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Original Article

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

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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 glyco-protein (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 the virion

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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, 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.

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.

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 Revert-Aid first strand cDNA synthesis Kit (Thermo Fisher Scientific, Canada). For cDNA synthe-

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

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

The M gene amplification using polymerase chain reaction (PCR). The M gene of IBV isolates was amplified using designed primers: IBMF4 (5' GGCTGCTGATGCTTGTTGTTT-AT 3') and IBMR4 (5′ GGGACCACAACCT-ACAACAA 3'). Each 20µl PCR reaction consisted of 10µl 2X PCR master mix (Sinaclon, Iran), 4 µl cDNA, 1µl of each primer (25 µM), and 1µl distilled water. This reaction was carried out using a BioRad thermal cycler with an initial denaturation step of 94°C for 3 min and then 35 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 80 s, with a final extension of 72°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 CromasPro (CromasPro Version 1.5). Multiple sequence alignments were performed with ClustalW, and a phylogenic 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. IBGK8 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, IBGK10, IBGK11, and IBGK12 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 α-helices and 12 β-strands, also 4 α-helices and 11 β-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 IBGK10 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.)	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	

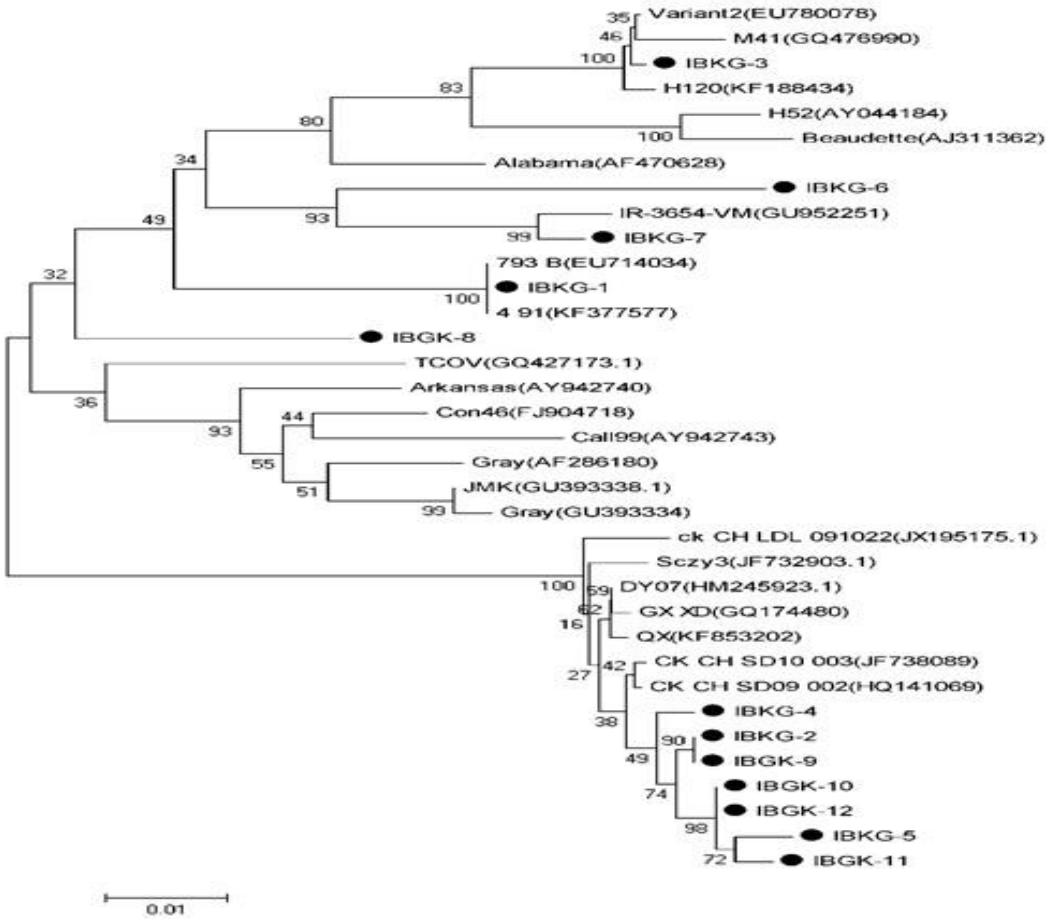

Fig. 1. Phylogenetic relationships of Iranian IBV isolates and selected reference strains based on the M gene sequences. The neighbor-joining method with the Kimura-2 parameter substitution model and1000 bootstrap replicates to assign a confidence level to branches constructed a phylogenetic tree. The 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.

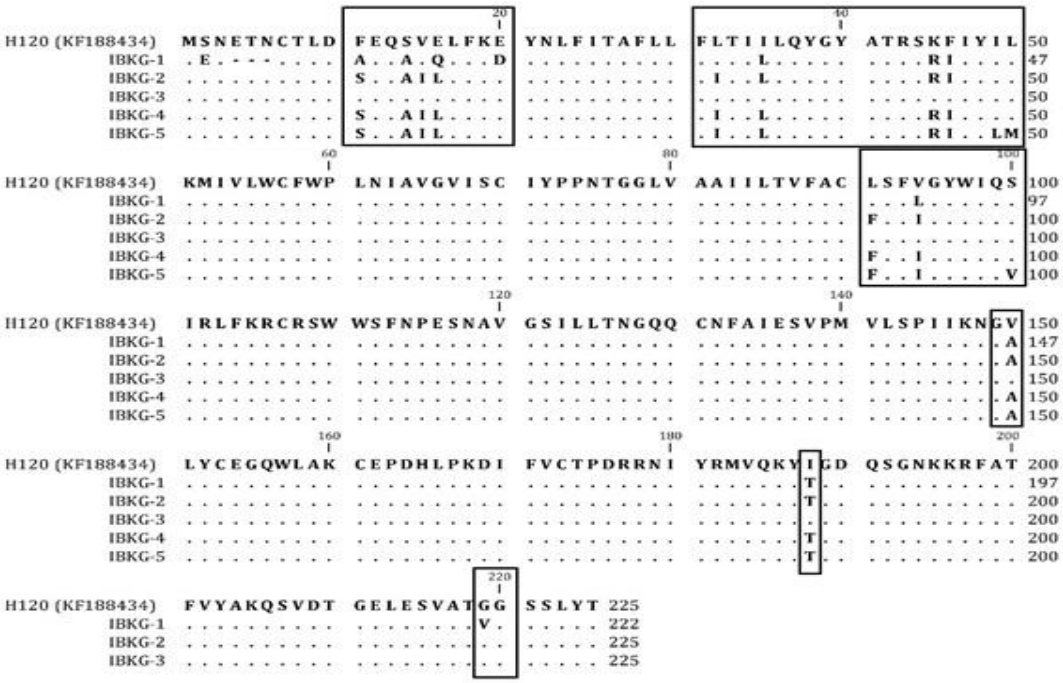

Fig. 2. Deduced amino acid and sequences of 5 Iranian IBV isolates compared to the H120 vaccine strain

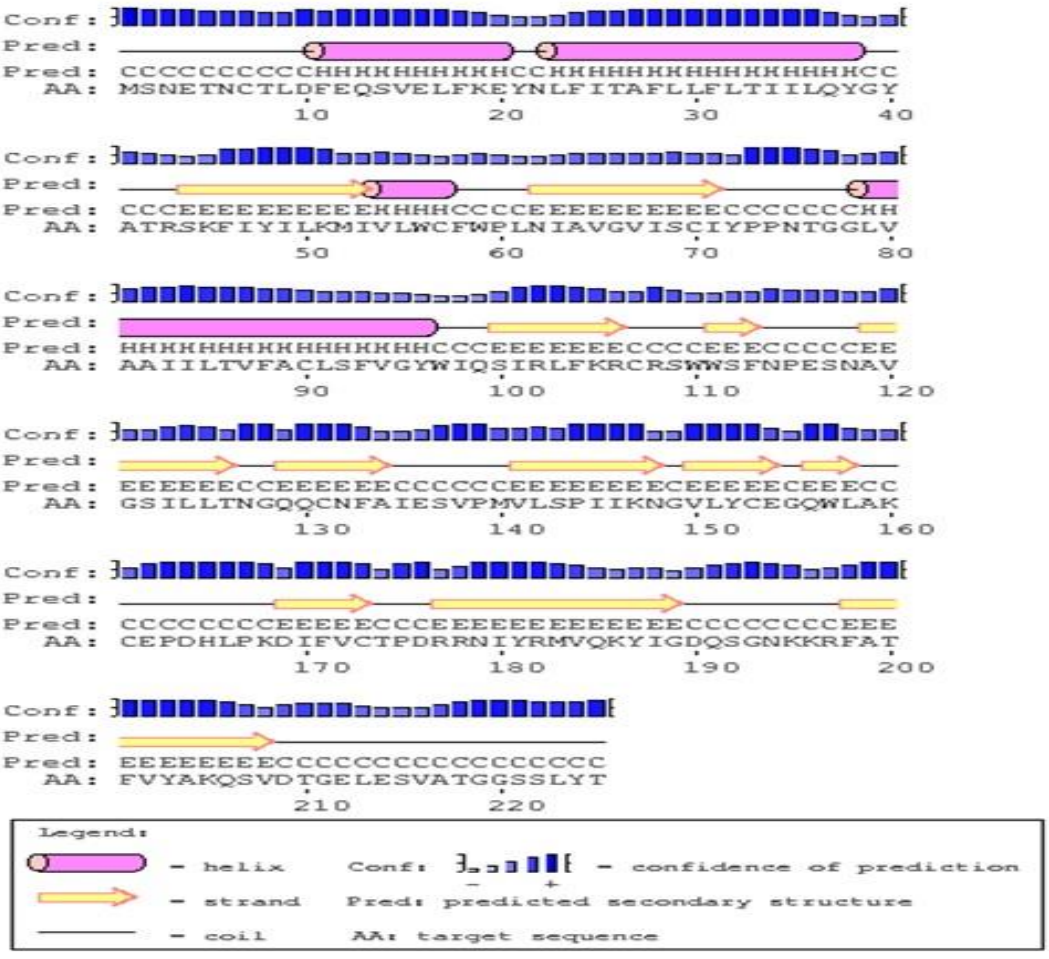

Fig. 3. M protein secondary structure of IBKG3 strain predicted by PSIPRED.

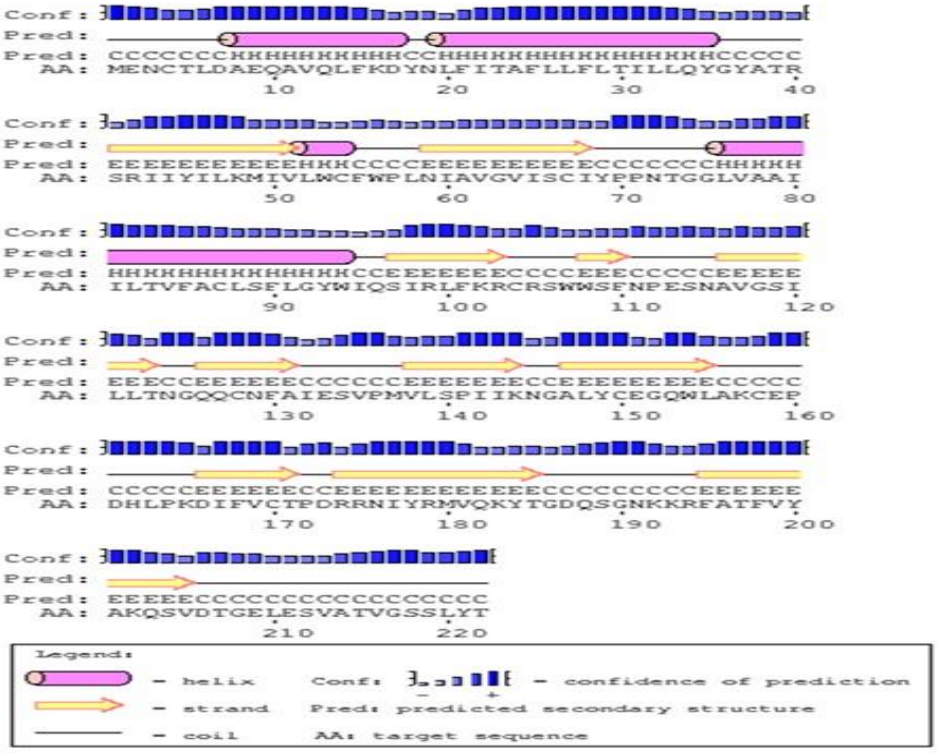

Fig. 4. M protein secondary structure of IBKG7 strain predicted by PSIPRED.

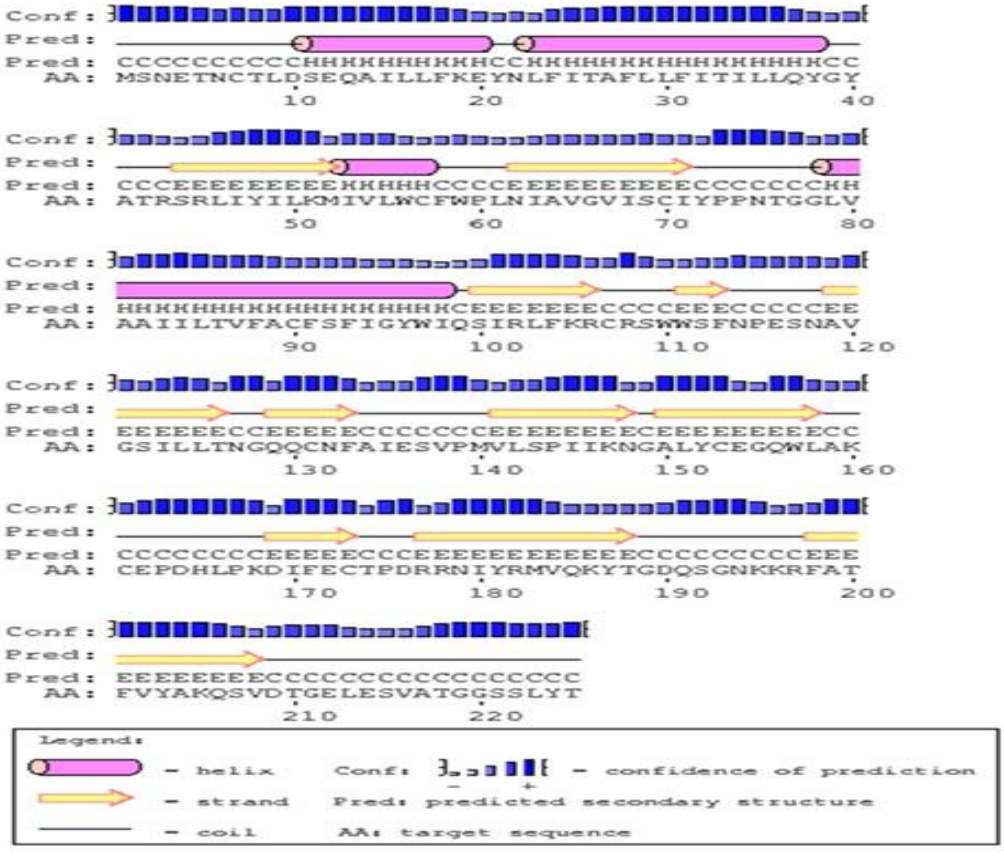

Fig. 5. M protein secondary structure of IBKG10 strain predicted by PSIPRED.

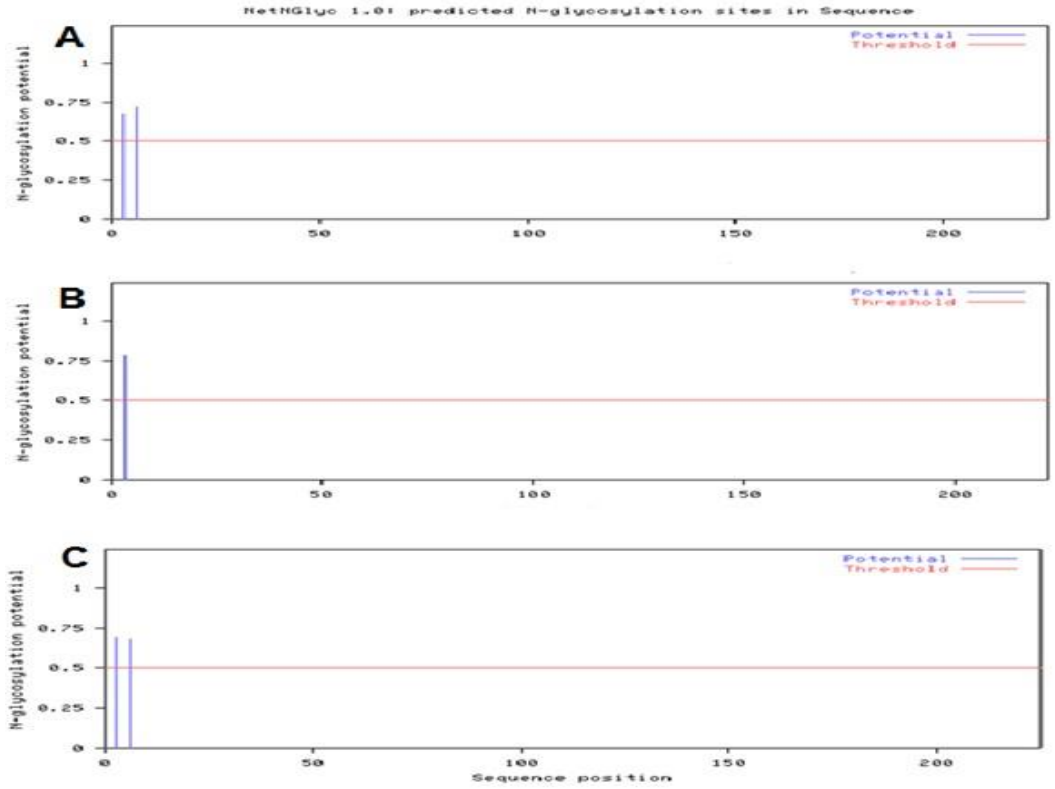

Fig. 6. Graphic representation of N-glycosylation site analysis of the M amino acid sequence of three strains, using NetNGlyc 1.0 computer program. Threshold=0.5. A; IBKG3, B; IBKG7, C; IBKG10.

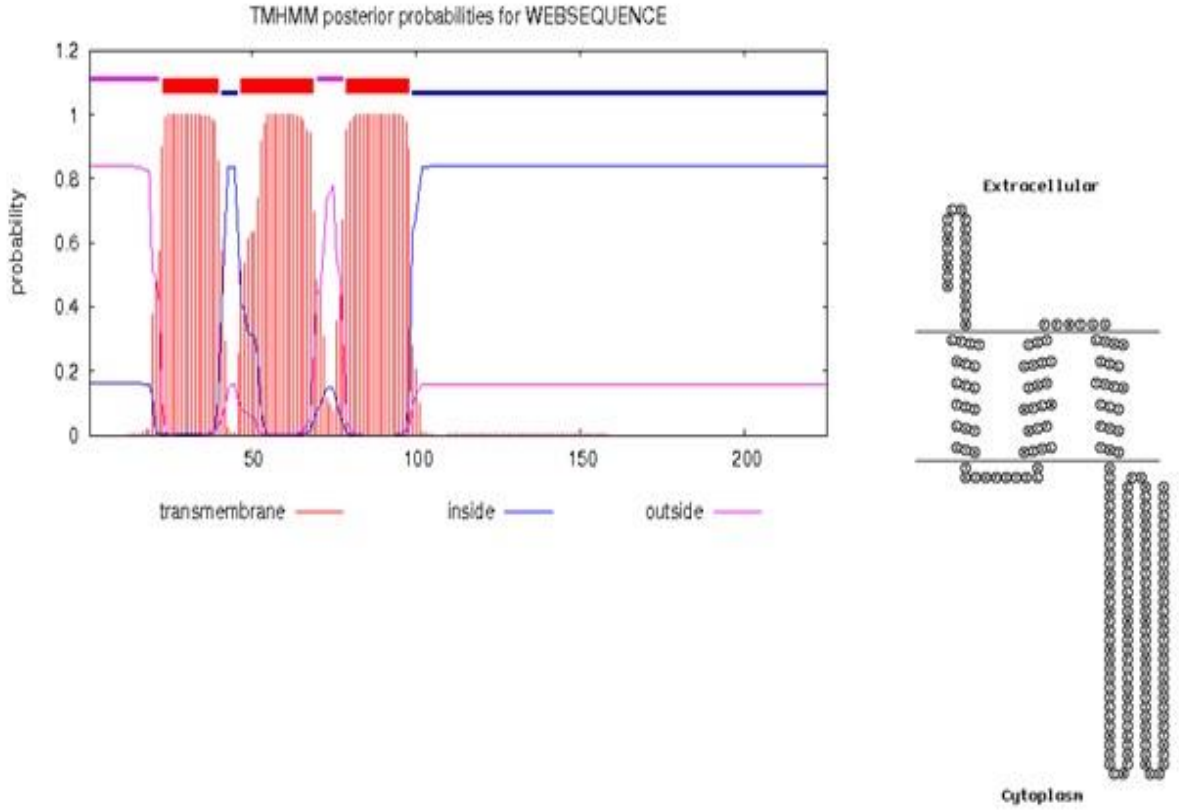

Fig. 7. a snake-like diagram of the predicted TM topology mode of IBKG3 strain.

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 IBGK10 had two Potential glyco-

sylation 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 variant2 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.

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