Molecular Characterization and Phylogenetic Analysis of the S1 Gene of Infectious Bronchitis Virus (IBV) Isolates from Broiler Farms in Markazi Province, Iran

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Abstract

Introduction: Infectious bronchitis (IB) is a contagious disease affecting chickens of any age and causes an extremely contagious disease that results in significant financial losses to the worldwide chicken industry. It affects both vaccinated and unvaccinated flocks globally. More information is needed about the recent IBV strains that have spread in Iran's Markazi province. **Objectives:** Therefore, this study was designed to isolate IBV from broiler farms showing respiratory distress in Iran's Markazi province and to analyze the evolutionary and molecular traits of the obtained isolates collected between 2017 and 2019.

Material and methods: In this investigation, tracheal and lung tissues were obtained from 42 poultry farms where chickens showed respiratory distress. These samples were inoculated into 9–11-day-old embryonated eggs, resulting in virus isolation from 10 of the examined farms. Afterwards, RT-PCR was applied to amplify fragments of the S1 gene from the newly detected isolates, and the obtained amplicons were sequenced and phylogenetically compared with a reference dataset.

Results: As determined by phylogenetic analysis, in total, 10 of 42 (23.80%) samples were evaluated as positive for Bronchitis virus. All ten isolates were sent for sequencing but three of them were failed in sequencing (two time) and finally, seven isolates were sequenced correctly. six isolates were in the GI-23 genotype from seven sequenced samples, and one isolate was in the GI-1 genotype. Therefore, GI-23 is the dominant genotype of IBV circulating in chicken farms of Markazi province, which has a low degree of homology with the vaccines used in the country.

Conclusion: Continuous monitoring of each region's circulating genotypes and compliance with health and biosecurity principles can be applied to reduce the complications of viral diseases involved in respiratory syndrome.

Keywords: Chicken, IBV, phylogenetic analysis, S1 gene

1. Introduction

Infectious bronchitis (IB) is a highly transmissible disease that can infect chickens at all ages and has led to considerable economic damage in poultry farms across Iran in recent years [1] .

Coughing and sneezing are respiratory signs of infectious bronchitis. Depression, huddling near heat sources , wheezing, nasal secretions, lethargy, tear discharge, and slightly swollen sinuses are further signs of this illness [2]. Moreover, kidney damage related to different types of bronchitis is one of the essential features of IB infection, especially in broiler chickens. Symptoms such as serous secretions and cheesy catarrh in the trachea, nose, and sinuses can also be mentioned[3]. This virus spreads through the respiratory tract and feces and can remain for several weeks or months inside the bird's body and for about 65 days in the environment [4]. The trachea is the target site for IBV replication, where viremia occurs and spreads to other tissues. Infectious bronchitis virus has been isolated from different tissues, such as the lungs, air sacs, esophagus, stomach, liver, spleen, kidney, etc. [5].Infectious bronchitis virus (IBV), together with other avian coronaviruses, belongs to the third group of coronaviruses (gamma coronaviruses) [6]. Coronavirus is enveloped, non-segmented, single-stranded, and positive-sense RNA, comprising approximately 27–32 Kb in size. The IBV genome encodes a large number of non-structural proteins as well as four major structural proteins containing spike

glycoprotein (S), small envelope protein (E), membrane glycoprotein (M), and nucleocapsid protein (N). The S protein has two glycopolypeptide components, S1 and S2. Subunit S1 has constant, variable, and highly variable regions, which divide bronchitis viruses into different serotypes and variants [7]. IBV strains could be distinguished by their S1 protein, which also became the main objective for genotype characterization [8].

IBV serotypes were first identified in Iran, and then the circulating genotypes of IBV were identified [9,10]. IBV is an endemic disease in Iran that has caused significant damage to the poultry industry. Accordingly, the present study aimed to isolate and characterize infectious bronchitis virus (IBV) from broiler flocks showing respiratory manifestations in the Markazi province of Iran, and its S gene was sequenced and phylogenetically evaluated.

2. Materials and Methods

2.1. Sampling and virus isolation

In the present work, tracheal and lung tissues were obtained from 42 vaccinated commercial broiler farms with respiratory symptoms in Markazi province, Iran, during 2017–2019. Information such as flock age, vaccination schedule, observed clinical signs, and the preliminary diagnosis provided by the farm veterinarian was recorded for each sample. Following collection, samples were immediately placed on ice and transferred to the Virology Laboratory of the Razi Vaccine and Serum Research Institute, Arak branch, where they were preserved at −70 °C prior to analysis. Each collected tissue was homogenized, and 0.2 ml of the resulting supernatant was used to investigate IBV infection through inoculation into the allantoic cavity of 9–11-day-old specific pathogen-free (SPF) embryonated eggs, in accordance with the WOAH guidelines[11] . The inoculated eggs were maintained at 37 °C, and embryo viability was checked daily for a period of five days. Embryos that died after 48 hours post inoculation (hpi) or remained alive until the end of incubation were subsequently chilled overnight at 4 °C Allantoic fluid was collected aseptically. Each sample was divided into two separate microtube and stored in a freezer at –70°C. Also, the embryos were investigated for abnormalities and lesions such as curled or dwarf emberyo, related to IB infection. Harvested allantoic fluids were re-inoculated into SPF embryonated chicken eggs for the second, third, and fourth passages to ensure viral propagation.

2.2. RNA extraction, cDNA synthesis and nested RT-PCR

Viral RNA was isolated from infected allantoic fluids with the RNX-plus™ reagent (Cinnagen, Iran) according to the manufacturer's instructions. The concentration and purity of the extracted RNA were assessed using a Nanodrop spectrophotometer, and the samples were kept at −70 °C until RT-PCR analysis. Complementary DNA (cDNA) synthesis was carried out using a commercial reverse transcription kit (BioFact, South Korea) and random hexamer primers. A portion of the S1 gene, including its hypervariable region 3, was amplified by nested PCR employing two specific primer pairs [12]. For the first PCR, SX1 (5'-CACCTAGAGGTTTGYTWGCATG-3') and SX2 (5'-TCCACCTCTATAAACACCYTTAC-3') primer set were used to amplify a 491 bp fragment. The PCR product was used as a template for the second PCR (nested PCR) in which SX3 (5´-TAATACTGGC/TAATTT TTCAGA-´3) and SX4 (5´´AATACAGATTGCT TACAACCACC-´3) primers were employed to amplify 392 bp fragment of the s1 gene. The amplification cycle consisted of an initial denaturation at 94 °C for 3 minutes, followed by 35 cycles of 94 °C for 45 seconds, 52 °C for 45 seconds, and 72 °C for 45 seconds, with a final extension at 72 °C for 10 minutes. RNA extracted from vaccine strains H120 and 793B served as positive controls in all PCR assays. The PCR products were electrophoresed on a 1.2% agarose gel prepared in tris acetate EDTA (TAE) and visualized using the gel documentation system - Image capture (Biometra, Germany).

2.3. RT-PCR analysis

Ten PCR-positive samples (second PCR round) were purified using a PCR purification kit (Favorgen, Taiwan) and submitted to Pishgam Company for bidirectional Sanger sequencing. The obtained sequences were processed and aligned using BioEdit software (version 7.2.5), and their homology was assessed against published IBV sequences available in the GenBank database through the BLAST tool of NCBI. A phylogenetic tree was generated following the latest classification framework of the IBV consortium as described by Awad et al. [13]. The seven S1 gene sequences obtained in this study were compared with the reference sequences reported by Valastro et al. (2016), which included 199 representative strains, the phylogenetic tree was constructed in MEGA version 10 using the Neighbor-Joining algorithm and the Maximum Composite Likelihood model, supported by 1,000 bootstrap replicates.

3. Results

3.1. RT-PCR Assay, Sequencing, and Sequence Homology Analysis

Out of the 42 collected samples, 30 were successfully isolated in SPF egg, which 10 of them was positive for IBV. The harvested allantoic fluids were subsequently used for further analyses. As anticipated, RT-PCR with primers SX1 and SX2 yielded a 491-bp fragment of the S gene (Figure 1)

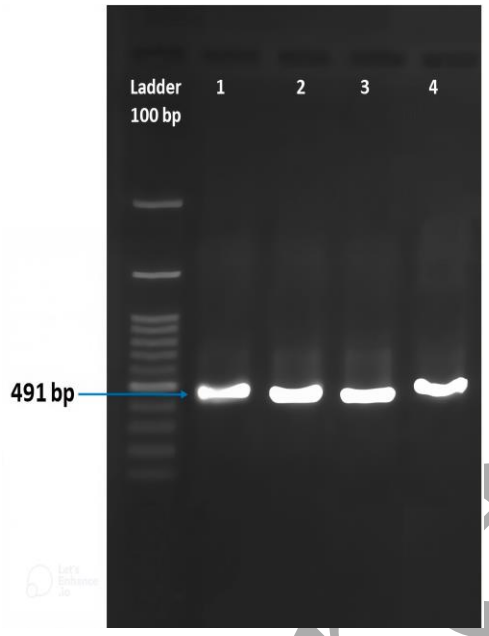

Figure 1: PCR product electrophoresis of bronchitis samples with SX1 and SX2 primers on 1.2% TAE gel

In total, 10 of 42 (23.80%) samples were evaluated as positive for Bronchitis virus. All ten isolates were sent for sequencing but three of them were failed in sequencing (two time) and finally, seven isolates were sequenced correctly. Nucleotide blast demonstrated high sequence similarity (99.07 to 99.69% nucleotide-level identity) in six isolates. Moreover, they had the most identity with the previously characterized variant 2 IBV (IRAN/IS1494 strain, accession No. MG233398) isolate from Iran (98.45 to 99.07% nucleotide-level identity). Also, one isolate had 100% identity with the Massachusetts genotype (IBV/Chicken/Iran/Mass/UTIVO-22/2014).

3.2. Phylogenetic Analysis

A phylogenetic tree was constructed following the latest IBV classification scheme proposed by the IBV consortium. In the phylogenetic tree, six isolates were clustered with the GI-23 genotype and variant 2 isolates (IS/1494/like) in a well-supported cluster (99% bootstrap value). One isolate was clustered with GI-1 genotype and together with isolates Massachusetts H120 vaccine virus (accession number: FJ888351.1) and Massachusetts M41 virus (96% bootstrap value) (Figure 2). The isolates obtained in the present study are indicated by the • symbol. Because of the large number of sequences and the complexity of the phylogenetic tree, only the subtree corresponding to the GI-23 genotype, which includes six isolates from this study, is presented in Figure 3.

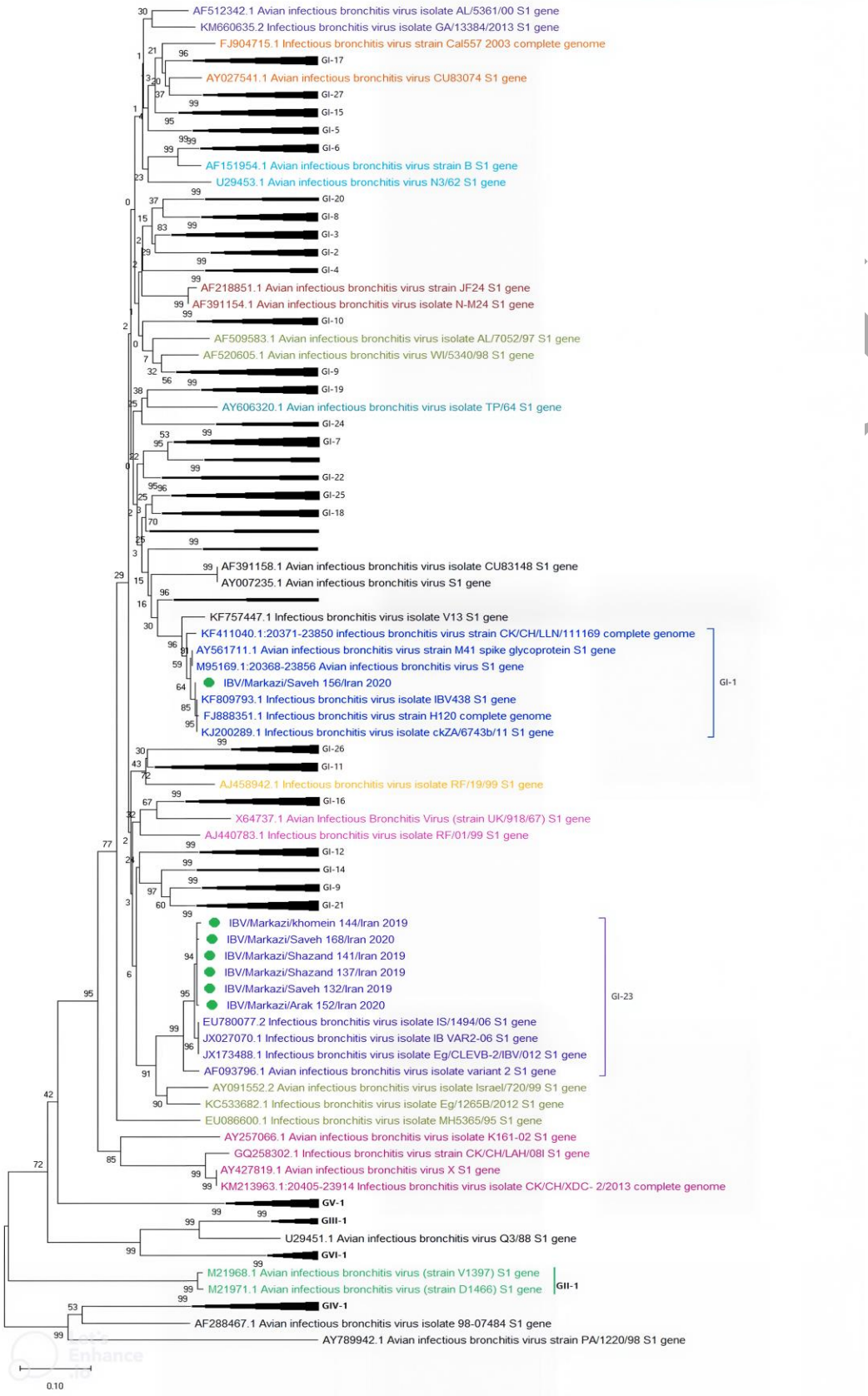
AF512342.1 Avian infectious bronchitis virus isolate AL/5361/00 S1 gene
KM660635.2 Infectious bronchitis virus isolate GA/13384/2013 S1 gene
FJ904715.1 Infectious bronchitis virus strain Cal557 2003 complete genome
GI-17
AY027541.1 Avian infectious bronchitis virus CU83074 S1 gene
GI-27
GI-15
GI-5
GI-6
AF151954.1 Avian infectious bronchitis virus strain B S1 gene
U29453.1 Avian infectious bronchitis virus N3/62 S1 gene
GI-20
GI-8
GI-3
GI-2
GI-4
AF218851.1 Avian infectious bronchitis virus strain JF24 S1 gene
AF391154.1 Avian infectious bronchitis virus isolate N-M24 S1 gene
GI-10
AF509583.1 Avian infectious bronchitis virus isolate AL/7052/97 S1 gene
AF520605.1 Avian infectious bronchitis virus WI/5340/98 S1 gene
GI-9
GI-19
AY606320.1 Avian infectious bronchitis virus isolate TP/64 S1 gene
GI-24
GI-7
GI-22
GI-25
GI-18
AF391158.1 Avian infectious bronchitis virus isolate CU83148 S1 gene
AY007235.1 Avian infectious bronchitis virus S1 gene
KF757447.1 Infectious bronchitis virus isolate V13 S1 gene
KF411040.1:20371-23850 infectious bronchitis virus strain CK/CH/LLN/111169 complete genome
AY561711.1 Avian infectious bronchitis virus strain M41 spike glycoprotein S1 gene
M95169.1:20368-23856 Avian infectious bronchitis virus S1 gene
IBV/Markazi/Saveh 156/Iran 2020
KF809793.1 Infectious bronchitis virus isolate IBV438 S1 gene
FJ888351.1 Infectious bronchitis virus strain H120 complete genome
KJ200289.1 Infectious bronchitis virus isolate ckZA/6743b/11 S1 gene
GI-1
GI-26
GI-11
AJ458942.1 Infectious bronchitis virus isolate RF/19/99 S1 gene
GI-16
X64737.1 Avian Infectious Bronchitis Virus (strain UK/918/67) S1 gene
AJ440783.1 Infectious bronchitis virus isolate RF/01/99 S1 gene
GI-12
GI-14
GI-9
GI-21
IBV/Markazi/khomein 144/Iran 2019
IBV/Markazi/Saveh 168/Iran 2020
IBV/Markazi/Shazand 141/Iran 2019
IBV/Markazi/Shazand 137/Iran 2019
IBV/Markazi/Saveh 132/Iran 2019
IBV/Markazi/Arak 152/Iran 2020
EU780077.2 Infectious bronchitis virus isolate IS/1494/06 S1 gene
JX027070.1 Infectious bronchitis virus isolate IB VAR2-06 S1 gene
JX173488.1 Infectious bronchitis virus isolate Eg/CLEVB-2/IBV/012 S1 gene
AF093796.1 Avian infectious bronchitis virus isolate variant 2 S1 gene
GI-23
AY091552.2 Avian infectious bronchitis virus isolate Israel/720/99 S1 gene
KC533682.1 Infectious bronchitis virus isolate Eg/1265B/2012 S1 gene
EU086600.1 Infectious bronchitis virus isolate MH5365/95 S1 gene
AY257066.1 Avian infectious bronchitis virus isolate K161-02 S1 gene
GQ258302.1 Infectious bronchitis virus strain CK/CH/LAH/08I S1 gene
AY427819.1 Avian infectious bronchitis virus X S1 gene
KM213963.1:20405-23914 Infectious bronchitis virus isolate CK/CH/XDC- 2/2013 complete genome
GV-1
GIII-1
U29451.1 Avian infectious bronchitis virus Q3/88 S1 gene
GVI-1
M21968.1 Avian infectious bronchitis virus (strain V1397) S1 gene
M21971.1 Avian infectious bronchitis virus (strain D1466) S1 gene
GII-1
GIV-1
AF288467.1 Avian infectious bronchitis virus isolate 98-07484 S1 gene
AY789942.1 Avian infectious bronchitis virus strain PA/1220/98 S1 gene
0.10

Fig.2 Phylogeny tree related to infectious bronchitis isolates based on the nucleotide sequence of the S1 gene drawn by MEGA 10 software using the neighbour-joining method with 1000 bootstrap. The analysis included seven isolates obtained in the present study along with a reference dataset of 199 sequences published by Valastro et al. The isolates from this study are indicated by a green circle. Six isolates were in the GI-23 group, and one was in the GI-1 group.

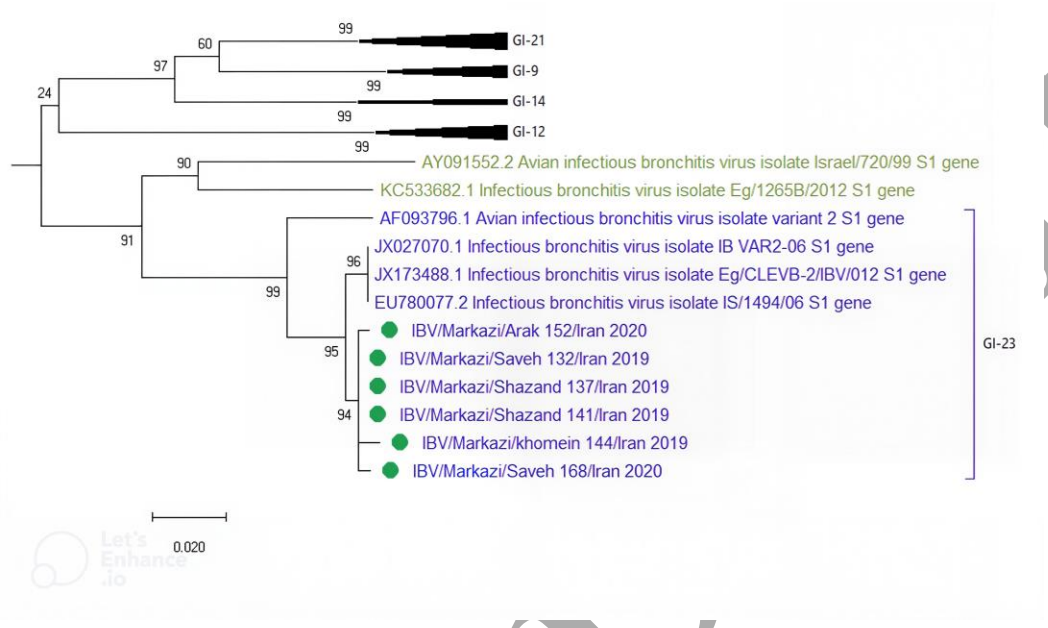

Figure 3: Subtree depicting the IBV isolates identified in this study within the GI-23 group. Green circles represent isolates from the current research.

4. Discussion

Infectious bronchitis has significantly caused respiratory diseases in broiler farms across the nation. Even with comprehensive control measures and vaccination efforts, instances of infectious bronchitis continue to be reported annually throughout the country [14]. As a result, IBV remains one of the poultry diseases with the most significant economic effect [6]. Global reports concern the emergence of various strains of IBV [14]. In Iran, multiple genotypes and serotypes of IBV have been documented in various regions, and analysis of the hypervariable region (HVR) of the S1 glycoprotein gene demonstrated that the recovered IBV strains belonged to four genotypes: QX, IS/1494, 793B, and Massachusetts[15]. In this study, 23.8% of the samples (10 out of 42) tested positive for the infectious bronchitis virus. Phylogenetic Analysis of bronchitis isolates from seven sequenced samples revealed that six of them belonged to the

GI-23 genotype, with variant 2 isolates (IS/1494/like), and one isolate was placed in the GI-1 genotype with Massachusetts, H120 vaccine virus and Massachusetts M41 virus. Recent research in other provinces has corroborated the findings of this study. According to the obtained results, the prevailing genotype of IBV circulating among poultry farms in Iran is Variant 2 (IS/1494/like), which falls within the GI-23 lineage[10,15]. Variant 2 (IS/1494/like) was first reported in occupied Palestine in 2001 and then spread to other parts of the world, including Libya, Egypt, Turkey, Afghanistan, Iraq, and Iran[13–16] . Hosseini et al. first reported the IS/1494 genotype in Iran in 2010 [9]. The frequency of this genotype has shown a continuous increase, making it the most widespread IBV strain currently detected in Iran. Gholami et al. showed that, among the 4 types of genotype 2 [IS/1494/06], 4/91, QX, and Massachusetts classified based on phylogenetic Analysis, type 2 [IS/1494/06] with the highest percentage of 66. 67%, and the rest, 4/91, QX and Massachusetts were 24.45%, 4.44% and 4.44%, respectively[10,15] A study was conducted on commercial chicken flocks infected with IBV in Razavi Khorasan province, and 11 viruses were identified. Six isolates belonged to the GI-23 genotype in the category of viruses, and one isolate was classified as the GI-1 genotype.

Reports suggest that the widespread presence of GI-23 lineage viruses may enhance the likelihood of genetic mutations and the emergence of novel viral variants[17]. Phylogenetic analysis revealed that eight isolates belonged to the GI-23 lineage (IS-Variant 2), two to the GI-1 lineage (Massachusetts), and one to the GI-12 lineage (793B). It was reported that seven Iranian-origin viruses within the GI-23 lineage possessed synonymous mutations (T954C and G1056A) and one non-synonymous mutation (C797T), none of which had been previously documented. These new genetic changes have occurred in two different areas in Razavi Khorasan. The authors emphasized that the widespread circulation of GI-23 lineage viruses in Iran could elevate the risk of viral mutations and the emergence of new variants; therefore, enhanced vaccination strategies and strict biosecurity practices are essential to limit viral dissemination[17]. In addition, the homology of variant 2isolates of the current study and the vaccines used in the country (4.91 and H120) were obtained from 82.52% to 83.13% and 82.35% to 82.97%, respectively. Therefore, variant 2 is the dominant strain of IBV circulating in chicken farms of the Markazi province, which has a low degree of homology with the vaccines used in the country. Other studies conducted in Iran also showed the predominance of IBV variant 2 in Iran and expressed its low level of homology with the used vaccines[9,18].

5. Conclusion:

In conclusion, the results of the present study indicate that, Continuous monitoring of circulating genotypes in each region, determining the dominant genotype, and developing vaccines that have more cross-protection with them, along with compliance with health and biosecurity principles, are strategies that can be applied to reduce the complications of infectious bronchitis.

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Author contribution

Study concept and design: V.V, S.E

Acquisition of data: V.V, S.E.

Analysis and interpretation of data: V. V.

Drafting of the manuscript: V.V, S.E, N.A, P.J.

Critical revision of the manuscript for important intellectual content: N.A, P.J.

Statistical analysis: V.V, S.E.

Administrative, technical, and material support: N.A, P.J.

Study supervision: S. E.

Ethical approval

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Conflict of interest

The authors have no competing interests to declare that are relevant to the content of this article.

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Data availability

Data will be made available on request.

ChatGPT (OpenAI, GPT-5 model) was used to edit and paraphrase the text in the Introduction, Methods, and Discussion sections. The AI did not contribute to data collection, analysis, or interpretation. All outputs were reviewed and verified by the authors.

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