

## Original Article

# Sequence and Phylogenetic Analysis of Membrane (M) Gene of Infectious Bronchitis Viruses Isolated in Iran during 2014 - 2015

Kami E<sup>1</sup>, Ghalyanchi Langeroudi A<sup>1\*</sup>, Madadgar O<sup>1</sup>, Nayyeri Fasaee B<sup>1</sup>, Hosseini H<sup>2</sup>, Najafi H<sup>3</sup>, Mousavi F S<sup>1</sup>

1. Department of Microbiology and Immunology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran
2. Poultry diseases Faculty of Veterinary Medicine, Azad University, Karaj, Iran
3. Department of Pathobiology, School of Veterinary Medicine, Shiraz University

### Abstract

**Background and Aims:** Avian infectious bronchitis virus (IBV) has a worldwide distribution and mutations occurring in the large viral genome of IBV have led to extensive antigenic variations among IBVs. This is the first study conducted to determine the complete membrane (M) gene sequences of different Iranian IBV genotypes.

**Materials and Methods:** The M gene of three 793/B (IBKG1,6,7), one Massachusetts (IBKG3), three Variant 2 (IBKG2,4,9), four QX (IBKG5,10,11,12) and one IR-1 (IBKG8) Iranian IBV isolates were sequenced and analyzed. Also, post-translational modifications of the M glycoprotein were predicted.

**Results:** Sequence analysis of the M gene indicated IBKG1, 6, 7, and 8 shared the highest homology to 793/B genotype. IBKG2, 4, 5, 9, 10, 11, and 12 showed the highest similarity to QX-like IBV. IBKG3 (Massachusetts) isolate shared 99/53% identities with H120 vaccine strain, respectively. Although M proteins are moderately well conserved within each coronavirus genus, the sequence differences of M gene reached to 12% among Iranian IBV isolates. Based on M gene analysis, IBKG-8 (IR-1 genotype) and all Variant2 IBVs, classified as a 793/B and QX IBV according to the M gene sequences, respectively.

**Conclusions:** It is the first characterization of IR-1 (Iranian local genotype) based on M gene. Also, the data shows M gene of variant 2 IBV strains are derived from QX genotypes. The results complete the Iranian IBVs genetic puzzle.

**Keywords:** M gene, Avian Infectious Bronchitis, Sequencing, Phylogenetic, Iran.

### Introduction

Avian infectious bronchitis (IB) is an economically important, highly contagious and acute upper respiratory tract disease of chickens, caused by the avian coronavirus infectious bronchitis virus (IBV). IBV is a positive-sense, single-stranded RNA virus belonging to the order Nidovirales,

family Coronaviridae, genus Gammacoronavirus [1, 2]. The IBV genome encodes four main structural proteins: phosphorylated nucleocapsid protein (N), membrane glycoprotein (M), spike glycoprotein (S) consisting of S1 and S2 and small membrane protein (E) [3]. The spike subunit 1 (S1) is highly variable in IBV and analysis of S1 using reverse transcription-polymerase chain reaction (RT-PCR) and sequencing has allowed for genotyping of IBV strains [4].

M protein is moderately well conserved within each coronavirus genus but diverges considerably across genera [5]. The M protein, a polytopic protein, is the most abundant component of corona virions. M protein gives

\* **Corresponding author:** Arash Ghalyanchilangeroudi  
Department of Microbiology and Immunology,  
Faculty of Veterinary Medicine, University of Tehran,  
Tehran, Iran.  
Email: arashghalyanchi@gmail.com  
Tel: 021- 61117154.

## Characterization of IBV M gene

the virion envelope its shape. The M monomer, which ranges from 25 to 30 kDa, is a polytopic membrane protein that is embedded in the envelope by three transmembrane domains. At its amino terminus is a very small ectodomain; the C-terminal endodomain of M accounts for the major part of the molecule and is situated in the interior of the virion or on the cytoplasmic face of intracellular membranes. Although it is inserted co-translationally into the endoplasmic reticulum (ER) membrane, the M protein does not bear an amino-terminal signal peptide. The ectodomain of M is modified by glycosylation, which is usually N linked [5]. The M protein of coronavirus plays a central role in virus assembly [6].

Reverse genetic studies and VLP assembly studies suggest that M protein promotes assembly by interacting with viral ribonucleoprotein (RNP) and S glycoproteins at the budding site [7-13], and by forming a network of M-M interactions that are capable of excluding some host membrane proteins from the viral envelope [14, 15]. M proteins interact through both the transmembrane domain and endodomain [14]. M can also interact with RNA that carries the genomic packaging signal [16]. Several IBV genotypes including Massachusetts;793/B, Variant2, QX,

The study is conducted to determine the whole M gene sequences of Iranian IBV isolates which had been previously genotyped based on S1 gene sequences to 1- Complete the puzzle of Iranian IBV characterization to have a full genome 2: Study phylogenetic-based on the M gene 3: Find the recombination events in the Iranian IBV genome 4: Use the data for future studies such as production of recombinant protein . 5: Have M data of IR-1 (As an Iranian specific and local genotype) as the first time. Viral RNA of twelve Iranian IBV isolates was extracted, and reverse transcription was performed. To amplify the “M” gene of IBV, the PCR was employed using specific primers. PCR products were sequenced and analyzed.

## Methods

**Strains history.** Twelve IBVs including circulating IBV genotypes (Massachusetts;793/B; QX; Variant2; IR1) were used in this study[17]. The genotypes of IBV were determined in the lab in the previous studies. The epidemiological information ( a type of flock; Location; Year) of 12 Iranian has been shown IBV isolates in table 1.

**Table 1: Epidemiology information for Iranian field IBV isolates used in this study**

IBV isolates	Flock	Year	Province	Organ used for virus isolation	Genotype(based on S1 gene analysis)
IBKG-1	Broiler	2014	Ghazvin	Trachea	793/B
IBKG-2	Broiler	2014	Isfahan	Trachea	Variant 2
IBKG-3	Broiler	2014	Ardebil	Kidney	Massachusetts
IBKG-4	Broiler	2014	Ghazvin	Trachea	QX
IBKG-5	Broiler	2015	Isfahan	Kidney	QX
IBKG-6	Broiler	2014	Azarbyjan-e- Sharghi	Trachea	793/B
IBKG-7	Broiler	2015	Isfahan	Trachea	793/B
IBKG-8	Broiler	2014	Isfahan	Trachea	IR-1
IBKG-9	Broiler	2015	Khorasane-e-Razavi	Kidney	Variant 2
IBKG-10	Broiler	2014	Khorasane-e-Razavi	Trachea	QX
IBKG-11	Broiler	2014	Azarbyjan-e- Sharghi	Kidney	QX
IBKG-12	Broiler	2015	Azarbyjan-e- Sharghi	Trachea	QX

IR-1 were reported, and each of the genotypes has its pathogenicity and immunogenicity characteristics. No data is available on the IBV M gene sequence and M protein topology of Iranian IBV isolates.

**RNA extraction and cDNA synthesis.** RNA was extracted using the Cinnagen kit (Sinaclon, Iran) according to manufacturer's instructions and then stored at -70. The cDNA was synthesized. cDNA synthesized using RevertAid first strand cDNA synthesis Kit

(Thermo Fisher Scientific, Canada). For cDNA synthesis, 1  $\mu$ l of random hexamer primer (Thermo Fisher Scientific, Canada) was added to 5  $\mu$ l of RNA, and the mixture was heated at 65  $^{\circ}$ C for 5 minutes. Fourteen  $\mu$ l of cDNA master mix containing 7.25  $\mu$ l of DEPC-treated water, 2  $\mu$ l of dNTP mix, 0.25  $\mu$ l of RiboLock RNase Inhibitor, 0.5  $\mu$ l of Revert Aid Reverse Transcriptase, and 4  $\mu$ l of 5X RT reaction buffer was added to each tube, resulting in a final volume of 20  $\mu$ l. Then, the mixture was incubated at 25  $^{\circ}$ C for 5 min, 42  $^{\circ}$ C for 60 min, 95  $^{\circ}$ C for 5 min, and 4  $^{\circ}$ C for 1 min. The cDNA was stored at -20  $^{\circ}$ C until use.

**The M gene amplification using polymerase chain reaction (PCR).** The M gene of IBV isolates was amplified using designed primers: IBMF4 (5' GGCTGCTGATGCTTGTTGTTTAT 3') and IBMR4 (5' GGGACCACAACCTACAACAA 3'). Each 20 $\mu$ l PCR reaction consisted of 10 $\mu$ l 2X PCR master mix (Sinaclon, Iran), 4  $\mu$ l cDNA, 1 $\mu$ l of each primer (25  $\mu$ M), and 1 $\mu$ l distilled water. This reaction was carried out using a BioRad thermal cycler with an initial denaturation step of 94 $^{\circ}$ C for 3 min and then 35 cycles of 94 $^{\circ}$ C for 30 s, 58 $^{\circ}$ C for 30 s and 72 $^{\circ}$ C for 80 s, with a final extension of 72 $^{\circ}$ C for 10 min. The size of PCR product was 1128 bp.

**Phylogenetic tree.** The PCR products were sequenced by Bioneer Corporation, Korea in both directions. Chromatograms were evaluated with FinchTV and consensus sequences were obtained using ChromasPro (ChromasPro Version 1.5). Multiple sequence alignments were performed with ClustalW, and a phylogenetic tree was constructed with MEGA 5 software using the neighbor-joining method with 1000 bootstrap replicates to assign confidence levels to branches [18]. The IBV M gene sequences were aligned and compared with the M gene of reference and vaccine strains.

M protein secondary structure determination  
CLC Main Workbench version 6.5 was used to translate. Secondary structure was predicted by PSIPRED (<http://bioinf.cs.ucl.ac.uk/psipred>)  
Prediction of glycosylation sites of M protein

Potential N-glycosylation sites were predicted by NetNGlyc 1.0 Server (<http://www.cbs.dtu.dk/services/NetNGlyc/>).  
Topology prediction of M protein  
Transmembrane localization data were searched using TMHMM (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>).

## Results

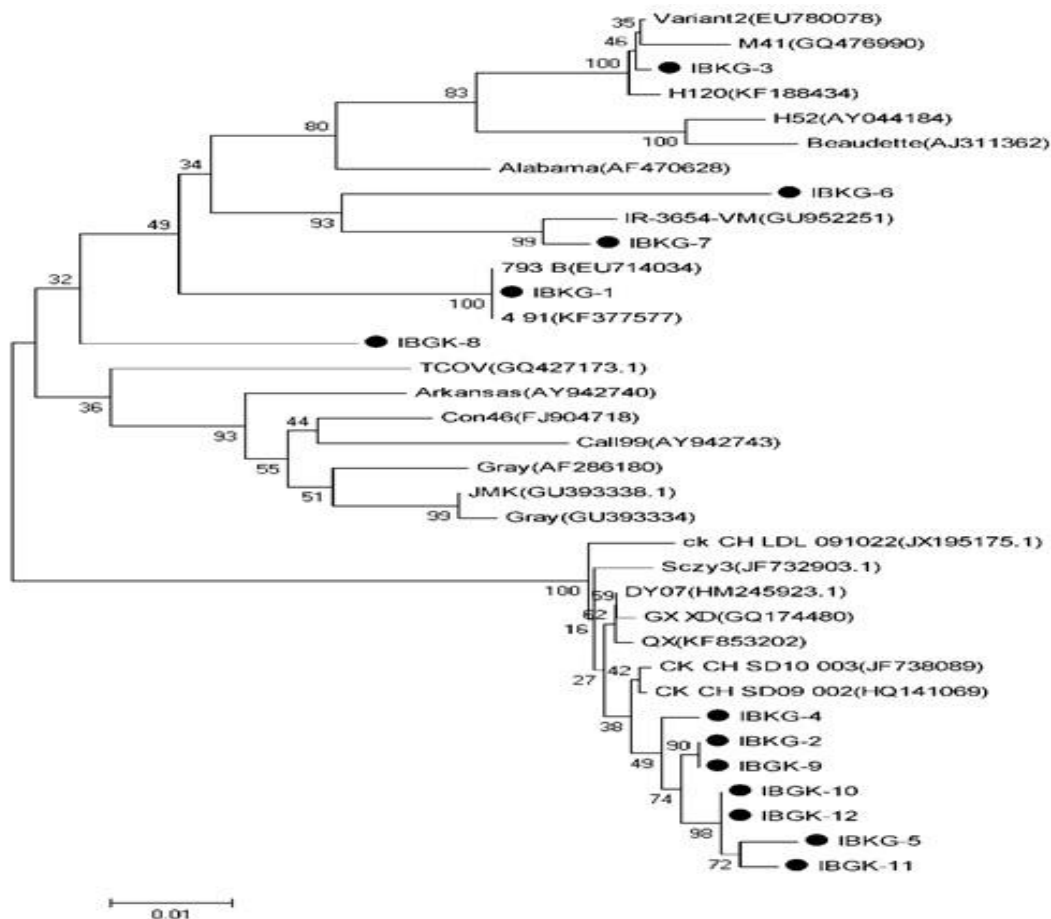
**Characterization, phylogenetic and clustering.** The M gene of Three 793/B (IBKG1,6,7), one Massachusetts (IBKG3), three variant 2 (IBKG2,4,9), four QX (IBKG5,10,11,12) and one IR-1 (IBKG8) Iranian IBV isolates were sequenced and analyzed. The isolates IBKG2, IBKG4, and IBKG9 which belonged to variant-2 genotype based on S1 gene analysis formed clusters by M gene analysis with QX genotype. IBKG8 from IR1 genotype was related to 793/B type. Strains IBKG1, IBKG6, and IBKG7, belonged to genotype 793/B based on both S1 and M gene analysis. Also, IBKG5, IBKG10, IBKG11, and IBKG12 from QX type, belonged to the same genotype of QX by M gene analysis (Figure 1 and Table 2). The sequences of the M gene of 12 Iranian IBV isolates shared 88.46 and 100% homologous identities.

**Structure determination, Glycosylation sites, and Topology prediction.** M protein of IBKG3 isolate composed of 4  $\alpha$ -helices and 12  $\beta$ -strands, also 4  $\alpha$ -helices and 11  $\beta$ -strands were identified in each of IBKG7 and IBKG10 M proteins. Data are shown in figures 3-5. Potential glycosylation sites of the M protein were determined in IBKG3, IBKG7 and IBKG10 strains. Two Sites of N-glycosylation were found in IBKG3 and IBKG10 while IBKG7 had one (Figure 6). The transmembrane domains are located between the 21st and the 41st amino acids, 52nd and the 72nd amino acids, 78th and the 98th amino acids.

## Characterization of IBV M gene

**Table 2:** Percent identity of whole nucleotide sequences of the M genes of some Iranian IBVs to those of IBV reference strains

Similarity	IBKG3	IBKG1	IBKG4	IBKG2	IBKG5	Variant2 (EU780078)	H120 (KF188434)	H52 (AY044184)	491(KF377577)	DY07(HM245923.1)	QX(KF853202)	CK CH SD10 003(JF738089)
IBKG3		93.92	88.81	89.36	88.67	99.84	99.53	96.22	93.92	89.00	89.18	88.64
IBKG1	93.92		90.82	91.00	90.31	94.09	94.09	92.91	100	91.18	91.35	91.35
IBKG4	88.81	90.826		99.38	98.60	88.62	88.26	88.14	90.82	99.07	98.91	99.53
IBKG2	89.36	91.00	99.38		98.92	89.18	88.82	87.95	91.00	99.07	98.91	99.22
IBKG5	88.67	90.31	98.60	98.92		88.48	88.12	86.87	90.31	98.29	98.13	98.45
Variant2(EU780078)	99.84	94.09	88.62	89.18	88.48		99.69	96.38	94.09	89.18	89.36	88.82
H120(KF188434)	99.53	94.09	88.26	88.82	88.12	99.69		96.70	94.09	89.17	89.35	88.81
H52(AY044184)	96.22	92.91	88.14	87.95	86.87	96.38	96.70		92.91	88.68	88.49	88.69
491(KF377577)	93.92	100	90.82	91.00	90.31	94.09	94.09	92.91		91.18	91.35	91.35
DY07(HM245923.1)	89.00	91.18	99.07	99.07	98.29	89.18	89.17	88.68	91.18		99.84	99.53
QX(KF853202)	89.18	91.35	98.91	98.91	98.13	89.36	89.35	88.49	91.35	99.84		99.38
CK CH SD10 003(JF738089)	88.64	91.35	99.53	99.22	98.45	88.82	88.81	88.69	91.35	99.53	99.38	



**Fig. 1.** Phylogenetic relationships of Iranian IBV isolates and selected reference strains based on the M gene sequences. The neighbor-joining method with the Kimura-2 parameter substitution model and 1000 bootstrap replicates to assign a confidence level to branches constructed a phylogenetic tree. The isolates used in this study highlighted with a black square. The scale bar represents the distance unit between sequence pairs. The sequences obtained from Gene Bank.

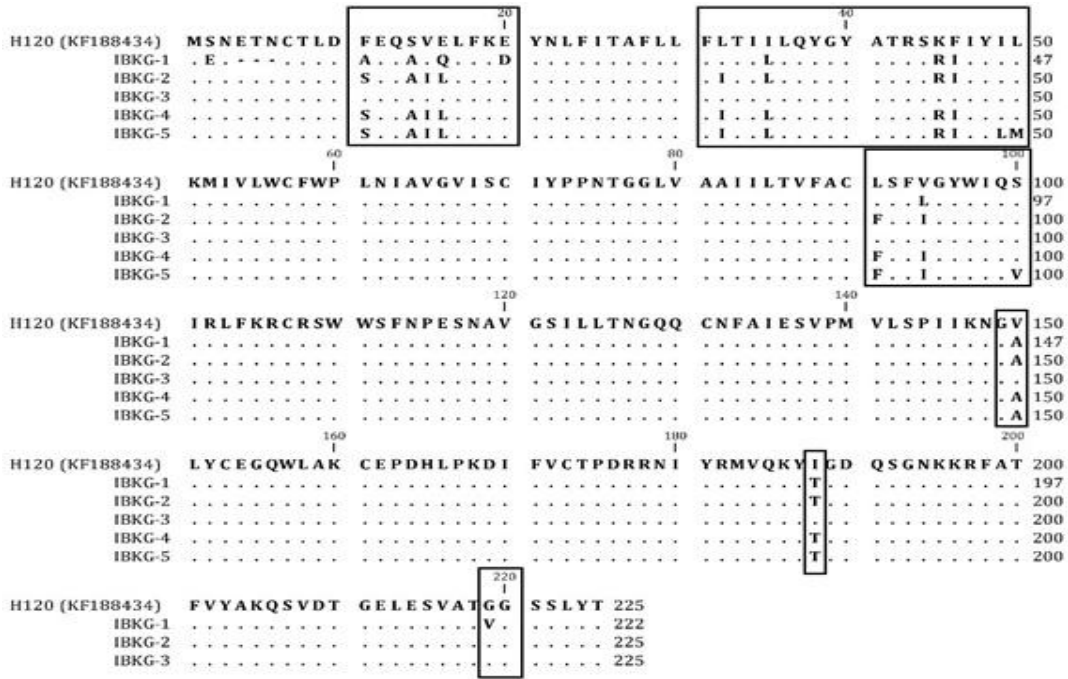


Fig. 2. Deduced amino acid and sequences of 5 Iranian IBV isolates compared to the H120 vaccine strain

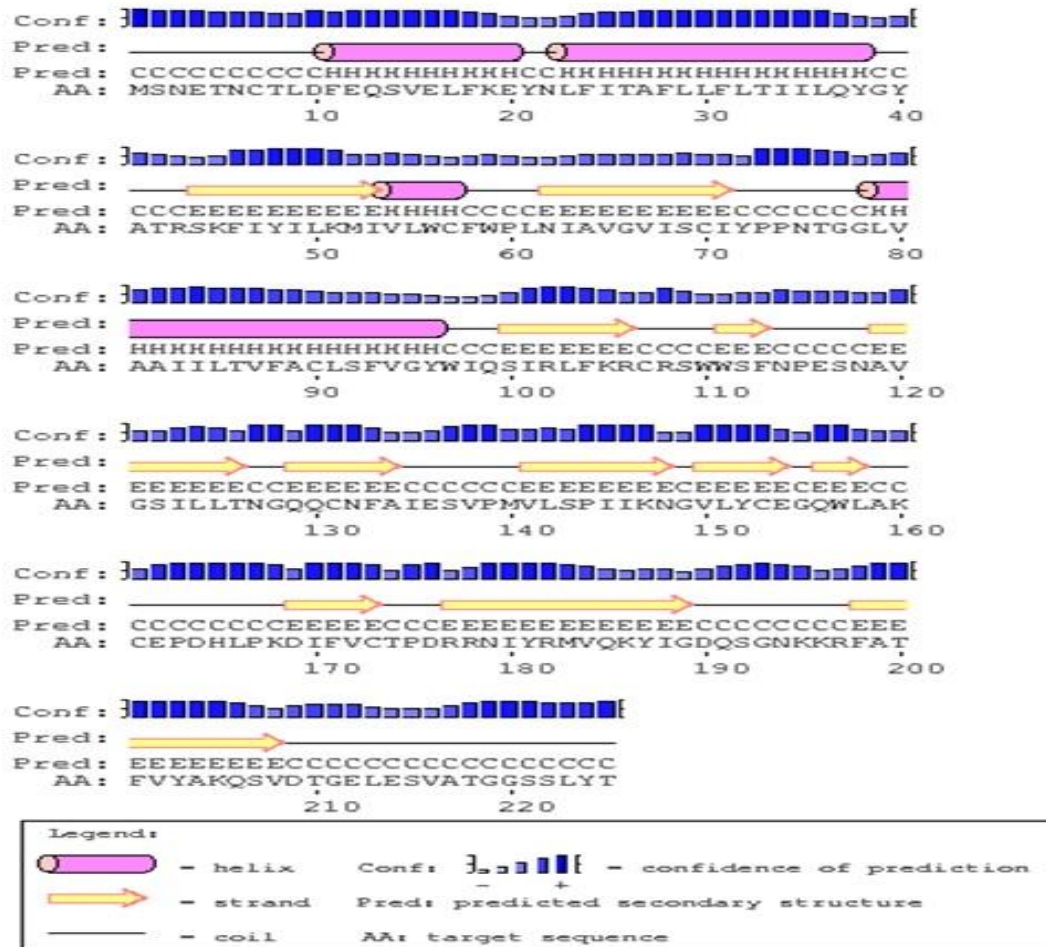


Fig. 3. M protein secondary structure of IBKG3 strain predicted by PSIPRED.

Downloaded from journal.isv.org.ir at 13:41 +0430 on Tuesday September 21st 2021



# Characterization of IBV M gene

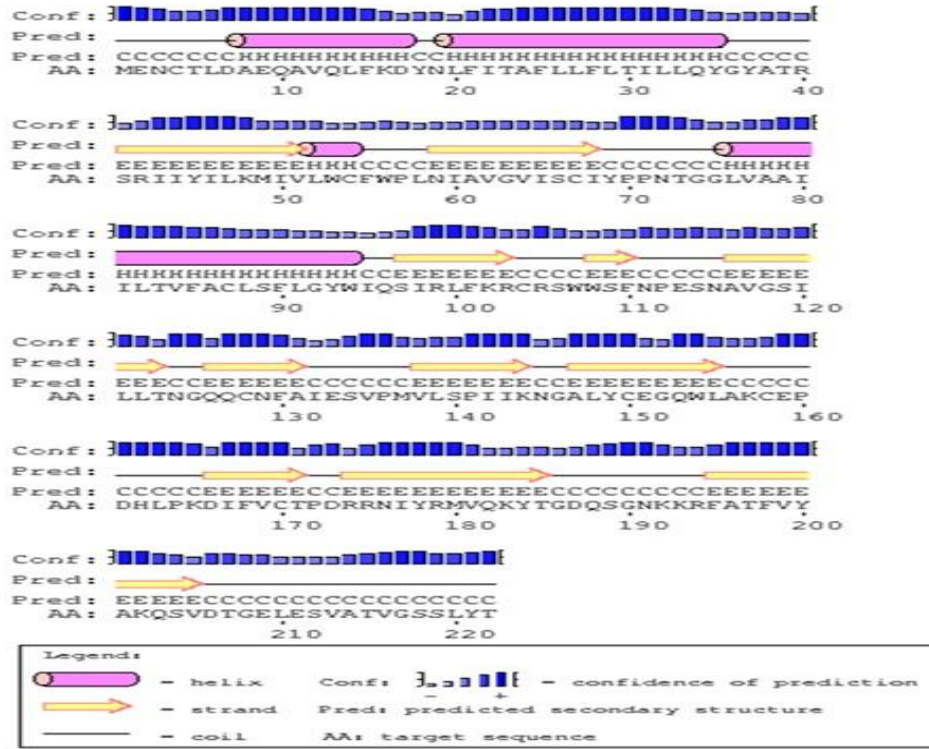


Fig. 4. M protein secondary structure of IBKG7 strain predicted by PSIPRED.

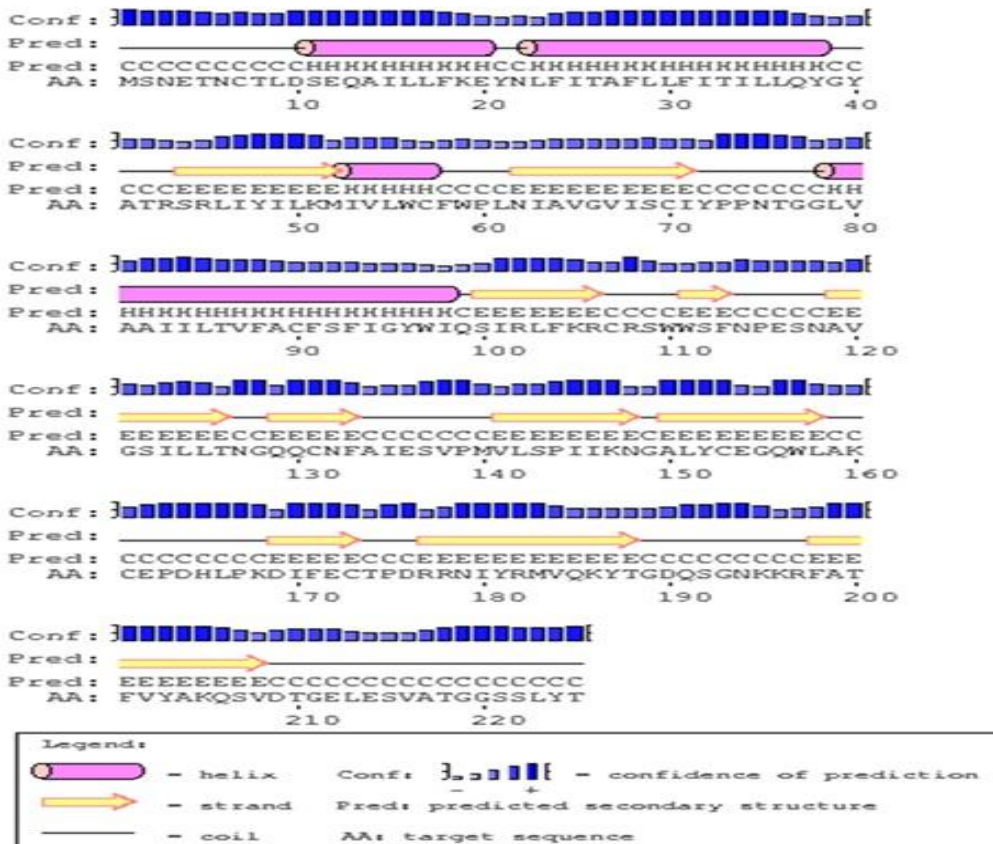
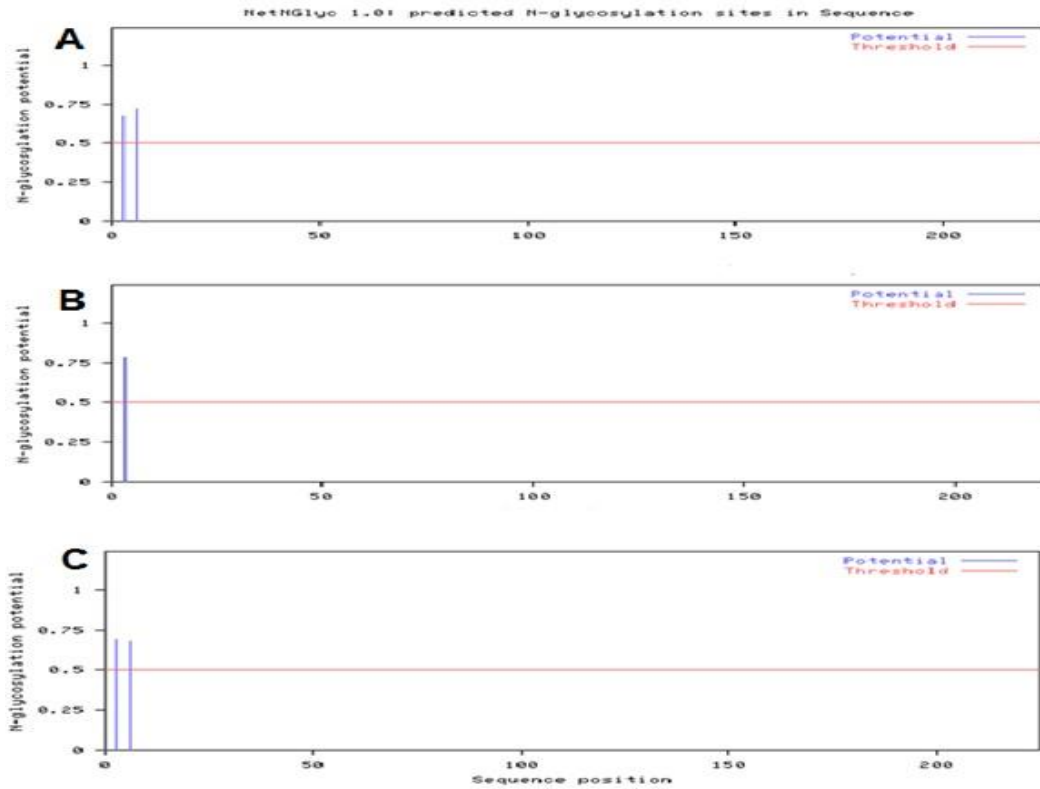
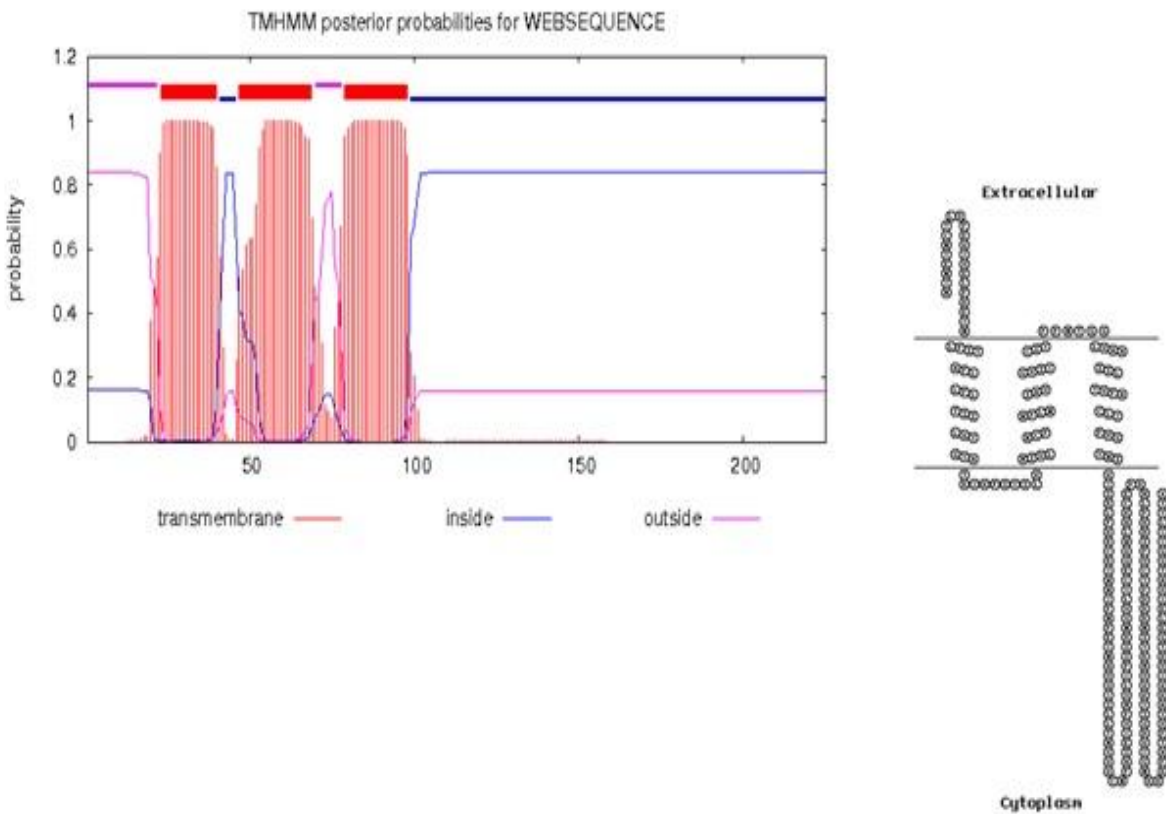


Fig. 5. M protein secondary structure of IBKG10 strain predicted by PSIPRED.



**Fig. 6.** Graphic representation of N-glycosylation site analysis of the M amino acid sequence of three strains, using NetNGlyc 1.0 computer program. Threshold=0.5. A; IBKG3, B; IBKG7, C; IBKG10.



**Fig. 7.** a snake-like diagram of the predicted TM topology mode of IBKG3 strain.

## Characterization of IBV M gene

The extracellular domains are located between the 1st and the 20th amino acids, 73th and the 77th amino acids, and the cytoplasmic domain is located between the 42nd and the 51st amino acids and 99th and the 225th amino acids. Transmembrane topology of the M protein of IBKG3 strain depicted in Figure 7 and 8.

## Discussion

Infectious bronchitis (IB) is a highly contagious upper respiratory tract disease of chickens [19]. IBV strains are continuously evolving through point mutations, insertions, deletions and genome recombination, leading to the emergence of new IBV serotypes or variants [20].

Virulent IBV genotypes that have a severe impact on chicken health and production have been reported in recent decades [21-24]. The spike subunit 1 (S1) is highly variable in IBV and analysis of S1 using RT-PCR and sequencing has allowed for genotyping of IBV strains [4]. Furthermore, the presence of deletions within the 3' end of gene 3 and the 5' end of M gene can be used to classify and differentiate Mass and non-Mass strains of IBV [20]. The M protein of coronaviruses is a structural membrane protein and plays a major role in the viral assembly process [25].

In the case of IBV strain Beaudette (Mass serotype), the M gene of which has been cloned and sequenced [26], the 30000 (30K) M glycopolypeptide has two glycans N-linked to asparagine residues at positions 2 and 5 from the N terminus of the mature M glycopolypeptide [27]. Electrophoretic analysis of the polypeptides of the recent isolates revealed that the serotypes had an M glycopolypeptide of either 27K or 30K, from which it was concluded that the numbers of glycans present were one and two respectively [28].

This study was conducted to determine the whole M gene sequences of Iranian IBV isolates which had been previously genotyped based on S1 gene sequences. 12 isolates were selected from five IBV genotypes of Massachusetts, 793/B, QX, variant2, and IR1. This is the first report on M gene and M protein of IBV in Iran. Sequence analysis of

the M gene indicated IBKG1, IBKG6, IBKG7, and IBKG8 shared the greatest homology to 793/B genotype. IBKG2, IBKG4, IBKG5, IBKG9, IBKG10, IBKG11 and IBKG12 showed the highest similarity to QX-type IBV. IBKG3 isolate shared 99/84% and 99/53% identities with variant2 and H120 genotypes, respectively. The homology of the M gene sequences of our 12 isolates was between 88.46 and 100%. Sequences analysis of M gene of IBKG1, IBKG4, IBKG5, IBKG6, IBKG7, IBKG10, IBKG11 and IBKG12 isolates indicated the same genotypes to that of the S1 phylogenetic tree shown. This agrees with previous reports (nosrati and zulperi) who did not find any considerable differences between IBV grouping based on S1 and M gene sequences.

Surprisingly the M gene sequence differences among our 12 isolates were between 1 and 12% which contravenes the fact states the M proteins are well conserved within each coronavirus genus. Our results agree with published data by Ren et al. who characterized the M gene of IBV Chinese strain HH06. The sequences of the M gene and protein share 83.9–97.9% and 83.6–96.5% identities, respectively, compared with 29 IBV reference strains derived from different regions or countries, which revealed that there are still significant variations between strains [25].

There are several reports especially focused on the M gene of coronaviruses. Cavanagh and Davis (1987) have sequenced 200 to 240 bases of the matrix (M) glycoprotein gene of 23 strains IBV representing the A (D207), B (D3896), C (D3128), D (D212), Massachusetts (Mass), UKI 1 and UK12 serotypes. As predicted from protein Mr studies the A/D, and B/C serotypes had two and one potential glycosylation sites respectively. The glycosylation sequence Asn-Cys-Thr was highly conserved. Overall, the exposed part of M exhibited a fourfold greater extent of amino acid variation than did the membrane-embedded sequence. The transcription-associated homology region sequence (CUUAACAA) in the 5' intergenic region was identical in all strains, but there was considerable variation as to its location [29].



Our results demonstrated the M proteins of IBKG3 and IBKG10 had two Potential glycosylation sites, and IBKG7 had one site of N-glycosylation.

Nosrati et al. analyzed the amino acid sequences of three structural proteins of M, N, and S1 for five Iranian IBVs isolated during 1998-2011. Similarity for M and S1 protein was lowest for IR-3654-VM and TW2575 / 98 isolate (0.862) and IR1061-PH and Georgia 1998 isolate/strain (0.41), respectively. In a phylogeny analysis, all proteins were distributed in three clusters. Iranian IBV belonged to Mass cluster in the phylogenetic tree of M and N proteins. However, S1 protein showed a close relationship with the California serotype and was distantly related to Mass serotype[30].

Zulperi and the colleagues (2009) in Malaysia analyzed the phylogenetic and the sequence of S1, M, S2, N gene of IBV isolates. Phylogenetic analysis of S1 gene and sequences analysis of M gene indicated that MH5365/95 and V9/04 belong to non-Massachusetts strain. However, both isolates share only 77% identity[31].

The results of this study showed that IR-1 (IBKG-8) is close to 793/B and is probably 793/B is one of the strains of the ancestors of this virus. To complete this hypothesis, it seems necessary to determine the complete sequence of this isolate. In addition, strains of variants 2 in this study were placed next to strain QX. Based on these results as well as other our unpublished studies, it is possible that with the high percentage, variant 2 may be created by the recombination of some IBV viruses with genotype QX. Also, probably the M gene sequences of the variant 2 in Gene bank is wrong.

## Conclusions

This was the first study on M gene and M protein of avian coronavirus in Iran. Results of this work can be used in further molecular and molecular epidemiology studies and completed the Iranian IBVs genetic puzzle. It is also applicable in monoclonal antibodies future development.

## References

1. Carstens E. Report from the 40th meeting of the Executive Committee of the International Committee of Taxonomy of Viruses. *Archives of virology*. 2009;154(9):1571-1574.
2. Cong F, Liu X, Han Z, , et al. Transcriptome analysis of chicken kidney tissues following coronavirus avian infectious bronchitis virus infection. *BMC genomics*. 2013;14(1):743.
3. Lai MM, Cavanagh D. The molecular biology of coronaviruses. *Advances in virus research*. 1997;48:1-100.
4. Kingham B, Keeler Jr C, Nix W, et al. Identification of avian infectious bronchitis virus by direct automated cycle sequencing of the S-1 gene. *Avian diseases*. 2000;325-335.
5. Masters PS, Perlman S. *Fields Virology*. Philadelphia, PA: Lippincott Williams & Wilkins. 2013:825-858.
6. Neuman BW, Kiss G, Kunding AH, et al. A structural analysis of M protein in coronavirus assembly and morphology. *Journal of structural biology*. 2011;174(1):11-22.
7. de Haan CA, Smeets M, Vernooij F, , et al. Mapping of the coronavirus membrane protein domains involved in interaction with the spike protein. *Journal of virology*. 1999;73(9):7441-7452.
8. Escors D, Camafeita E, Ortego J, , et al. Organization of two transmissible gastroenteritis coronavirus membrane protein topologies within the virion and core. *Journal of virology*. 2001;75(24):12228-12240.
9. Kuo L, Masters PS. Genetic evidence for a structural interaction between the carboxy termini of the membrane and nucleocapsid proteins of mouse hepatitis virus. *Journal of virology*. 2002;76(10):4987-4999.
10. Narayanan K, Maeda A, Maeda J, , et al. Characterization of the coronavirus M protein and nucleocapsid interaction in infected cells. *Journal of virology*. 2000;74(17):8127-8134.
11. Nguyen V-P, Hogue BG. Protein interactions during coronavirus assembly. *Journal of virology*. 1997;71(12):9278-9284.
12. Opstelten DJ, Raamsman M, Wolfs K, , et al. Envelope glycoprotein interactions in coronavirus assembly. *The Journal of cell biology*. 1995;131(2):339-349.
13. Sturman LS, Holmes K, Behnke J. Isolation of coronavirus envelope glycoproteins and interaction with the viral nucleocapsid. *Journal of virology*. 1980;33(1):449-462.

## Characterization of IBV M gene

14. De Haan CA, Vennema H, Rottier PJ. Assembly of the coronavirus envelope: homotypic interactions between the M proteins. *Journal of virology*. 2000;74(11):4967-4978.
15. Neuman BW, Joseph JS, Saikatendu KS, et al. Proteomics analysis unravels the functional repertoire of coronavirus nonstructural protein 3. *Journal of virology*. 2008;82(11):5279-5294.
16. Narayanan K, Chen C-J, Maeda J, et al. Nucleocapsid-independent specific viral RNA packaging via viral envelope protein and viral RNA signal. *Journal of virology*. 2003;77(5):2922-2927.
17. Najafi H, Langeroudi AG, Hashemzadeh M, et al. Molecular characterization of infectious bronchitis viruses isolated from broiler chicken farms in Iran, 2014-2015. *Archives of virology*. 2016;161(1):53-62.
18. Tamura K, Peterson D, Peterson N, et al. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol*. 2011;28(10):2731-2739.
19. Jackwood MW. Review of infectious bronchitis virus around the world. *Avian diseases*. 2012;56(4):634-641.
20. Cavanagh D, Davis P, Cook J. Infectious bronchitis virus: evidence for recombination within the Massachusetts serotype. *Avian Pathology*. 1992;21(3):401-408.
21. Gough R, Randall C, Dagless M, et al. A new strain of infectious bronchitis virus infecting domestic fowl in Great Britain. *Veterinary record*. 1992;130(22):493-494.
22. Kahya S, Coven F, Temelli S, et al. Presence of IS/1494/06 genotype-related infectious bronchitis virus in breeder and broiler flocks in Turkey. *Ankara universitesi veteriner fakültesi dergisi*. 2013;60:27-31.
23. Meir R, Rosenblut E, Perl S, et al. Identification of a novel nephropathogenic infectious bronchitis virus in Israel. *Avian diseases*. 2004;48(3):635-641.
24. Yu L, Jiang Y, Low S, et al. Characterization of three infectious bronchitis virus isolates from China associated with proventriculus in vaccinated chickens. *Avian diseases*. 2001:416-424.
25. Ren X, Yin J, Ma D, Li G. Characterization and membrane gene-based phylogenetic analysis of avian infectious bronchitis virus Chinese strain HH06. *Virus Genes*. 2009;38(1):39-45.
26. Boursnell M, Brown T, Binns M. Sequence of the membrane protein gene from avian coronavirus IBV. *Virus research*. 1984;1(4):303-313.
27. Cavanagh D. Coronavirus IBV glycopolypeptides: size of their polypeptide moieties and nature of their oligosaccharides. *Journal of General Virology*. 1983;64(5):1187-1191.
28. Cavanagh D, Davis P. Coronavirus IBV: Relationships among recent European isolates studied by limited proteolysis of the Virion Glycopolypeptides. *Avian Pathology*. 1987;16(1):1-13.
29. Cavanagh D, Davis PJ. Evolution of avian coronavirus IBV: sequence of the matrix glycoprotein gene and intergenic region of several serotypes. *J Gen Virol*. 1988;69(Pt 3):621-629.
30. Nosrati M, Tahmorespoor M, Nassiry M. Genetic Analysis of Three Structural Proteins in Iranian Infectious Bronchitis Virus Isolate. *Iranian Journal of Applied Animal Science*. 2013;3(3):617-623.
31. Zulperi ZM, Omar AR, Arshad SS. Sequence and phylogenetic analysis of S1, S2, M, and N genes of infectious bronchitis virus isolates from Malaysia. *Virus genes*. 2009;38(3):383-391.