Archives of Razi Institute, Vol. 77, No. 5 (2022) 1611-1619

DOI: 10.22092/ARI.2022.356836.1924

Original Article

Comparative Genetic Features of *S1* Gene from Six Massachusetts and Variant Avian 2 Infectious Bronchitis Viruses Isolated in Iran

Motamed, N[1] *, Bashashati, M[2]

1. Department of Poultry Vaccine Research and Production, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran
2. Department of Poultry Disease Research and Diagnostic, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran

Received 24 November 2021; Accepted 17 January 2022
Corresponding Author: n.motamed@rvsri.ac.ir

Abstract

Infectious bronchitis (IB) disease, avian Infectious Bronchitis disease in one of the major cause of respiratory problems and economic loss in poultry industry, even in developed countries with good biosecurity practice. Since the first isolation of the virus in 1931, a lot of serotypes and genotypes of the virus have been reported around the world. The GI-1 lineage, including Massachusetts (Mass) serotype viruses, is one of the most widely spread types worldwide. Moreover, the GI-23 lineage with a growing incidence rate was reported approximately 20 years ago in the Middle East, with no or little homologues vaccine use. The genotype was previously restricted to the Middle East; now, there is evidence that it has spread to European countries, raising concerns regarding potential outbreaks. In the present study, our attempt was to phylogenetically analyze the S1 gene of six isolates from Massachusetts and variant 2 genotypes, which were isolated from broiler and broiler breeder flocks in Iran. The variant 2 viruses were compared to other reported variant 2 viruses from neighboring countries and they had more than 98% identity with the latest reported Iranian variant 2. In addition, Three Mass type viruses were similar to vaccine strains which may be shows continuous circulation of vaccine viruses in the field. This event can cause increasing the risk of their mutation or even reversion to virulence after several passages in natural host, furthermore circulating viruses may recombinant with virulent field viruses and cause emergence of new variants. Considering the variable nature of IB viruses in which few changes lead to important differences, continuous epidemiological surveillance along with clinical studies of new isolates, are crucial to a better understanding of their pathogenicity and subsequent disease control.

Keywords: Spike glycoprotein, Phylogenetic analysis, the Middle East, Lineage

1. Introduction

Infectious bronchitis (IB) is among the most contagious viruses and, after the highly pathogenic avian influenza, is the second disease that economically damages the countries with developed poultry industries worldwide.

The virus can infect chickens of all ages and it is one of the important viral causes of respiratory complexes in poultry. Infection signs include depression, sneezing, coughing, gasping, loss of weight, increased feed conversion ratio, condemnation in broilers till drop in egg production, and decreased inner/outer egg quality in layers. When the kidneys are affected, increased water intake, depression, scouring, and wet litter are commonly observed.

The situation can get worse if secondary co-infections such as bacterial or mycoplasma infections occur, in which case the mortality rate may be

increased by up to 30%. Vaccination has been among the most effective ways of controlling IBV since the 1940s by Van Rockel. However, due to the continuous emergence of numerous variant viruses, which may result in cross-protection to not confer, failure of vaccinations has begun to occur (1).

The causative agent is an enveloped, single-stranded positive-sense 27.6 kbp RNA virus belonging to genus Gammacoronavirus, Orthocoronavirinae subfamily from Nidovirales order. According to the last international committee on taxonomy of viruses classification, the genus can be divided into three subgenus and the IBVs are located in the Igacovirus subgenus (2, 3).

Two-thirds of the first 5' of the virus genome belongs to polyprotein 1a, 1ab, which encodes 15 non-structural proteins (nsp). The rest of it encodes four structural proteins, including spike (S), envelope (E), membrane (M), and nucleocapsid (N), as well as at least four accessory proteins: 3a, 3b, 5a, and 5b. In addition to S1 glycoprotein, it is thought that nsp, accessory protein, and nucleocapsid may act as tools in the immune modulatory and virulence mechanism of the virus. Spike glycoprotein is cleaved into S1 and S2 post-translationally. The S1 is involved in virus-cell attachment, inducing neutralizing antibodies, antigenicity, and divergence of the virus. Generally, genotyping is according to the S1 gene sequence (4). A few changes in amino acids in S1 may cause new variants or serotype formations that can easily escape from vaccine-derived immunity and cause infection in chickens. Like RNA viruses, IBVs lack good proofreading despite having exoribonuclease (ExoN) nsp14, and as a result, their mutation rate is high. Recombination between two different IBVs when infecting a host simultaneously is a confirmed mechanism of IB divergence (5). The S2 subunit anchors S1 glycoprotein to the cell membrane and involves host cell-virus membrane fusion. According to Cavanagh (6) only a few amino acid substitutions cause 2% sequence divergence in the S1 gene and alter the serotype and pathogenicity. The S1 glycoprotein is the most important structural protein that induces serotype-specific neutralizing antibodies, protection, and diversity. Three hyper-variable regions (HVR) in S1 gene sequences have been detected. Based on deduced amino acid residues, HVRs positions are 56-69, 117-131, and 274-387aa (7, 8). Many studies did not analyze complete S1 sequences and instead only analyzed various partial S1 sequences targeting one or two of the HVR regions, resulting in different classifications or nomination of isolates. When the analysis is partial, the virus may have more or less similarity with other members of a specific genotype than the complete S1 gene analysis. Classification based on S1 gene sequences results in genotype. On the contrary, serotyping is a classification method based on antigenic characterization, which may be totally different from genotype. In addition, prediction of immunity from genotyping is risky (1). Most phylogenetic studies focus on S1 nucleotides or their amino acids sequences analysis.

As there are few S1 full gene sequences in the Middle East reports and continuous surveillance is critical to draw the virus epidemiological map and interpret evolutionary trends (9), the present study was conducted to investigate the genetic characterization of the avian coronavirus infectious bronchitis virus (AvCoV-IBV) S1 gene and phylogenic analysis of some of the most recently IBV isolates from Iranian poultry flocks.

2. Materials and Methods

2.1. Studied Viruses

Viruses were selected from the latest comprehensive epidemiological survey of respiratory pathogens in Iranian poultry farms with chicken flocks suffering from respiratory problems. Six virus isolates were selected from broiler and broiler breeder flocks in Hamadan, Yazd, Mazandaran, and Markazi provinces, Iran. Each with a history of live attenuated IBV vaccination, respiratory signs, and mortality due to IBVs in the molecular evaluation. Positive tissue samples by Reverse transcription polymerase chain reaction

(RT-PCR) targeting nucleocapsid gene, including trachea or lungs, were homogenized with sterile phosphate-buffered saline to prepare a 20% tissue suspension, then clarified by low-speed centrifuge for 10 min. Following inoculation of 0.2 ml of the supernatant into the allantoic cavity of 9-11-days-old Specific pathogen free (SPF) embryonated chicken eggs, then incubated in 37°C for 48-72h. First we inoculated samples in eggs then we detected the virus by molecular test and then sequencing of complete genome.

2.2. Molecular characterization

2.2.1. Primer Design and RT-PCR

Viral RNA extraction was done on positive IBV allantoic fluids using a High Pure Viral RNA kit (Roche, Germany) according to the company's instructions. In addition, a BioFact™ 2X RT-PCR Pre-Mix kit was used for cDNA synthesis. Two primer pairs were used in the present study, the first pair flanked the conserved region of the nucleocapsid gene as previously reported (10). However, on the other hand, to complete S1 genotyping and better molecular characterization, a pair of primer flanking full-length *S1* gene coding regions was designed by extraction and alignment of multiple IBV complete S1 gene sequences as previously submitted in GenBank from all over the world. (https://www.ncbi.nlm.nih.gov) designed oligonucleotide sequences that were sent to Sinaclone Co. for synthesis.

The polymerase chain reaction was done in 25µl reaction mixture, containing 15µl nuclease-free water, 1µl of 10 mM dNTP Mix, 2.5 µl of 10xPCR Buffer, 1µl 25mM MgCl2, 1µl of 10 µM each S1 forward and reverse primers and 3 µl cDNA, 0.25 µl of 2.5u/ µl Pfu DNA Polymerase (biotech rabbit, Gmbh). The thermal profile for S1 gene amplification is: 94°C 2min, followed by 35 cycles of 94°C/ 20 sec; 53°C /30 sec; 72°C /4 min and finally 72°C /5 min for the last elongation. Final PCR products were electrophoresed on a 1% agarose gel and positive samples were used for cloning and further processing.

Given that the majority of the reported IBV S1 sequences from Iran were partially analyzed, a previously published nested PCR reaction set using two pairs of primers was applied (Primers set and their target positions are shown in table 1).

Table1. Oligonucleotide sequences used in the present study

Primers	Primers 5´-3´	Location	PCR product size/Reference	Target region	Reference
N103-F N102-R	CCTGATGGTAATTTCCGTTG ACGCCCATCCTTAATACCTT	26368-26387* 26705-26724	~357bp	Nucleocapsid gene	(10)
IB-S1-P-F IB-S1-P-R	ATTTACAAACCTCTGCTTATAG CAAAACCTGCCATAACTAACAT	20227-20248* 22016-22037	~1820bp	S1 gene	In house
SX1-SX2 SX3-SX4	CACCTAGAGGTTTGCTA/T GCAT TCCACCTCTATAAACACCC/T TT TAATACTGG C/TAATTTTTCAGA AATACAGATTGCTTACAACCAC	1148-1168 1075-1096	490bp 390bp	S1 gene	(11)

*GenBank accession number MK937830 infectious bronchitis virus strain M41

2.2.2. Partial and Complete *S1* Gene Sequencing and Phylogenetic Analysis

Synthesized amplicons were purified with a High Pure PCR Purification kit (Roche, Germany), after ligation in pJET1.2/blunt vector (according to instructions of Clone JET PCR cloning kit, Thermofisher instructions) and transformed into the chemically competent *E. coli* Top10.

Since the pJET1.2/blunt vector harbors ampicillin resistance factor, *E.coli* colonies on culture media containing ampicillin were considered as positive clones. The colony PCR was carried out for

confirmation using S1 amplification primers. Then nucleotide sequences from purified positive clones were sent to a commercial company for analysis in both directions with pJET1.2 clone kit primers (Faza Pajooh, Tehran, Iran). Obtained chromatograms and the forward and reverse nucleotide sequences were edited and aligned in the final consensus sequence using Bioedit software (Version 7.2). Subsequently, they were compared with each other and the sequences published in the GenBank database with the help of the Basic Local Alignment Search Tool (BLAST).

All FASTA format nucleotide sequences were imported in MEGA 7 software for phylogenic analysis. The reference sequence dataset for IB classification was obtained from Valastro, Holmes (12) study and used for the topology construction of the S1 phylogenic tree. The phylogenic tree was constructed by the neighbor-joining method with 1,000 bootstrap replicates in MEGA 7 software.

3. Results

Based on complete S1 sequences, BLAST and phylogenetic analysis of six virus isolates compared with other IBV S1 sequences, three of the viruses were related to GI-23 lineage, so-called as IS-1494 like viruses, and three isolates belonging to Massachusetts (Mass) type or in other words, GI-1 lineage. Full S1 sequences were submitted to Genbank (isolates names, place, and date of collection with their NCBI accession numbers are described in table 2).

The phylogenetic tree constructed by the six full S1 gene sequences and reference dataset of Valastro, Holmes (12) is shown in figure 1.

Since there were few full S1 sequences of IB variant-2 and Massachusetts group viruses from neighboring and the Middle East countries, an extra phylogenetic analysis based on partial S1 nucleotide sequences consisting of HVR3 was built (Figure 2). By partial S1 phylogenic analysis, viruses were clustered in two lineages GI-1 and GI-23 as like as full-length S1 analysis; as shown in figure 2, the IS-1494 isolates in this study were clustered with other IS-1494 partial S1 sequences collected from Iran and neighboring countries, including Iraq, Egypt, Turkey, Israel, and Poland.

The full sequence similarity between IS-1494 viruses in this study was 99 to 100%. Moreover, they had more than 98.88% nucleotide similarity to IS-1494 like virus previously reported from Iran in 2015 (MG233398), 92.78% with 2017 Poland isolate (MK581207), and 87% with the Middle East 1998 isolate (AF093796).

Sequence similarity (Table 3) in our GI-1 lineage isolates was 99.76 to 100%, their nucleotides identity with RaziH120 and RaziH52 vaccine strain were 99.47 to 99.88%. However, there was more than 97% similarity with Mass41, indicating vaccine origin viruses were circulating in poultry flocks. The least identity was for Iowa virus (GU303337). The realized isolates had more than 99.8% average identity within the genotype and less than 80.5% between the genotypes.

Table 2. Virus isolates used in this study and GenBank accession number

Isolate	Host	Location	Genotype	Accession number
IBV/chicken/Hamedan/RIBV-1/2017 (RIBV-1)	Broiler	Hamedan	GI-23	OK626290
IBV/chicken/Yazd/RIBV-2/2017 (RIBV-2)	Broiler	Yazd	GI-23	OK626291
IBV/chicken/Markazi/RIBV-3/2019 (RIBV-3)	Broiler	Markazi	GI-23	OK626292
IBV/chicken/Markazi/RIBV-4/2019 (RIBV-4)	Broiler	Markazi	GI-1	OK626293
IBV/chicken/Mazandaran/RIBV-5/2020 (RIBV-5)	Broiler	Mazandaran	GI-1	OK626296
IBV/chicken/Mazandaran/RIBV-6/2020 (RIBV-6)	Broiler Breeder	Mazandaran	GI-1	OK626297

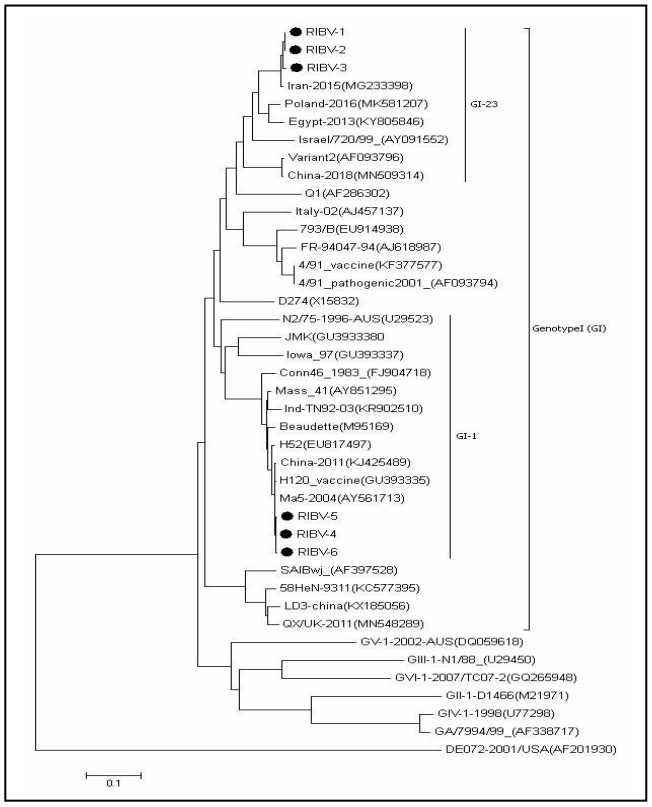

Figure 1. Phylogenetic analysis of partial S1 gene of IBV isolates with other viruses extracted from the GenBank. Isolates of this study are indicated by black circles. Horizontal lines show the number of variations in nucleotides per position. The phylogenetic tree was constructed by the Neighbor-joining statistical method and the Maximum Composite Likelihood model with Bootstrap 1,000 using MEGA 7 software.

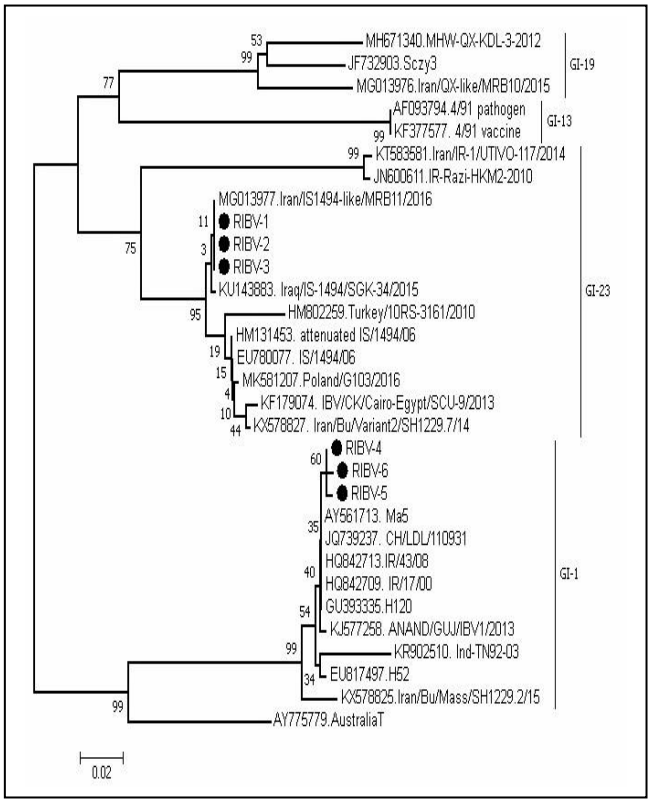

Figure 2. Phylogenetic analysis of partial S1 gene of IBV isolates with other viruses extracted from the GenBank. Isolates of this study are indicated by black circles. Horizontal lines show the number of variations in nucleotides per position. The phylogenetic tree was constructed by the Neighbor-joining statistical method and Maximum Composite Likelihood model with Bootstrap 1,000 using MEGA 7 software.

Table 3. Nucleotide similarity of IBV isolates across with various infectious bronchitis Genotype

1RIBV-1	100																
2RIBV-2	99.88	100															
3RIBV-3	99	98.94	100														
4RIBV-4	80.41	80.52	80.35	100													
5RIBV-5	80.23	80.35	80.17	99.76	100												
6RIBV-6	80.29	80.41	80.23	99.82	99.58	100											
7 793B	79.53	79.47	79.53	78.35	78.18	78.24	100										
8 QX	79	79.12	79.17	79.23	79.06	79.12	79.47	100									
9 Pol-IS-1494	92.78	92.84	92.6	80.64	80.46	80.52	80.11	79.12	100								
10 IR-IS-1494	98.88	98.82	98.59	80.58	80.41	80.46	79.58	79.23	92.96	100							
11 H120Razi	80.46	80.58	80.41	99.7	99.47	99.53	78.47	79.29	80.82	80.64	100						
12 H52razi	80.52	80.64	80.46	99.88	99.64	99.7	78.47	79.35	80.76	80.7	99.82	100					
13 4/91vaccine	79.53	79.64	79.64	79.41	79.23	79.35	93.43	80.52	80.05	79.76	79.53	79.53	100				
14 Mass41	80.58	80.58	80.52	97.82	97.59	97.77	78.94	79.23	80.76	80.76	97.82	97.94	79.94	100			
15 Conn46	79.94	80.05	79.76	94.89	94.66	94.83	78.41	78.53	80.52	80.17	94.89	95.01	79.47	95.83	100		
16 IOWA	79.06	79.06	79.17	82.58	82.4	82.4	76.89	77.18	78.94	79.12	82.63	82.69	77.47	82.99	81.93	100	
17 Q1	83.87	83.81	83.98	78.24	78.06	78.18	79.47	78.29	82.69	83.98	78.29	78.35	79.64	78.94	78.41	77.94	100
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**

Based on deduced amino acid sequences, cleavage recognition site -the furin recognition motif-containing multiple basic amino acids in spike glycoprotein located at S1 533-537 aa residues, for the three Variant-2 viruses were similar to each other and the same as the last Iranian reported virus (accession no. MG233398) and expressed multiple basic amino acids in S1 C-Terminal harboring Arg-Arg-Thr-Arg-Arg ($RRTRR_{537}$/S).

Based on Jackwood, Hilt (13), (14) findings, mentioned residues in the 3 GI-1 viruses were the same as previously reported cleavage recognition site from the United States viruses Mass 41, Beaudette, and C1013, European virus H120, China virus D41, and Central American virus V8147, including Arg-Arg-Phe-Arg-Arg ($RRFRR_{537}$/S).

4. Discussion

Repeated outbreaks of AvCoV-IBV variants in different countries have been causing economic burdens on poultry industries globally. Due to mutation or recombination of wild and vicinal circulating IBVs, frequent and rapid genetic and antigenic diversities occurs. Consequently, vaccination efforts became less effective and the conventional serotyping method became less practical in the characterization of emerging IBVs. As a result, most laboratories shifted to molecular and genotype identification of AvCoV-IBV, which nowadays is mostly performed by S1 gene sequencing. However, since the virus has a variable nature and occasionally new variants cause outbreaks, it is critical to understand the genetic and antigenic trend to design strategic plans for controlling and vaccination.

The First report on IB infection in Iran dates back to 1994 by Modiri Hamadan, Ghalyanchilangeroudi (15) when the widespread use of vaccines in our country started. Despite widespread vaccination, a large number of IBV infections in the poultry industry are frequently reported (15-18). The six studied isolates were related to two distinct genetic groups, namely Mass type and IS-1494 like viruses, which are in accordance with other Iranian studies (18, 19). Until now, a dozen types of IBVs have been reported from our region, consisting of Mass type, 4/91, QX, and variant 2 like viruses, whereas there are rarely reports of other viruses, such as Italy-02, D1466, D274, and so on, which are specific to American or European genotypes, indicating the fact that some genotypes have worldwide distribution while others are specific to some geographical zones.

Despite the high prevalence of the virus, even in developed countries, there is no common determinate classification method, similar to what exists for avian influenza or Newcastle disease agents. For the purpose of genotyping and phylogenetic analysis, we used Valastro, Holmes (12) proposed IBV classification method, comprising six different genotypes and 32 lineages based on the complete S1 gene of the IBV. Unfortunately, the S1 full gene sequence for IB viruses isolated from the Middle East was mostly based on a partial S1 sequence (12). Because of this, we constructed two phylogenetic trees: first based on partial S1, including the HVR3 region, and second, a complete S1 gene phylogenic evaluation. Considering two partial and complete S1 phylogenic trees, all six isolates were clustered in the same lineages, as shown in figures 1 and 2.

So-called IS-1494 like viruses (variant 2) group were isolated first in 1998 (Callison 2001), from The Middle East, causing respiratory and renal pathogenicity. This group of viruses were called as a Middle-East restricted lineage; However, in 2016, a new IS-1494 isolate from Poland has reported clustering with IS-1494 group, as well as the latest report of its isolation from broiler flocks in Germany in 2019 (11, 20).

According to our study, IS-1494 like viruses were close to other Iranian variant 2 viruses in 2015, with more than 98% and 87% nucleic acid identity with the first isolate from the Middle East. In addition, based on partial S1 sequences, they had close relation with the other Middle East isolates. Migratory birds, and maybe illegal trade between neighboring countries are considered as the reasons for similar virus genotype isolations (2). However, it is worth mentioning that in

the partial analysis we can observe three GI-23 isolates are closer to MG2333398 reported in 2015 than other GI-23 viruses reported from Iranian studies expressing virus evolution while circulating in the region. The first Iranian IS-1494-like virus was detected in 2010 as the second most frequent IBV isolate from 2010 to 2014 (16) when then it turned to be the most predominant virus type in Iranian poultry flocks (19) with a remarkably increased appearance at about two times more than previous reports (19). Until recently, IS-1494 type vaccines have not been used in our poultry industry, inferring increased occurrence. However, according to Modiri Hamadan, Ghalyanchilangeroudi (15), vaccination with two different serotypes after 2006, including Mass type and 793/B serotype, reduced the prevalence rate of two 793/B and QX genotypes. Furthermore, epidemiological studies in Italy and Spain showed that there was a causal relationship between the two types of vaccination and a decrease in QX prevalence (21).

According to partial and full-length S1 phylogenic analysis, three Mass-type viruses were clustered in GI-1 lineage with H120 vaccine virus, indicating that these 3isolates are vaccine-derived viruses. Considering the importance of vaccination in the disease control and shedding of the virus in the environment under multiple environmental selection pressures might result in their mutation (5). Our three GI-1 viruses were isolated from IB disease outbreaks as there are a lot of reports of Mass type virus isolation in regions using this kind of vaccine type, including our country (15, 19, 22), highlighting the risk of continuous circulation of vaccine-derived viruses in poultry flocks and the consequent emergence of new mutants. Molecular and clinical supplementary studies should be performed to investigate their pathogenicity and genetic variation. However considering that recombination events might occur between vaccinal and wild viruses, exchanging their genetic materials, and generally spike glycoprotein is not solely virulence factor and researchers should investigate nucleocapsid, accessory, and some other nonstructural genes which contribute to pathogenicity and immune escape. Since there is no definite indicator for differentiation between vaccine and field viruses based on molecular characteristics, it is difficult to interpret these isolates any further. Callison, Jackwood (23) explained that only little changes in the virus S1 gene are sufficient for the divergence of the virus virulence. Amarasinghe, De Silva Senapathi (24) reported that minor genetic diversity in IBV isolates may lead to substantial differences in pathogenicity in susceptible hosts. They showed that two Massachussete isolates with less than 1% variation were different in virulence and their difference was not just in the S1 gene but also in other open reading frames Jackwood, Hilt (12) suggested that the spike glycoprotein of infectious viruses, has to be cleaved at the C-terminal of the S1 and N-terminal of the S2 glycoproteins nominating as cleavage recognition site composed of five basic amino acids (533-537aa). Analyzing more than 50 different IB strains from different continents, they recommended that IBV host cell range or pathogenicity does not correlate with its cleavage site traits, as it does for Orthocoronavirinae and paramyxoviruses viruses, whereas the geographic region is an exception. Consequently, we investigated the C-terminal of the S1 gene in the six isolates and it was in accordance with frames Jackwood, Hilt (12) suggestion, as well as with other isolates reported in the Middle East (17).

In conclusion, despite vaccination against the disease, different IB genotypes are present in poultry farms and are imposing economic losses on all chicken ages. The GI-23 viruses are going to be more predominant in the region and are not limited to the Middle East anymore, as they are spreading to other continents as recently reported from some European countries. The GI-23 (Variant 2) and GI-1 viruses are continuously circulating and evolving in the country. Co-circulation of vaccine-derived and field viruses in poultry flocks is a warning sign for the advent of new different variants, followed by mutation or recombination (5), which may

breach from vaccination immunity and result in the emergence of new IB outbreaks.

Authors' Contribution

Study concept and design: N. M. and M. B.
Acquisition of data: N. M.
Analysis and interpretation of data: M. B. and N. M.
Drafting of the manuscript: N. M.
Critical revision of the manuscript for important intellectual content: M. B. and N. M.
Statistical analysis: N. M. and M. B.

Ethics

This study was approved by the ethics committee of Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran the

Conflict of Interest

The authors declare that they have no conflict of interest.

Grant Support

The current study was supported by a grant from the Agricultural Research, Education and Extension Organization NO. 13-18-1851-063-97020-971042.

References

1. de Wit JJ, Cook JKA, van der Heijden HMJF. Infectious bronchitis virus variants: a review of the history, current situation and control measures. Avian Pathol. 2011;40(3):223-35.
2. Rahman MM, Talukder A, Chowdhury MMH, Talukder R, Akter R. Coronaviruses in wild birds – A potential and suitable vector for global distribution. Vet Med Sci. 2021;7(1):264-72.
3. Wille M, Holmes EC. Wild birds as reservoirs for diverse and abundant gamma- and deltacoronaviruses. FEMS Microbiol Rev. 2020;44(5):631-44.
4. De Wit JJ. Detection of infectious bronchitis virus. Avian Pathol. 2000;29(2):71-93.
5. Lin S-Y, Chen H-W. Infectious Bronchitis Virus Variants: Molecular Analysis and Pathogenicity Investigation. 2017;18(10):2030.
6. Cavanagh D. Coronaviruses in poultry and other birds. Avian Pathology. 2005;34(6):439-48.
7. Lee C-W, Hilt DA, Jackwood MW. Typing of Field Isolates of Infectious Bronchitis Virus Based on the Sequence of the Hypervariable Region in the S1 Gene. J Vet Diagn Invest. 2003;15(4):344-8.
8. Montassier H. Molecular epidemiology and evolution of avian infectious bronchitis virus. Braz J Poult Sci. 2010;12(2):87-96.
9. Al-Jallad T, Kassouha M, Salhab M, Alomar A, Al-Masalma M, Abdelaziz F. Molecular characterization of isolated infectious bronchitis viruses from affected vaccinated broiler flocks in Syria. BMC Vet Res. 2020;16(1):449.
10. Loa CC, Lin TL, Wu CC, Bryan TA, Hooper TA, Schrader DL. Differential detection of turkey coronavirus, infectious bronchitis virus, and bovine coronavirus by a multiplex polymerase chain reaction. J Virol Methods. 2006;131(1):86-91.
11. Lisowska A, Sajewicz-Krukowska J, Fusaro A, PikuBa A, DombDska-Blicharz K. First characterization of a Middle-East GI-23 lineage (Var2-like) of infectious bronchitis virus in Europe. Virus Res. 2017;242:43 - 8.
12. Valastro V, Holmes EC, Britton P, Fusaro A, Jackwood MW, Cattoli G, et al. S1 gene-based phylogeny of infectious bronchitis virus: An attempt to harmonize virus classification. Infect Genet Evol. 2016;39:349 - 64.
13. Jackwood M, Hilt D, Callison S, Lee C-W, Plaza H, Wade E. Spike Glycoprotein Cleavage Recognition Site Analysis of Infectious Bronchitis Virus. Avian Dis. 2001;45:366-72.
14. Yamada Y, Liu DX. Proteolytic activation of the spike protein at a novel RRRR/S motif is implicated in furin-dependent entry, syncytium formation, and infectivity of coronavirus infectious bronchitis virus in cultured cells. J Virol. 2009;83(17):8744-58.
15. Modiri Hamadan A, Ghalyanchilangeroudi A, Hashemzadeh M, Hosseini H, Karimi V, Yahyaraeyat R, et al. Genotyping of Avian infectious bronchitis viruses in Iran (2015–2017) reveals domination of IS-1494 like virus. Virus Res. 2017;240:101-6.
16. Hosseini H, Fard M, Charkhkar S, Morshed R. Epidemiology of Avian Infectious Bronchitis Virus Genotypes in Iran (2010–2014). Avian Dis. 2015;59.
17. Mousavi FS, Ghalyanchilangeroudi A, Hosseini H, Nayeri Fasaei B, Ghafouri SA, Abdollahi H, et al. Complete genome analysis of Iranian IS-1494 like avian infectious bronchitis virus. Virus Dis. 2018;29(3):390-4.

18. Saadat Y, Bozorgmehri Fard MH, Charkhkar S, Hosseini H, Shaikhi N, Akbarpour B. Molecular characterization of infectious bronchitis viruses isolated from broiler flocks in Bushehr province, Iran: 2014 - 2015. Vet Res Forum. 2017;8(3):195-201.
19. Najafi H, Ghalyanchilangeroudi A, Hashemzadeh M, Madadgar O, Karimi V, Farahani R, et al. Pathogenicity characteristics of an Iranian variant-2 (IS-1494) like infectious bronchitis virus in experimentally infected SPF chickens. Acta Virol. 2016;60:393-9.
20. Fischer S, Klosterhalfen D, Wilms-Schulze Kump F, Casteel M. Research Note: First evidence of infectious bronchitis virus Middle-East GI-23 lineage (Var2-like) in Germany. Poult Sci. 2020;99(2):797-800.
21. Franzo G, Legnardi M, Tucciarone CM, Drigo M, Martini M, Cecchinato M. Evolution of infectious bronchitis virus in the field after homologous vaccination introduction. Vet Res. 2019;50(1):92.
22. Gholami F, Karimi V, Ghalyanchi Langeroudi A, Hashemzadeh M, Vasfi Marandi M. Genotyping of Infectious bronchitis viruses isolated from broiler chicken farms in Iran during 2015-2016 .Iran J Vet Med. 2018;12(1):9-17.
23. Callison SA, Jackwood MW, Hilt DA. Molecular Characterization of Infectious Bronchitis Virus Isolates Foreign to the United States and Comparison with United States Isolates. Avian Dis. 2001;45(2):492-9.
24. Amarasinghe A, De Silva Senapathi U, Abdul-Cader MS, Popowich S, Marshall F, Cork SC, et al. Comparative features of infections of two Massachusetts (Mass) infectious bronchitis virus (IBV) variants isolated from Western Canadian layer flocks. BMC Vet Res. 2018;14(1):391.