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Genetic Analysis of Three Structural Proteins in Iranian Infectious Bronchitis Virus Isolate

Research Article

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ABSTRACT

Infectious bronchitis virus (IBV) is a contagious pathogen in fowl that results in economic loss in the poultry industry. In this study, the amino acids sequences of three structural proteins M, N, and S1 for five Iranian IBV isolated during 1998-2011 have been analyzed. Conserved and variable regions, hydrophobic characteristics and identity matrix were determined after alignment by Bioedit ver 7.0.4.1. The phylogenetic tree was obtained by using the neighbor-joining method within MEGA4. 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. For the N protein, highest similarity was found between Ur1 / 09 and Arkansas DPI (0.948). Four conserved regions for M and N proteins were recognized. In the S1 protein three hypervariable regions were detected in 52-90, 124-150 and 265-315 residues. In a phylogeny analysis all proteins were distributed in three clusters. Iranian IBV belonged to Mass cluster in phylogeny tree of M and N proteins. But S1 protein showed a close relationship with the California serotype and was distantly related to Mass serotype. The results showed that the Iranian IBV isolate was probably diverted from Mass strain that might be brought to Iran as a vaccine strain.

KEY WORDS diversity, genome, infectious bronchitis virus, phylogeny.

INTRODUCTION

Avian infectious bronchitis virus causes respiratory tract disease in chickens, which results in reducing weight gains and egg production. IBV is the first Coronavirus discovered (Beaudette *et al*. 1937). More than 20 serotypes within the IBV are recognized worldwide. The Massachusetts (Mass)-type was first isolated in Europe in the 1940 s (Roussan *et al*. 2009). Massachusetts strains such as M41 and H120 have been widely used for immunization in many countries as Iran (Chen *et al*. 2010). The IBV genome is single-stranded RNA with 27.6 kb in length and positive polarity (Shen *et al*. 2003; Xu *et al*. 2007). It encodes four structural proteins such as S, M, N and E glycoprotein (Park *et al*. 2005). The S protein is a dimer or trimer that attaches the virus to the receptor on host cells for releasing the viral genome into the cell (Corse and Machamer. 2003). It is divided into two subunits, S1 and S2 (Michael *et al*. 2003). The S1 subunit makes up the head of the S protein. Various S1 serotypes are produced by insertions and deletions in nucleotides or RNA recombination (Lee *et al*. 2004; Ren *et al*. 2009; Linlin *et al*. 2010). In spite of the fact that serotyping is generally based on the S protein, the N and M proteins are also important for immunogenic studies (Williams *et al*. 1992).

The IBV's N protein is a phosphorylated protein with 409 amino acids. It is highly conserved, especially in the middle, among IBV strains (Parker and Masters 1990; Chen *et al*. 2005). N protein reorganize viral RNA to form a helical ribonucleoprotein complex, that plays a role in genome

replication, transcription, translation, and formation of the nucleocapsid (Zhou and Collisson 2000; Jayaram *et al*. 2005; Spencer and Hiscox 2006).

Similarly, the N protein plays a role in packaging and correct folding of the RNA molecule, so it may be useful for a vaccine synthesis (Williams *et al*. 1992). M protein is a transmembrane protein. It is the most abundant glycoprotein in the virus and in cell that is infected (Maeda *et al*. 2001). It plays a role in interferon induction (Ren *et al*. 2009) and contains a Golgi target signal that triggers the concentration of the E protein into the Golgi (Corse and Machamer, 2003).

Co-expression of M and E proteins cause formation virion that has interferon induction features. In contrast, expression of the M protein alone does not induce virion production (Maeda *et al*. 2001). The first report in Iran for predominance of IBV infection in chicken was in 1994 (Aghakhan *et al*. 1994).

Since then, the IBV has showed its presence almost all over the country. Until now, no significant study has been carried out for genetic diversity and the origin of IBV in Iran. Therefore, the present study was planning to investigate protein sequencing and phylogenetic analysis of IBV isolates in Iran.

MATERIALS AND METHODS

Amino acid sequence of structural protein in five Iranian IBV published in Genbank during 1998-2011 were used in this study (Table 1). The sequences comprised the structural proteins of 11 reference strains that had complete genomes sequence in NCBI and had been used in other research as reference sequence. For Iranian IBV, five sequences for N, M and S1 protein were retrieved from Genbank. Table 1 summarizes the source and the accession numbers of all sequences used.

The amino acid sequences were aligned with Bioedit ver 7.0.4.1 and conserved and variable regions, hydrophobic characteristics and identity matrix (similarity) were determined.

Hydrophobic characterization was performed with Kyte and Doolittle means hydrophobicity profile with Bioedit. The phylogenetic trees were obtained with the neighbor-joining method in the MEGA4 software with 1000 bootstrapping replicates.

RESULTS AND DISCUSSION

The amino acid sequences of N, M and S1 proteins according to accession number in table 1 were obtained from Genbank. They were aligned and compared with Bioedit and Mega4 software. Phylogeny tree (Figure 1), similarity (Table 2, 3) conserved and variable regions and hydrophobic characteristics were determined in this study (Table 4).

Phylogeny analysis

Phylogeny analysis in four proteins demonstrated three clusters (Figure1). Iranian IBVs isolate belonged to Mass cluster in M and N protein. For the N protein, the CQ04-1, GX-YL9 from China and TW2575 / 98 from Taiwan localized in two independent clusters. In the M protein, CQ04-1 from the Iran isolate co-localised in the Mass cluster but GX-YL9 and TW2575 / 98 were independent clustered together. For the S1 protein, Iran, Asian, Arkansas DPI and Cal557 2003 strain / isolates were in the same cluster.

Similarity

For the N protein, Iran isolates had 91-95% similarity with all other strains (Table2). Similarity was lowest between Beaudette and CQ04-1 (89.2%) and highest among Mass41 strains (99.7%). For the M protein, identity matrix showed that IR-3654-VM (Iran) and CQ04-1 (China) has highly similarity with cal 557 2003 (Table 2). With regard to the S1 protein, the similarity matrix showed 70-73% relationship among Iran and other isolates, but those had lowest similarity with Georgia 1998 strain (0.41) (Table 3).

Conserved regions

Four conserved regions were observed at residues 87-102, 161-175, 310-336 and 374-391 for the N protein whereas for the M protein these regions were found as 55-91, 152-166, 168-188 and 190-207 residues. The conserved sequences are presented in Table4. For S1 subunit, no conserved region was detected.

Hypervariable regions

Three hypervariable regions were recognized at residues 52-90, 124-150 and 265-315 for the S1 protein, but none were found for the other proteins.

Gaps

No gaps were detected in N protein sequences in all strains / isolates. M protein sequences showed one gap at the N-terminal end. In addition, another gap as long as 8 residues was detected in the Iran isolate in amino acids 218-226. Regarding the S1 protein four gaps with mean 4-7 residues were located at residues 74-80, 151-155, 301-305 and 351-354.

Variability in coronaviruses genome was related to two origins; the first one is spot mutations as a result of non proof-reading capacity in RNA polymerases and the second one is as recombination events in the viral genome. This represents a mechanism for antigenic and pathogenic evolution (Lai 1992; Shoshtari *et al*. 2008).

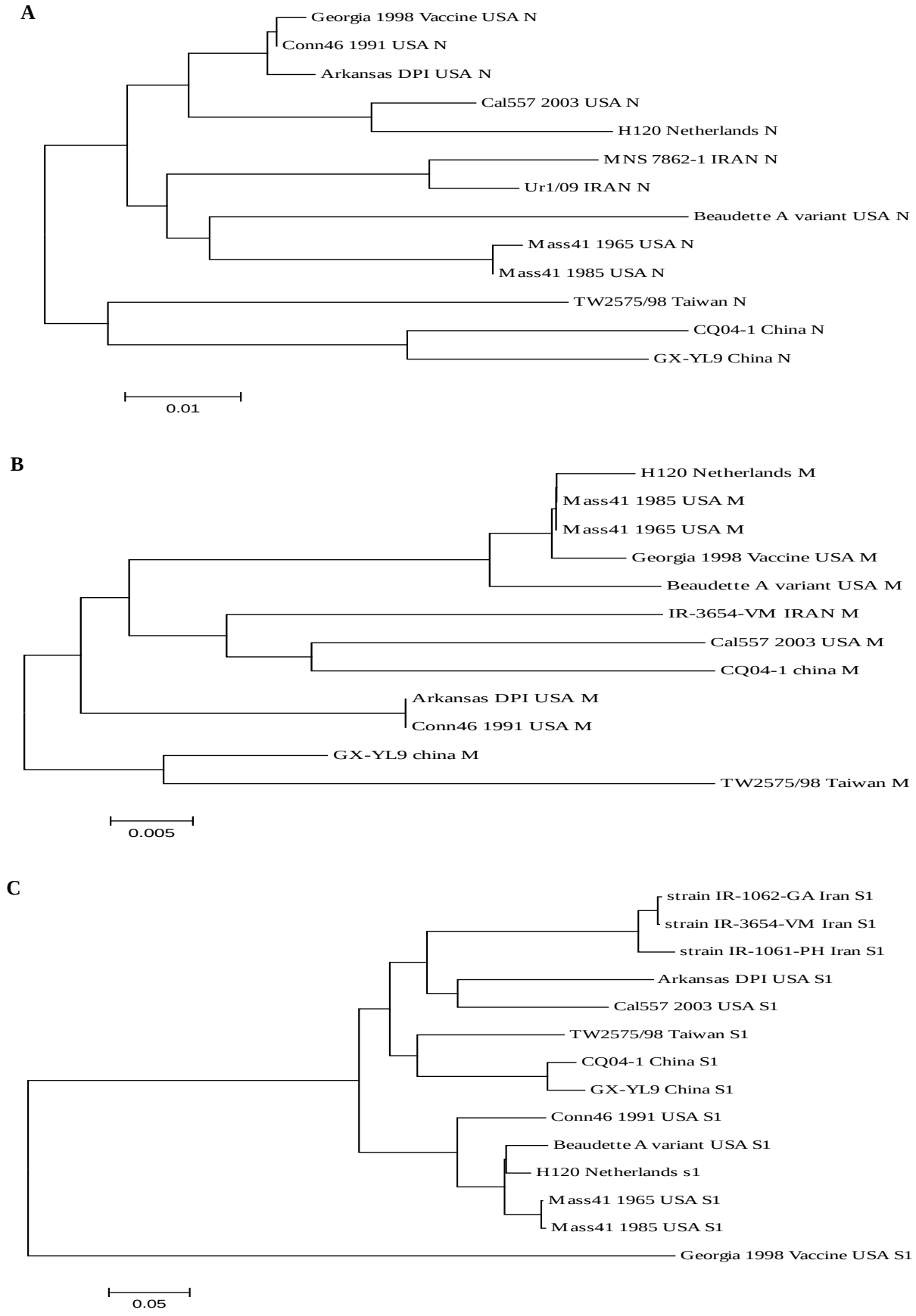

Figure 1 Phylogenic trees based on the three structural protein N (a), M (b), and S1 (c) of IBV isolates constructed by Neighbor-Joining method with the MEGA 4 (1000 Bootstrap replicates, each)

Table 1 IBV protein structural sequence published in Genbank

Strain / isolate	membrane (M) glycoprotein	nucleocapsid (N) protein	Spike (S1) glycoprotein	Serotype / isolate
Arkansas DPI USA	ADP06455	ADP06452	ADP06459	-
Beaudette USA	AAY24437	AAY24440	AAY24433	IBV-p65
Cal557 2003 USA	ADA83491	ADA83492	ADA83490	California
CQ04-1 China	ADI54959	ADI54962	ADI54955.	CQ04-1
Georgia1998Vaccine USA	ADP06488	ADP06485	ADP06492	-
GX-YL9 China	AEB00719	AEB00722	AEB00715	chick embryo
H120 Netherlands	ACQ55234	ACQ55237	ACQ55230	-
Mass41 1965 USA	ADA83541	ADA83544	ADA83537	Massachusetts
TW2575 / 98 Taiwan	ABG36791	ABG36794	ABG36787	TW2575/98
Conn46 1991 USA	ADA83531	ADA83534	ADA83527	Connecticut
Mass41 1985 USA	ADA83571	ADA83574	ADA83567	Massachusetts
MNS 7862-1 Iran	-	AEF98428	-	-
Ur1/09 Iran	-	AEF98429	-	-
IR-3654-VM Iran	ADF78097	-	-	-
IR-1061-PH Iran	-	-	AAS48626	793/B
IR-1062-GA Iran	-	-	AAS48625	793/B
IR-3654-VM Iran	-	-	AAS48624	793/B

Table 2 Comparison of the amino acid sequences of the N (upper triangular) and M (lower triangular) protein of 13 Iranian and non-Iranian IBV strains

Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. Arkansas DPI	****	0.938	0.965	0.924	0.992	0.921	0.955	0.953	0.933	0.995	0.955	0.941	0.948	-
2. Beaudette	0.928	****	0.924	0.892	0.938	0.894	0.911	0.933	0.907	0.941	0.936	0.919	0.929	-
3. Cal557 2003	0.933	0.915	****	0.916	0.963	0.921	0.97	0.941	0.916	0.965	0.943	0.929	0.938	-
4. CQ04-1	0.915	0.92	0.933	****	0.921	0.955	0.904	0.899	0.916	0.924	0.902	0.914	0.914	-
5. Georgia 1998	0.933	0.982	0.933	0.92	****	0.924	0.953	0.955	0.936	0.997	0.958	0.943	0.951	-
6. GX-YL9	0.915	0.924	0.911	0.933	0.928	****	0.914	0.909	0.914	0.926	0.911	0.909	0.916	-
7. H120	0.933	0.982	0.924	0.92	0.991	0.928	****	0.926	0.904	0.955	0.929	0.916	0.931	-
8. Mass41 1965	0.937	0.986	0.928	0.924	0.995	0.933	0.995	****	0.916	0.958	0.997	0.936	0.938	-
9. TW2575 / 98	0.924	0.92	0.92	0.911	0.924	0.937	0.924	0.928	****	0.938	0.919	0.911	0.907	-
10. Conn461991	0997	0.928	0.928	0.915	0.933	0.915	0.933	0.937	0.924	****	0.96	0.946	0.953	-
11. Mass41 1985	0.937	0.986	0.937	0.924	0.995	0.933	0.995	0.997	0.928	0.937	****	0.938	0.941	-
12. MNS 7862-1	-	-	-	-	-	-	-	-	-	-	-	****	0.977	-
13. Ur1/09	-	-	-	-	-	-	-	-	-	-	-	-	****	-
14. IR-3654-VM	0.893	0.897	0.905	0.889	0.888	0.884	0.888	0.893	0.862	0.893	0.893	-	-	****

Table 3 Comparison of the amino acid sequences of the S1 protein in 14 Iranian and non-Iranian IBV strains

Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. Arkansas DPI	****	0.722	0.776	0.705	0.428	0.703	0.720	0.714	0.738	0.714	0.714	0.702	0.714	0.717
2. Beaudette	0.722	****	0.737	0.731	0.460	0.725	0.959	0.944	0.746	0.868	0.941	0.707	0.710	0.713
3. Cal557 2003	0.776	0.737	****	0.714	0.440	0.709	0.745	0.731	0.716	0.728	0.731	0.728	0.731	0.734
4. CQ04-1	0.705	0.731	0.714	****	0.445	0.959	0.734	0.734	0.784	0.720	0.734	0.718	0.732	0.730
5. Georgia1998	0.428	0.460	0.440	0.445	****	0.434	0.468	0.460	0.435	0.442	0.460	0.414	0.425	0.422
6. GX-YL9	0.703	0.725	0.709	0.959	0.434	****	0.731	0.728	0.787	0.728	0.725	0.721	0.732	0.730
7. H120	0.720	0.959	0.745	0.734	0.468	0.731	****	0.959	0.754	0.892	0.956	0.710	0.710	0.713
8. Mass41 1965	0.714	0.944	0.731	0.734	0.460	0.728	0.959	****	0.740	0.880	0.997	0.704	0.713	0.715
9. TW2575 / 98	0.738	0.746	0.716	0.784	0.435	0.787	0.754	0.740	****	0.752	0.740	0.726	0.729	0.732
10. Conn46 1991	0.714	0.868	0.728	0.720	0.442	0.728	0.892	0.880	0.752	****	0.877	0.704	0.710	0.713
11. Mass41 1985	0.714	0.941	0.731	0.734	0.460	0.725	0.956	0.997	0.740	0.877	****	0.701	0.710	0.713
12. IR1061-PH	0.702	0.707	0.728	0.718	0.414	0.721	0.710	0.704	0.726	0.704	0.701	****	0.953	0.950
13. IR1062-GA	0.714	0.710	0.731	0.732	0.425	0.732	0.710	0.713	0.729	0.710	0.710	0.953	****	0.997
14. IR3654-VM	0.717	0.713	0.734	0.730	0.422	0.730	0.713	0.715	0.732	0.713	0.713	0.950	0.997	****

The first study in genetic diversity among IBV serotypes was reported by RNA fingerprint analyses in 1981 (Clewley *et al*. 1981). However, research on phylogenetic relationships was published in 1989 in nucleotide sequence analyses (Kusters *et al*. 1989).

Nowadays, phylogenetic analyses are imperative in evolutionary studies in viruses.

In this research we studied phylogeny relationship of three structural proteins in IBV isolate of Iran with some reference strains.

In Phylogeny analysis of S1 protein, Iranian and also Asian isolate were distantly related to Mass serotype. In phylogenetic tree of nucleocapsid protein, Iranian IBV belonged to Mass type but China and Taiwan isolates (GX-YL9, CQ04-1 and TW2575 / 98) formed two independent clusters different from the Mass type.

Table 4 Conserved regions in the N and M structural proteins

Protein	Position	Conserve sequence
N	87-101	VPDAWYFYYTGTGPAA
N	161-175	NRGRSGRSTAASSAA
N	310-336	DPQFDNYVKICDQCVDGVGTRPKDDEP
N	374-391	DKALTSDEERNNAQLEFD
M	55-91	VLWCFWPLNIAVGVISCIYPPNTGGLVA AIILTVFAC
M	152-166	LYCEGQWLAKCEPDH
M	168-188	PKDIFVCTPDRRNIYRMVQKY
M	190-207	GDQSGNKKRFATFVYAKQ

Evidence in hand showed that the N protein is more conserved, but the S1 glycoprotein has changed due to mutation and recombination between IBV serotypes (Williams *et al*. 1992; Jayaram *et al*. 2005; Chen *et al*. 2005). This might indicate that the origin of Iranian IBV isolate was different to isolates in China and Taiwan. Since the N protein that is conserved belongs to different groups, but during evolution because of recombination among this isolate in a near area, S1 protein be closed together. In M protein, Asian isolate divided into two groups. Iran isolate with CQ04-1 of China was in a same cluster with Mass type but TW2575/98 and GX-YL9 isolates were differing from them. Phylogeny analysis in three proteins, dedicated that Iran isolate might be diverted from Mass type.

Similarity score for N protein, among Iran strain with Asian isolates and American strains were 91% and 93-95% respectively. Total variation in identity among all isolates/strains was 2-10%. It was shown that the N protein was highly conserved. According to other research, the N protein has 94-99% identity among various strains and its highly immunogenic (Ndifuna *et al.* 1998). High similarities among Iran isolate (IR-3654-VM) and other reference strains were detected for M protein (86-90%). This protein affects the viral assembly process in coronaviruses, which explain the pressure to be conserved (Zhang *et al*. 2010).

Molecular studies have indicated that a few changes in amino acid sequence of the S1 subunit can produce new IBV serotypes (Liu *et al*. 2003). The length of S protein varied among 535-548 amino acids. It contains a signal peptide and an arginine-rich cleavage site between S1 and S2 (Sapats *et al*. 1996; Linlin *et al*. 2010). Molecular data for S1 protein showed that Iranian IBVs had 95-99% identity within groups and had over 70-71% homology with American strains in this group, Mass type varied 1-5% within each other. The IBV vaccine strains applied in Iran are mainly correlated with the Mass type (Haqshenas *et al*. 2005). As the similarity among strain related to cross-protection observed between them (Adzhar *et al*. 1995) so low similarity between the Iranian IBV isolate and the Mass serotype caused that vaccination cannot stop the outbreak of IBV. This could explain why IB has still occurred in vaccinated flocks in Iran.

Three hypervariable regions were recognized in 52-90, 124-150 and 265-315 residues of S1 protein. Amino acid variations between residues 52-90 and 124-150 reported herein, were similar to HVR I (residues 56-69) and HVR II (residues 117-131) of Mass and European strains reported previously (Kusters *et al*. 1989; Wang *et al*. 1994). Change in amino acid at HVR may cause changes in epitopes sequence and protein folding, which results in distinct host cell antibody (Wang and Huang 2000).

As for the M protein, four common conserved regions were detected, three of which (152-166, 168-188 and 190-207 residues) belonged to the end-domain. It is believed that the C-terminal end-domain of the M protein interacts with the N and S proteins, which has prominent role in the formation assembly of the virus (Zhang *et al*. 2010). The first conserved region was hydrophobic, while all others were hydrophilic. Four conserved regions were recognized in the N protein sequence. The first one (87-102 residues) might represent a critical sequence in coronavirus replication (Park *et al*. 2005). Some studies reported that residues between 91 and 171 interact with viral RNA by maintaining a certain structure of the peptide (Zhou and Collisson 2000). Zhang *et al*. (2010) found that interaction of the N and M proteins take place in the residues 168-225 of the M protein and the residues 150-210 of the N protein. All four conserved regions in the N protein were hydrophilic. The result for conserved region in M and N protein are the same as for the Mass serotype.

Some gaps in S1 and M protein were detected. These gaps might emerge as a result of mutation by deletion or insertion, which might eventually cause changes in IBV serotypes (Liu *et al*. 2003).

CONCLUSION

In conclusion, the present study has demonstrated that Iranian IBV strains are genetically diverse and are under continuing evolution. It seems they might derive originally from Mass type strains as the result of mutation or recombination with vaccine strain. Nonetheless, during evolution circulating IBV strains have evolved and gain genetic distance from vaccine strains. This result obtained from a few protein sequence of Iranian IBV that we found in NCBI. For many of IBV strain we don't have a sequence in Genbank, so further study are need in sequencing of Iranian

IBV isolate to better stop outbreaks of IBV infections in commercial flocks.

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