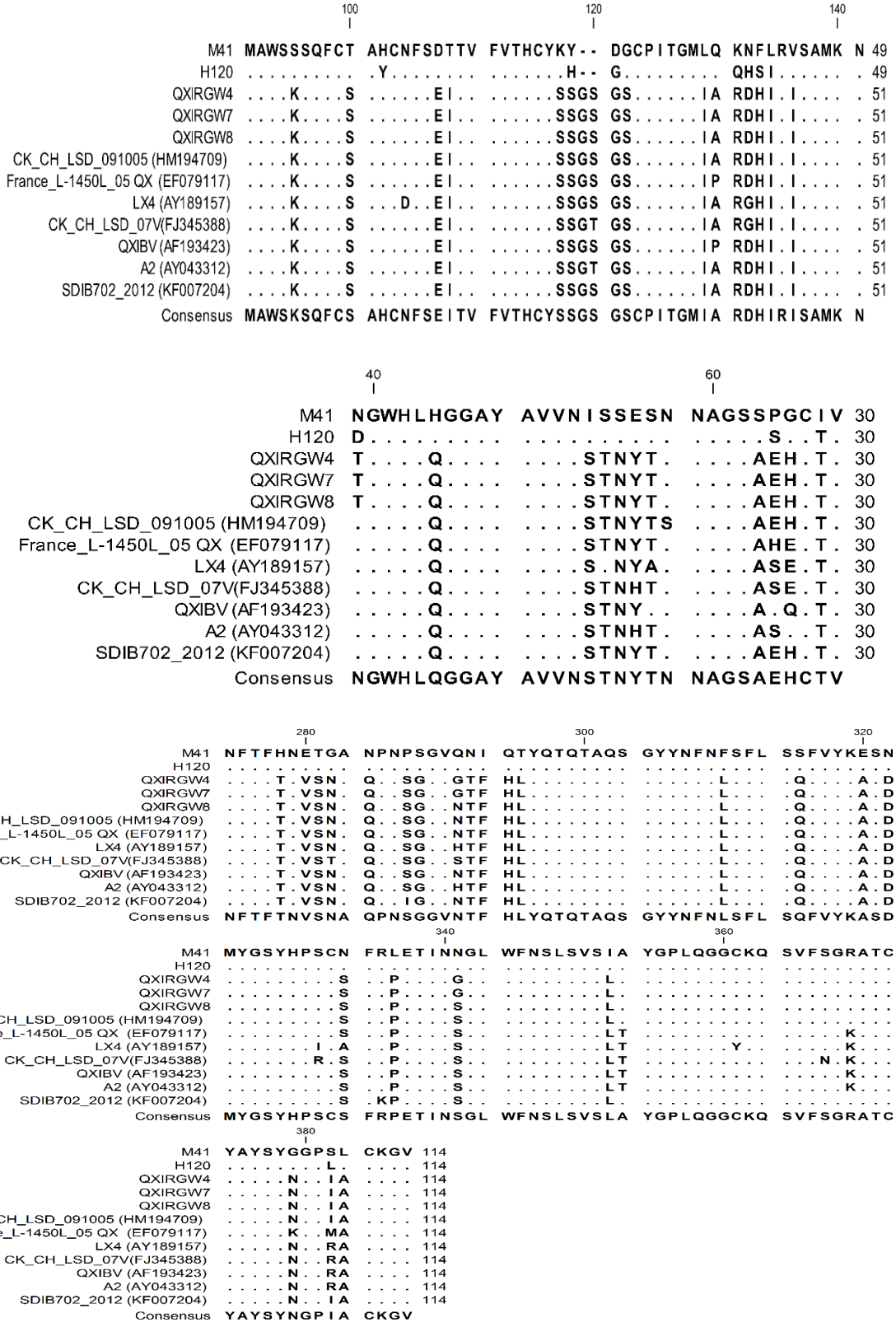


Fig. 5. A,B,C shows Hyper variable regions 1 (aa 38-67) ,2(aa 91-141),3 (aa 274–387) of S1 protein of Infectious Bronchitis Viruses (IBV) respectively (Base on M41 AA numbering system). QXIBV 4, 7, 8 are Iranian QX IBV isolates.

[Downloaded from journal.isv.org.ir on 2024-03-29]

[DOI: 10.21859/isv.10.4.18]

(proventriculitis), diarrhea and loss of body weight in 25- to 70-day-old chickens occurred in chicken flocks in Qingdao, China [16]. However, it was not recognized as a novel type of IBV at that time. In Europe, the first report of the presence of a similar nephropathogenic IBV was found in the Netherlands between 2003 and 2004 [17]. In Iran, Bozorgmehri-Fard et al. detected Iran/QX/H179/11 strain in 2011 and Iran/QX/H255/12, Iran/QX/H281/12, and Iran/QX/H284/12 strains in 2012. QX-type isolates of this study had more than 96% homology to these Iranian strains [18]. Hosseini et al (2015) described QX-type IBVs as 6th genotype in Iranian poultry farms (6.5%) during 2010-2014 [12]. Najafi et al (2015) reported QX-type IBVs were the third most predominant genotype in circulation in Iranian chicken flocks,2014-2015.In their survey , the IBV QX-type isolates shared more than 96% nucleotide similarity (Partial S1 sequence) with original QX (AF193423), and UTIVO-103 and UTIVO-105 had 99% sequence similarity with Iran/QX/H255/12 (KP310038). UTIVO-14 had 96% sequence similarity with Iran/QX/H179/11 (KP310037) [19]. In 2009, based on genetic characterization of Iraqi isolates, a putative novel IB variant not related to the QX-like strain was revealed. The phylogenetic analysis showed that the Kurdistani-Iraqivirus was closely related (98.9%) to QX-strains collected in China between 2009 and 2010. Lower nucleotide similarity (95.4 %) was revealed with the unique QX-type virus sequence from the Middle East deposited in Gen Bank and isolatedin Israel in 2004 (IS/1201 isolate, accession number DQ400359) [20]. In early 2011, five IBV isolates obtained from respiratory disease outbreaks in Iraq, Jordan, and Saudi Arabia were characterized .The isolates were found to be IBV strain CK/CH/LDL/97I [21]. QX-type IBVs isolated in this study shared more than 96% nucleotide similarity with original QX (AF193423). A retrospective analysis on IBV strains isolated in Russia showed some isolates genetically related QX-type in 2001 [17].

Worthington et al. stated that QX-like IBV was the dominant genotype in Germany (2003), Holland, and Belgium [22]. Since then, the prevalence of the so-called QX-like strains has been reported in Italy (2005), Slovenia (1990-2005), Poland (2006), UK (2008), Hungary (2008) and Swiss [23-28]. Due to their similarity to QX, these IBV viruses were termed European QX-like viruses, and clinical signs associated with them include bad egg quality, false layers among mature hens, and poor growth of broilers. According to the results of Abro et al (2012), sequence analysis based on the S1 gene revealed that the European QX-like genotype of IBV is currently predominating in Europe and has been circulating in the continent for over a decade [29]. QX IBV was detected in the southern part of the African continent (Zimbabwe) in 2011 [20]. From 1999 to 2004, Liu et al (2004) investigated five chicken flocks in four provinces in China and found that the QX-like variant (taking the LX4 strain as representative) is a novel genotype of nephropathogenic [30]. Sun et al (2011) did classified LX4 IB like viruses to two clusters (I, II) as first time. Seventy-eight isolates of IBV were obtained from different field outbreaks in China in 2009 and genotyped with 34 reference strains. Four genotypes of IBV and three new isolates were identified by phylogenetic analysis and BLAST searches of the entire S1 gene. Genotype I included 57 out of the 78 isolates that were grouped with reference strains LX4 and QX. The strains in this group included field viruses isolated between 1997 and 2009. Phylogenetic analysis comparing the complete S1 gene sequences of the 62 isolates and reference viruses published previously in the GenBank database revealed that the isolates in this group could be further grouped into two separated genetic clusters, identified as cluster I and cluster II, represented by LX4 and QX, respectively. The minimum amino acid divergence observed between isolates of the two clusters was 96.5%. Interestingly, two of the three South Korean QX-like viruses, three QX-like strains from The Netherlands and two from France fell into the same group as QXIBV by S1 gene phylogenetic analysis [31]. Furthermore, the viruses isolated in 2009 clustered in the QX

[Downloaded from journal.isv.org.ir on 2024-03-29]

[DOI: 10.21859/isv.10.4.18]

cluster closely with strains isolated before 2003. In study of Ma et al (2012) on genetic diversity of Chinese IBVs, they reported complement the previous finding that the LX4 genotype can be grouped into two clusters [32] . Based on S1 phylogenetic analysis or comparison of the partial S1 genes for amino acid similarity, Chinese QX-like IBVs had a close relationship with strains from the Netherlands, France, Slovakia, Greece and Hungary. However, by the partialS1 gene analysis, Russian QX-like IBVs showed high divergence from Chinese QX-like strains. In addition, QX IBV was reported in south east of Asia. Since 1995 in Japan, four different genetic groups have beenidentified: Japan (JP)-I, JP-II, JP-III (LX4), and JP-IV. The JP-III group falls into the China LX4 group (QX IBV)[33, 34].In Korea , the IBV isolates are divided to three genetic groups : Korea (K)-I, K-II (LX4-type), and K-III (LDL-type) .QX group located to KII group [35].

Because of the irreversible effects of the QX strain on reproduction, further studies should be performed on the pullet and layer flocks in Iran. Therefore, in the case of high prevalence and false layer issues, a suitable vaccine and vaccination program are recommended, particularly in the layer and breeder pullet farms. Therefore, further detailed investigation of the genome is needed to clarify the evolution of Iranian IBV isolates. In addition, to safeguard against the sudden emergence of new predominant strains, it is important to keep continuous monitoring of the IBV strains circulating in the field.

Acknowledgment

This study was conducted under grant of research council, University of Tehran .The authors gratefully acknowledge Mr.Behrooz Asadi for his extensive technical supports.

Conflict of Interest

NO conflict of Interest

References

1. Fellahi, S., et al., Phylogenetic analysis of avian infectious bronchitis virus S1 glycoprotein regions reveals emergence of a new genotype in Moroccan broiler chicken flocks. Virology journal, 2015. 12(1): p. 1.
2. Jackwood, M.W., Review of infectious bronchitis virus around the world. Avian Dis, 2012. 56(4): p. 634-41.
3. Sjaak de Wit, J.J., J.K. Cook, and H.M. van der Heijden, Infectious bronchitis virus variants: a review of the history, current situation and control measures. Avian Pathol, 2011. 40(3): p. 223-35.
4. Bochkov, Y.A., et al., Molecular epizootiology of avian infectious bronchitis in Russia. Avian Pathology, 2006. 35(5): p. 379-393.
5. Kumar, K.S., et al., Genotypic characterization of infectious bronchitis viruses from India. Indian journal of biotechnology, 2007. 6(1): p. 41.
6. Cavanagh, D., M.M. Elus, and J.K. Cook, Relationship between sequence variation in the S1 spike protein of infectious bronchitis virus and the extent of cross-protection in vivo. Avian Pathol, 1997. 26(1): p. 63-74.
7. Jackwood, M.W., et al., Spike glycoprotein cleavage recognition site analysis of infectious bronchitis virus. Avian Dis, 2001. 45(2): p. 366-72.
8. Aghakhan, S., et al., Studies on avian viral infections in Iran. Archives de l'Institut Razi, 1994(44/45): p. 1-10.
9. Akbari Azad, G., M. Vasfi Marandi, and H. Keyvani Aminae, Molecular analysis of three Iranian isolates belonged to 793/B serotype of Infectious bronchitis viruses. J. Vet. Res, 2007. 62: p. 69-80.
10.Seyfi Abad Shapouri, M., et al., A survey of the prevalence of infectious bronchitis virus type 4/91 in Iran. Acta Veterinaria Hungarica, 2004. 52(2): p. 163-166.
11.Najafi, H., et al., Molecular characterization of infectious bronchitis viruses isolated from broiler chicken farms in Iran, 2014-2015. Archives of Virology: p. 1-10.
12.Hosseini, H., et al., Epidemiology of Avian Infectious Bronchitis Virus Genotypes in Iran (2010-2014). Avian Diseases, 2015.
13.Mahmood, Z.H., R.R. Sleman, and A.U. Uthman, Isolation and molecular characterization of Sul/01/09 avian infectious bronchitis virus, indicates the emergence of a new genotype in the Middle East. Veterinary microbiology, 2011. 150(1): p. 21-27.

[Downloaded from journal.isv.org.ir on 2024-03-29]

[DOI: 10.21859/isv.10.4.18]

14.Tamura, K., et al., MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol, 2011. 28(10): p. 2731-9.
15.Najafi, H., et al., Molecular characterization of infectious bronchitis viruses isolated from broiler chicken farms in Iran, 2014-2015. Archives of Virology, 2015: p. 1-10.
16.Yudong, W., et al., Isolation and identification of glandular stomach type IBV (QX IBV) in chickens. Chinese Journal of Animal Quarantine, 1998. 15(1): p. 1-3.
17.Terregino, C., et al., Pathogenicity of a QX strain of infectious bronchitis virus in specific pathogen free and commercial broiler chickens, and evaluation of protection induced by a vaccination programme based on the Ma5 and 4/91 serotypes. Avian Pathology, 2008. 37(5): p. 487-493.
18.Bozorgmehri-Fard, M., S. Charkhkar, and H. Hosseini, Detection of the Chinese Genotype of Infectious Bronchitis Virus (QX-type) in Iran. Iranian Journal of virology, 2014. 7(1&2): p. 21-24.
19.Najafi, H., et al. Molecular and clinical study on prevalence of feline herpesvirus type 1 and calicivirus in correlation with feline leukemia and immunodeficiency viruses. in Veterinary Research Forum. 2014. Faculty of Veterinary Medicine, Urmia University, Urmia, Iran.
20.Amin, O.G., et al., Circulation of QX-like infectious bronchitis virus in the Middle East. Vet Rec, 2012. 171(21): p. 530.
21.Ababneh, M., et al., Presence of Infectious Bronchitis Virus Strain CK/CH/LDL/97I in the Middle East. ISRN veterinary science, 2012. 2012.
22.Worthington, K.J., R. Currie, and R.C. Jones, A reverse transcriptase-polymerase chain reaction survey of infectious bronchitis virus genotypes in Western Europe from 2002 to 2006. Avian Pathology, 2008. 37(3): p. 247-257.
23.Benyeda, Z., et al., Comparison of the pathogenicity of QX-like, M41 and 793/B infectious bronchitis strains from different pathological conditions. Avian Pathology, 2009. 38(6): p. 449-456.
24.Valastro, V., et al., QX-type infectious bronchitis virus in commercial flocks in the UK. Vet Rec, 2010. 167(22): p. 865-6.
25.Sigrist, B., et al., Detection of Avian coronavirus infectious bronchitis virus type QX infection in Switzerland. J Vet Diagn Invest, 2012. 24(6): p. 1180-3.
26.Krapež, U., et al., Molecular analysis of infectious bronchitis viruses isolated in Slovenia between 1990 and 2005: a retrospective study. Virus genes, 2010. 41(3): p. 414-416.
27.Terregino, C., et al., Pathogenicity of a QX strain of infectious bronchitis virus in specific pathogen free and commercial broiler chickens, and evaluation of protection induced by a vaccination programme based on the Ma5 and 4/91 serotypes. Avian Pathol, 2008. 37(5): p. 487-93.
28.Beato, M., et al., Evidence of circulation of a Chinese strain of infectious bronchitis virus (QXIBV) in Italy. Veterinary record, 2005. 156(22): p. 720-720.
29.Abro, S.H., et al., Characterization and analysis of the full-length genome of a strain of the European QX-like genotype of infectious bronchitis virus. Archives of virology, 2012. 157(6): p. 1211-1215.
30.Liu, S. and X. Kong, A new genotype of nephropathogenic infectious bronchitis virus circulating in vaccinated and non-vaccinated flocks in China. Avian Pathology, 2004. 33(3): p. 321-327.
31.Sun, C., et al., Phylogenetic analysis of infectious bronchitis coronaviruses newly isolated in China, and pathogenicity and evaluation of protection induced by Massachusetts serotype H120 vaccine against QX-like strains. Avian Pathol, 2011. 40(1): p. 43-54.
32.Sun, C., et al., Phylogenetic analysis of infectious bronchitis coronaviruses newly isolated in China, and pathogenicity and evaluation of protection induced by Massachusetts serotype H120 vaccine against QX-like strains. Avian Pathology, 2011. 40(1): p. 43-54.
33.Mase, M., et al., A novel genotype of avian infectious bronchitis virus isolated in Japan in 2009. Journal of Veterinary Medical Science, 2010. 72(10): p. 1265-1268.
34.Mase, M., et al., Phylogenetic analysis of avian infectious bronchitis virus strains isolated in Japan. Archives of virology, 2004. 149(10): p. 2069-2078.
35.Lim, T.-H., et al., An emerging recombinant cluster of nephropathogenic strains of avian infectious bronchitis virus in Korea. Infection, Genetics and Evolution, 2011. 11(3): p. 678-685.

[Downloaded from journal.isv.org.ir on 2024-03-29]

[DOI: 10.21859/isv.10.4.18]