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Original Article

Nucleotide Sequence Analysis of *S1* Gene among Iranian Avian Infectious Bronchitis Viruses Isolated during 2001-2002

Salarpour [1], A., Toroghi [1,*], R., Momayez [2], R.

1. Department of Veterinary Research and Biotechnology, Razi Vaccine and Serum Research Institute, Agricultural Research, Education, and Extension Organization, Mashhad, Iran
2. Department of Poultry Disease Research and Diagnosis, Razi Vaccine and Serum Research Institute, Agricultural Research, Education, and Extension Organization, Karaj, Iran

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Corresponding Author: r.toroghi@rvsri.ac.ir

ABSTRACT

Infectious bronchitis (IB) virus genome codes for four structural proteins, among which the S1 subunit of spike glycoprotein comprises the major epitopes to induce neutralizing antibodies. This study involved the comparison of the full S1 sequences of five IB viruses, namely two Massachusetts and three 793/B serotypes, isolated from IB outbreaks during 2001-2002, with all other Iranian and foreign 793/B isolates and 10 known serotypes. Analysis of S1 subunit showed three unique amino acid changes at positions 349 (V to L), 392 (T to N), and 393 (Q or R to T or K or S) for the Iranian 793/B isolates, compared to those of the foreign 793/B isolates reported before 2006 (onset of vaccination with 793/B vaccine in Iran). They were used as amino acid markers for the differentiation of Iranian 793/B isolates for years. Sequence alignment of the Iranian isolates with those of the foreign ones reported after 2006 demonstrated that amino acids 392 and 393 were no longer considered as amino acid markers, and only the change in amino acid 349 still remained specific to the Iranian 793/B isolates. Phylogenetic tree sequence analysis revealed that the Iranian 793/B isolates were closely related indicating that they came from a single source, more probably from France. There was a very close correlation between the first detection of 793/B serotype and the time of French chicken meat importation. Moreover, it was shown that one of the Massachusetts isolates was completely identical with the H120 vaccine strain. Furthermore, the other Massachusetts isolate with two amino acid changes at positions 64 (G to E) and 95 (S to R) was very similar to this vaccine strain. It seems that the latter isolate is a passaged chicken H120 vaccine strain.

Keywords: Infectious bronchitis virus, 793/B serotype, S1 gene, Amino acid marker, Phylogenetic analysis

Analyse de la Séquence Nucléotidique du Gène S1 Parmi les Virus de la Bronchite Infectieuse Aviaire Isolés en Iran entre 2001 et 2002

Résumé: Le génome du virus de la bronchite infectieuse (BI) code quatre protéines structurelles, parmi lesquelles la sous-unité S1 de la glycoprotéine de Spike comprend les principaux épitopes permettant d'induire des anticorps neutralisants. Cette étude a pour objectif de comparer les séquences S1 complètes de cinq virus de BI, à savoir deux sérotypes Massachusetts et trois sérotypes 793/B, isolés des foyers d'infection épidémique de BI entre 2001 et 2002, avec tous les autres isolats 793/B iraniens et étrangers ainsi que 10 sérotypes connus. L'analyse de la sous-unité S1 a montré trois changements uniques des acides aminés aux positions 349 (V à L), 392 (T à N) et 393 (Q ou R à T ou K ou S) pour les isolats iraniens 793/B, comparés rapport à ceux des isolats étrangers 793/B signalés avant 2006 (début de la vaccination par le vaccin 793/B en Iran). Ils ont été utilisés comme acides aminés marqueurs afin de différencier les isolats iraniens 793/B pendant des années. L'alignement des séquences des isolats iraniens avec celles des isolats étrangers signalés après 2006 a montré que les acides

aminés 392 et 393 n'étaient plus des acides aminés marqueurs et que seulement le changement de l'acide aminé 349 demeurait spécifique aux isolats iraniens 793/B. La reconstruction de l'arbre phylogénétique a partir des séquences obtenues a révélé que les isolats iraniens 793/B étaient étroitement liés, indiquant qu'ils provenaient d'une source unique, plus probablement de France. Il existait en effet une corrélation très étroite entre la première détection du sérotype 793/B et la periode de l'importation de la viande de poulet française. De plus, il a été démontré qu'un des isolats du Massachusetts était complètement identique à la souche vaccinale H120. De plus, l'autre isolat du Massachusetts avec deux changements d'acide aminé aux positions 64 (G à E) et 95 (S à R) était très similaire à cette souche vaccinale. Il semble que ce dernier soit une souche vaccinale H120 de poulet ayant subi plusieurs passagesé.
Mots-clés: Virus de la bronchite infectieuse, Sérotype 793/B, Gène S1, d'Acide aminé marqueur, Analyse phylogénétique

INTRODUCTION

Infectious bronchitis (IB) is an acute and highly contagious respiratory disease, which causes tracheal rale, sneezing, and coughing in chickens. This disease is of economic importance due to its accompaniment with weight loss and feed deficiency. Coinfection with other infectious agents results in airsacculitis and a decline in egg quality and quantity. Highly contagious nature of the disease and concurrent infections with several serotypes have made the disease very complex and expensive to control through vaccination. The IB virus is located in group 3 coronavirus (i.e., gammacoronavirus) and Coronaviridae family. The virus is round and polymorphic in shape and has a membrane of 120 nm in diameter with club-shaped projections with a length of 20 nm. The IB virus virion has four major structural proteins, including spike (S), membrane glycoprotein (M), nucleoprotein (N), and envelope (E). The S glycoprotein splits into S1 (535 amino acids) and S2 subunits (627 amino acids) at the arginine-rich region called cleavage site. The S1 and S2 subunits are attached together through noncovalent bonds. Antibodies induced against S1 play a role in hemagglutination inhibition and virus neutralization (Cavanagh, 1983; Kant et al., 1992). The S2 subunit strengthens the S protein structure by membrane anchoring. The S1 is responsible for virus-cell attachment and genetic variation of the virus (Kwon et al., 1993). The genetic variation of the virus exists not only among different serotypes, but also among the strains of a serotype. One of the reasons for such virus genetic variation is the frequent mutation of the virus, which can occur in the form of point mutation, deletion, insertion, and recombination. These mutations are responsible for the evolution and survival of the virus. Genetic and antigenic variations in S1 glycoprotein are the results of the virus response to immune pressure caused by the implementation of vaccination programs and other management protocols against the disease. In many cases, IB outbreaks in a region result from the emergence of IB virus serotypes, which are serologically different from the applied vaccine. There are many serotypes of IB virus, including Massachusetts, Connecticut, Arkansas, Gray, T, and 793/B (4.91) (Cavanagh, 1983). Many different systems have been proposed for the classification of IB viruses. These systems can be categorized as functional and nonfunctional methods. Functional methods focus on virus biology, while nonfunctional methods focus on viral genome. Real-time polymerase chain reaction (RT-PCR), RT-PCR-restriction fragment length polymorphism, and nucleotide sequencing are considered as nonfunctional methods for virus classification. On the other hand, the identification of protectotypes and serotypes is carried out by functional methods (Jackwood et al., 2001). Nucleotide sequencing and deduced amino acid sequences of viral proteins are applicable to assess molecular epidemiology and identify the conserved and variable

regions of the proteins. These assessments, along with epitope mapping by means of monoclonal antibodies, can give valuable information on amino acid markers. The first isolated IB viruses in Iran were located in Massachusetts serotype with no evidence of the presence of IB virus variants. However, during the time, the dominance of Massachusetts serotype was replaced with new serotypes. Studies in different parts of Iran demonstrated that 793/B serotype as the most dominant serotype in Iran was responsible for IB outbreaks before using 793/B vaccine in the country (Seyfi Abad Shapouri et al., 2004). Following the occurrence of 793/B serotype in this country, vaccination against this serotype was launched in 2006 (Hosseini et al., 2015). New serotypes have emerged in Iran over time. Recent studies have revealed the presence and dominance of seven distinct phylogenetic groups, including IS/1494-like, 793/B-like, Massachusetts, QX-like, IS/720-like, IR-1, and IR-2 viruses, with the domination of IS/1494-like virus (Najafi et al., 2016; Modiri Hamadan et al., 2017). The 793/B serotype has existed in Iran for years and is still one of the most important IB virus serotypes in our country. Regarding this, it is important to compare the characteristics of the Iranian 793/B isolates and more deeply study the genomic changes of this serotype over time. The present study involved the investigation of five IB viruses isolated from early IB outbreaks in Iran, two cases of which were confirmed to be Massachusetts and three others were non-Massachusetts. Based on the specific RT-PCR, the three non-Massachusetts isolates belonged to 793/B serotype. The sequencing of S1 gene was used to differentiate the Iranian isolates of 793/B serotype from the foreign 793/B isolates in our laboratory for years. The 793/B viruses reported in this study, along with three other Iranian 793/B isolates (Hosseini et al., 2015), are the only Iranian 793/B viruses that have been sequenced before using 793/B commercial vaccine in 2006 in Iran. The current study was targeted toward comparing the changes of S1 gene among the Iranian 793/B viruses over time before and after 2006. Our aim was to assess the use of S1 gene sequencing for the differentiation of the Iranian 793/B from the foreign isolates. The results of this study could render some information regarding the likely origin of 793/B serotype in Iran.

MATERIAL AND METHODS

Viruses. Five IB viruses isolated from the broiler flocks of different parts of Iran in 2001 were used in this study (Table 1).

Table 1. Characteristics of the viruses used in this study

Isolate	GenBank accession number	Serotype	Province
IR/773/2001	MH252985	739/B	Ardebil
IR/520/2002	MH252990	739/B	Tehran
IR/794/2002	MH236618	739/B	Tehran
IR/680/2002	MH252988	Massachusetts	Tehran
IR/801/2001	MH252989	Massachusetts	Gilan

Real-time polymerase chain reaction. Viral RNA was extracted using the viral RNA extraction kit (Roche, Germany) according to the manufacturer's instructions for the amplification of the full sequence of *S1* gene in our five IB isolates. The RT-PCR reaction was performed using the primers adopted by Callison et al. (2001) (5′-TGAAACTGAACAAAAGAC-3′ as forward and 5′-CATAACTAACATAAGGGCAA-3′ as reverse primers). The RT was performed by mixing 4 µL of 5X AMV enzyme buffer, 15 pmol of mentioned reverse primer, 20 µM of each dNTPs, 20 units of RNase inhibitor, 2 µg RNA, 20 units of AMV enzyme (Roche, Germany), and nuclease-free distilled water up to 20 µL. The mixture was incubated at 42 °C for 1 h, and then at 95 °C for 3 min. Finally, PCR was carried out using Expand High Fidelity PCR System (Roche, Germany) by mixing 10X buffer containing 1.5 µmol $MgCl_2$, 15 pmol of each primer, 20 µM of each dNTPs, 3 µL complementary DNA, 2.6 units of Taq and Tgo enzymes, and nuclease-free distilled water up to 50 µL. The reaction mixture was subjected to 35 cycles of 94 °C, 45 °C, and 72 °C for 1, 1.5, and 3 min, respectively, followed by a final extension at 72 °C for 10 min. The

PCR products (1720 bp) were confirmed according to their size in 1% agarose gel electrophoresis, along with 100 bp DNA marker. Purification of PCR products was performed using the PCR purification kit (Roche, Germany) following the manufacturer's instruction. Nucleotide sequencing was carried out using the mentioned primers and those used by Worthinton et al. (2008) as internal primers (i.e., SX1 and SX2). Nucleotide sequences were analyzed in MEGA software (version 6).

Analysis of S1 nucleotide and deduced amino acid sequences. MEGA software (version 6) was applied to align and analyze *S1* nucleotide and deduced amino acid sequences of our five isolates, along with 91 partial and complete coding sequences of the other Iranian and foreign 793/B serotypes, and also for the complete coding sequence of 10 known serotypes retrieved from GenBank. Furthermore, the investigation of nucleotide and amino acid differences was accomplished using CLC Main Workbench (version 5.5). Table 2 lists the isolates and the accession numbers. The mentioned nucleotide sequences were subjected to pairwise comparison. Since our aim was to track amino acid changes in the native Iranian isolates and given the possibility of having 793/B viruses with vaccine origin, all of the reported Iranian 793/B viruses of field and vaccine origins were grouped based on amino acid 95. In this regard, in case of the presence of serine or alanine at this position, the virus was considered as field or vaccine origins, respectively.

Phylogenetic analysis. Phylogenetic analysis was performed using *S1* sequences of our five isolates, along with all other full coding sequences (i.e., 1,617 nucleotides and 539 amino acids) retrieved from GenBank. The analysis was performed in MEGA software (version 6) using the neighbor-joining method with 1,000 bootstrap replicates (Tamura et al., 2011).

RESULTS

Analysis of nucleotide and amino acid sequences. Sequence alignment of the Iranian and foreign 793/B isolates reported before 2006 demonstrated three nucleotide and amino acid changes that were specific and unique to the Iranian isolates (Figure 1). The differences were located at nucleotide positions 1045 (G to T), 1175 (C to A), and 1177 (C to A), as well as the amino acid positions 349 (V to L), 392 (T to N), and 393 (Q or R to T, K or S), respectively. None of these changes were observed in the foreign 793/B isolates reported before 2006; therefore, we considered the mentioned amino acids as amino acid markers for the differentiation of Iranian 793/B isolates from the foreign ones. Among the Iranian 793/B isolates reported after 2006, IR/491/08 was considered to be a vaccine virus because of having alanine at position 95 and showing 99.9% similarity to 793/B vaccine. All three amino acid markers (i.e., 349, 392, and 393) in the isolate IR/491/08 were different from those of the Iranian 793/B isolates. Investigation of the *S1* sequence changes and three amino acid markers among the Iranian field 793/B isolates reported after 2006 demonstrated that they had the mentioned three unique amino acid markers similar to the Iranian 793/B isolates reported before 2006. Comparison of the Iranian isolates of 793/B with the foreign ones demonstrated that two amino acid markers (i.e., 392 and 393) did not remain unique and specific to the Iranian 793/B isolates anymore. The amino acid marker 392 was seen in Spain/92/35, Spain/92/185, Spain/95/193, Spain/96/330, Spain/98/315, Spain/98/328, and Spain/99/327; in addition, amino acid marker 393 was observed in Spain/98/315. These isolates were reported in 2006, 2008, and 2013. Analysis of the sequences demonstrated that amino acid 349 was still unique to the Iranian 793/B isolates and was not seen in any of the foreign 793/B isolates. Study of Iranian 793/B isolates showed that these isolates could be divided into two groups. One group included viruses without deletion and the other one included viruses with deletions. Viruses IR-3654-VM, IR-1062-GA, and IR-1061-PH had 6, 6, and 15 nucleotide deletions at positions 164-169, 164-169, and 156-170, which resulted in the deletion of amino acids at positions 58-60, 58-60, and 53-57, respectively.

Table 2. Accession number of isolates and serotypes

Isolate	GenBank accession Number
IR-3654-VM	AY544776
IR-1062-GA	AY544777
IR-1061-PH	AY544778
IR/803/03	HQ842711
IR/14/07	HQ842712
IR/19/08	HQ842714
IR/491/08	HQ842715
IR/525/99	HQ842708
IR/512/99	HQ842707
793/B.08IR	KX789488
Chicken/Iran/793-B/UTIVO-48/2015	KX702145
Chicken/Iran/793-B/UTIVO-42/2015	KX702138
Chicken/Iran/793-B/UTIVO-47/2014	KX702146
IBV/Chicken/Iran/793B/UTIVO-1/2014	KT583572
IBV/Chicken/Iran/793B/UTIVO-86/2014	KT583577
Chicken/Iran/793-B/UTIVO-29/2015	KX702139
Chicken/Iran/793-B/UTIVO-106/2015	KX702140
Chicken/Iran/793-B/4-91Vaccine(IR)/2015	KX702143
Chicken/Iran/793-B/UTIVO-11/2014	KX702147
IBV/Chicken/Iran/793B/UTIVO-18/2014	KT583573
IBV/Chicken/Iran/793B/UTIVO-21/2015	KT583574
IBV/Chicken/Iran/793B/UTIVO-25/2015	KT583575
IBV/Chicken/Iran/793B/UTIVO-48/2014	KT583576
Chicken/Iran/793-B/IB88Vaccine(IR)/2015	KX702144
IBV/Chicken/Iran/793B/UTIVO-80/2015	KT583578
IBV/Chicken/Iran/793B/UTIVO-108/2015	KT583579
Chicken/Iran/793-B/UTIVO-85/2015	KX702136
Chicken/Iran/793-B/UTIVO-83/2015	KX702137
Chicken/Iran/793-B/UTIVO-82/2014	KX702141
Chicken/Iran/793-B/1-96Vaccine(IR)/2015	KX702142
Chicken/Iran/793-B/UTIVO-109/2014	KX702148
strain 4/91 (JN600614), 4/91 mutant	FJ445217
FR-85131-85	AJ618985
FR-CR88061-88	AJ618986
FR-94047-94	AJ618987
UK-1233-95	AJ618984
UK/7/91	Z83975
UK/2/91	Z83976
UK/3/91	Z83977
UK/793/B Attenuated	AF093793
UK/793/B/ Pathogenic	AF093794
UK/5/91	Z83978
UK/7/93	Z83979
4/91 vaccine	KF377577
CK/CH/FJ/FS1310-1	KX107643
CK/CH/FJ/FS1410	KX107647
CK/CH/FJ/PT1301	KX107649
CK/CH/FJ/ZZ1406-1	KX107653
CK/CH/FJ/ZZ1406-2	KX107654
CK/CH/GD/HY1410	KX107662
CK/CH/GD/LZ1311	KX107666
CK/CH/GD/XX1301-2	KX107675
CK/CH/GD/XX1411-1	KX107680
CK/CH/GD/XX1412-4	KX107685
CK/CH/GD/XX1412-5	KX107686
CK/CH/GD/XX1501-2	KX107689
CK/CH/GX/GL1301-1	KX107692
CK/CH/GX/GL1311-1	KX107700
CK/CH/GX/GL1311-2	KX107701
CK/CH/GX/NN1301-4	KX107719
CK/CH/GX/NN1306	KX107723
CK/CH/GX/ZS1301-4	KX107737
CK/CH/GX/ZS1412	KX107738
CK/CH/HuB/HC1303-1	KX107740
CK/CH/HuB/HC1303-2	KX107741
CK/CH/HuB/HC1303-3	KX107742
CK/CH/HuB/HC1303-4	KX107743
CK/CH/HuB/HC1304-1	KX107744
CK/CH/HuB/HC1304-2	KX107745
CK/CH/HuB/HC1402-3	KX107755
CK/CH/HuB/HC1408-2	KX107759
CK/CH/HuB/WH1304-1	KX107779
CK/CH/JS/ZJ1502	KX107826
CK/CH/YN/SL1301-1	KX107836
CK/CH/YN/SL1301-2	KX107837
CK/CH/YN/SL1501	KX107841
CK/CH/GX/GL1303-2	KX107697
4/91(UK)	JN192154
Spain/92/35	DQ386091
Spain/92/185	DQ386092
Spain/95/193	DQ386093
Spain/96/330	DQ386094
Spain/98/315	DQ386095
Spain/98/328	DQ386096
Spain/99/327	DQ386097
Spain/00/336	DQ386098
CK/CH/SCTQ/120718	KU364615
GUJ/IBV-2015	KR815570
IBV97/1986/Morocco	KM594213
IBV32/2011/Morocco	KM594234
H120 Vaccine strain	M21970
Ark99 strain	M99482
Gray strain	L14069
Holte strain	L18988
JMK strain	L14070
D274 strain	X15832
D1466 strain	M21971
QX strain	DQ167148
Variant 2	AF093796
IS/720/99	AY091552

The IR/512/99 and IR/525/99 were the viruses which showed deletions in these regions (nucleotide 163-174). Mentioned nucleotide deletions were observed in none of the other Iranian 793/B viruses. In addition, the deletion of nucleotide positions 162-170 was seen in one of the foreign 793/B viruses named Spain/99/327. Among the Iranian 793/B viruses, the highest percent identity of nucleotide sequences was between IR/512/99 and IR/525/99 (99.9%) and between IR/794/2002 and IR/803/03 (99.94%). The IR/491/08 showed the highest difference with the other Iranian 793/B viruses. Furthermore, IR-1062-GA and FR-94047-94 (97.4%) had the highest percent identity in the nucleotide sequences of the Iranian 793/B viruses, compared to that in the other 793/B viruses. On the other hand, IR/14/07 and IR/19/08 showed the lowest percent identity with UK/7/93, which was 93.3%. None of the viruses were completely identical

in nucleotide sequences, except IR/680/2002, which was completely identical to H120 vaccine virus. The IR/801/2001 was very similar to H120 vaccine virus with the percent identity values of 99.8% and 99.6% at nucleotides and amino acid levels, respectively. The IR/801/2001 had two nucleotide changes, compared to H120 vaccine virus, which were located at positions 191 (G to A) and 283 (A to C), resulting in amino acid substitutions at positions 64 (G to E) and 95 (S to R), respectively. The difference between the nucleotide sequences of the Iranian 793/B isolates and H120 vaccine strain was 25-26%. The Iranian 793/B isolates had a nucleotide difference of 22-60% with the other 10 serotypes.

Phylogenetic analysis. Nucleotide phylogenetic tree showed that D1466 was placed in a completely different branch, compared to the other viruses. Other viruses were located in two sub-branches. One branch included variant 2 and IS/720/99, and the other branch entailed other viruses. One of the French isolates named FR-94047-94 had the highest proximity to the Iranian 793/B isolates. In addition, IR/491/08 showed the highest proximity to both 793/B attenuated vaccines, and also 793/B pathogenic virus. The Iranian 793/B isolates (except IR/491/08) were placed in a different branch from the UK isolates. The IR/680/2002, IR/801/2001, and H120 vaccine strains were placed in a common branch. The closest serotype to 793/B viruses was QX strain. The 793/B viruses and H120 vaccine strain were at a far distance from each other. Figure 2 illustrates the phylogenetic tree based on nucleotide sequences. Phylogenetic tree based on amino acid sequences demonstrated that D1466 was placed independently from other viruses. The 793/B viruses were located in two main sub-branches. In this regard, the Spain isolates (i.e., Spain/95/193, Spain/96/330, and Spain/98/315) were placed in a different sub-branch from other 793/B isolates, including Iranian, French, and UK viruses. The IR/680/2002, IR/801/2001, and H120 vaccine strains were placed in a common branch and showed the farthest distance to 793/B viruses. In the amino acid phylogenetic tree, variant 2 and IS/720/99 were located closer to 793/B viruses, compared to those

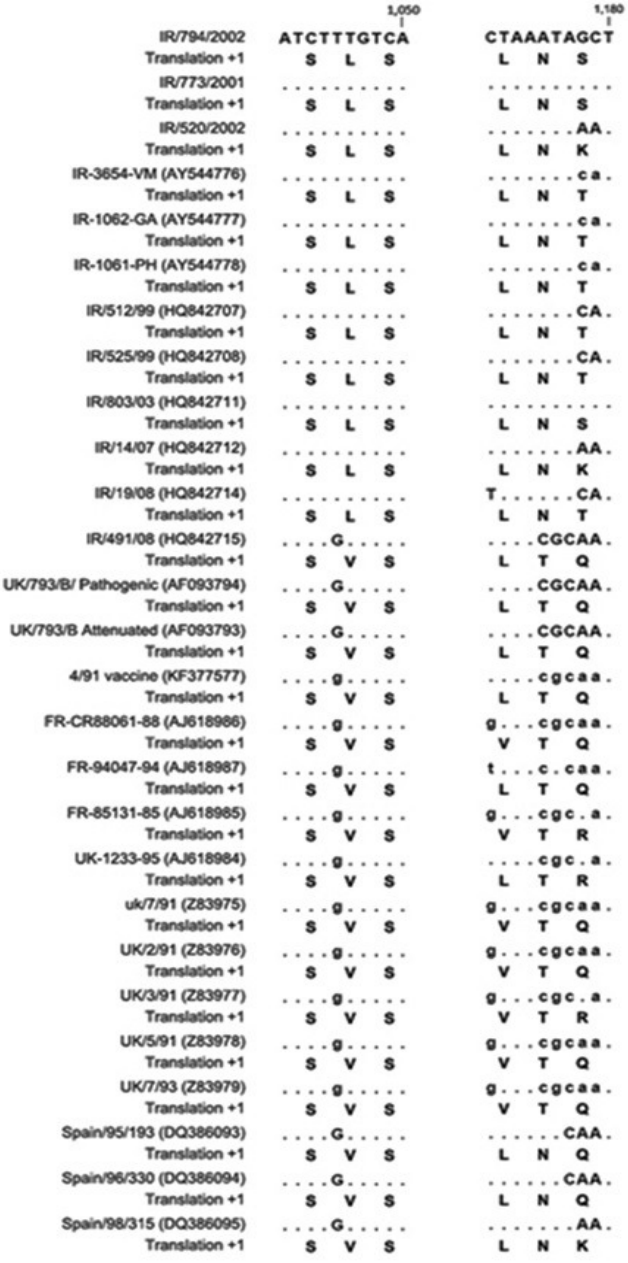

Figure 1. Comparison of the nucleotide and amino acid sequences of S1 belonging to our three 793/B isolates, along with the complete coding sequences of the Iranian and some foreign 793/B serotypes retrieved from GenBank (Only those parts in which we introduced amino acid markers for the Iranian isolates of 793/B serotype are shown in the figure.)

in the nucleotide phylogenetic tree. Other relationships among the 793/B viruses in the amino acid phylogenetic tree were similar to those in the nucleotide phylogenetic tree. Figure 3 depicts the phylogenetic tree based on amino acids.

DISCUSSION

The IB is one of the major respiratory diseases of chickens, which is prevalent in all countries and causes high economic losses. The IB virus has many serotypes and variants which usually show low cross-immunity. Therefore, different serotypes have been used in vaccination programs in Europe, America, and many other countries for many years (Cavanagh et al., 1992). Variation in IB viruses results from mutations in S1 glycoprotein as the most important surface protein, which is a reliable target gene for the classification of different serotypes and variants of the virus. The S1 protein of IB virus has three hypervariable regions (HVR). They are located at amino acid positions 56-69, 117-131, and 274-387 (Lee et al., 2003; Montassier, 2010). This protein contains antigenic sites that induce neutralizing antibodies. By means of monoclonal antibodies, five antigenic sites were mapped, which could induce the production of neutralizing antibodies and were located at amino acid positions 24-61, 132-139, and 291-398. Some of these antigenic sites are located at or in proximity to HVRs, and it is believed that HVRs, especially HVRI, have an important role in inducing neutralizing antibodies (Ignjatovic and Sapats, 2005; Lin et al., 2005). The IB disease has been prevalent in Iran for many years. Massachusetts serotype was the first and the only isolate of IBV detected in Iran. However, the commercial flocks in this country were affected by other IB virus serotypes entering the country over time. The existence of the disease in many flocks with respiratory problems showed the possibility of the presence of new serotypes. The isolated viruses from IB outbreaks were identified and classified as 793/B serotype by means of the serologic and molecular methods (Seyfi Abad Shapouri et al., 2004). Some other studies were performed in many parts of Iran, which confirmed the presence of 793/B serotype (Seyfi Abad Shapouri et al., 2004). The results of a study conducted in 2004 were indicative of the relatively high prevalence (42.8%) of 793/B serotype in Iran (Seyfi Abad Shapouri et al., 2004). The prevalence of this serotype has been variable during different time periods; in this regard, this rate was reported as 8.4% in a study performed during 2010-2014 (Hosseini et al., 2015). Additionally, these rates were obtained as 21% and 19.3% in 2014-2015 and 2017, respectively (Najafi et al., 2016; Modiri Hamadan et al., 2017). This serotype still seems to be one of the most predominant serotypes of IB virus in Iran despite using 793/B commercial vaccine (Modiri Hamadan et al., 2017). In the current study, five IB viruses, which had been already isolated from the different provinces of Iran during 2001-2002, were sequenced and compared with other Iranian and foreign 793/B viruses and some other serotypes. Nucleotide sequences showed that the Iranian 793/B viruses isolated before 2006 underwent changes at some positions, which were found to be unique to the Iranian viruses. These nucleotide changes resulted in amino acid substitutions at positions 349 (V to L), 392 (T to N), and 393 (Q or R to T, K or S). All Iranian 793/B isolates reported before 2006 owned these amino acids. Therefore, it seems that these valuable amino acids could be considered as markers for the differentiation of the Iranian 793/B isolates up to 2006. Since it has been found that Massachusetts vaccine cannot produce a suitable protective immunity against 793/B serotype, one possibility is that the mentioned unique amino acid changes have resulted from the passaging of the virus in susceptible birds in the absence of suitable protective immunity. However, this hypothesis should be confirmed by future studies. The Iranian field 793/B isolates reported after 2006 also had the three unique amino acid markers. The two mentioned marker amino acids were also found in some foreign 793/B isolates, including Spain/92/35, Spain/92/185, Spain/95/193,

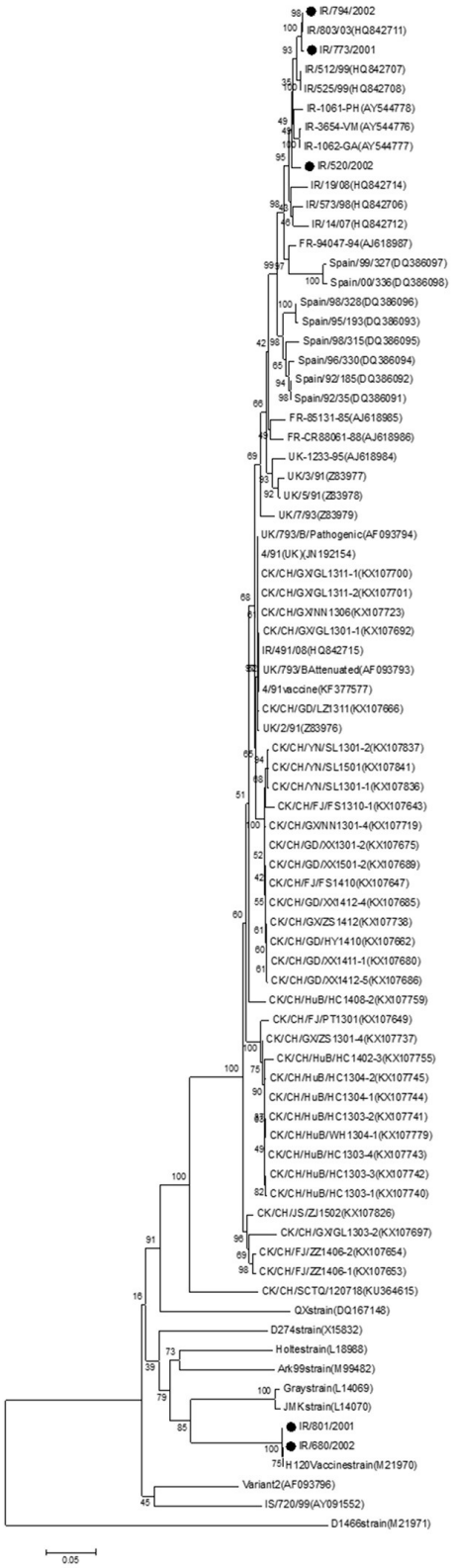

Figure 2. Nucleotide phylogenetic tree by using the neighbor-joining method with 1000 bootstrap replicates (using MEGA6 software) (Isolates which belonged to our laboratory are shown by black dots.)

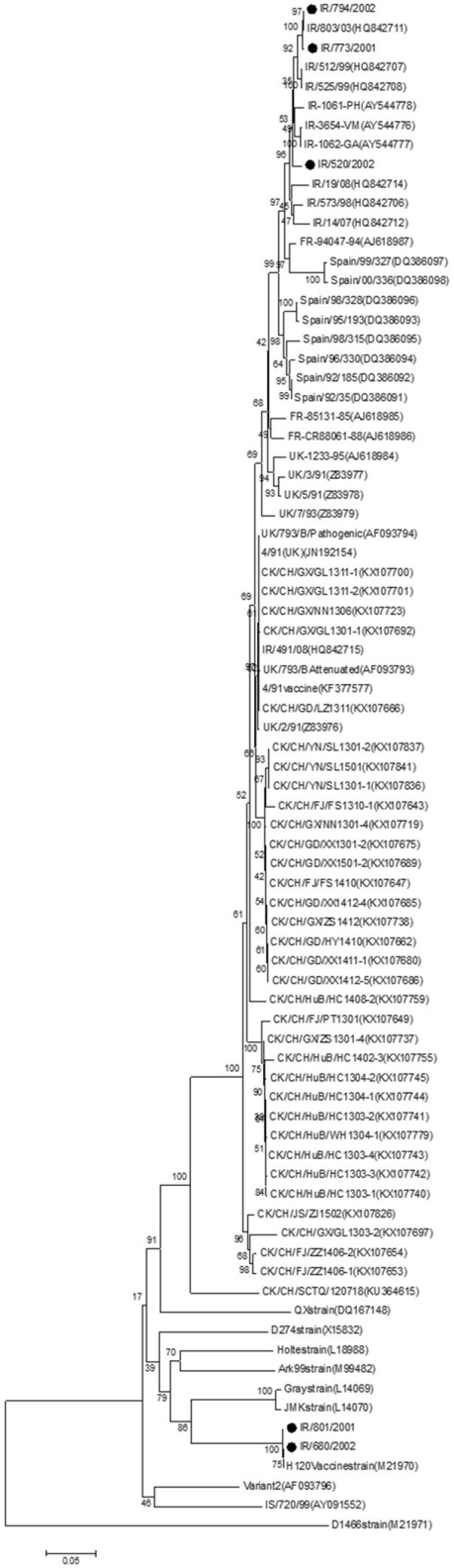

Figure 3. Amino acid phylogenetic tree by using the neighbor-joining method with 1000 bootstrap replicates (using MEGA6 software) (Isolates which belonged to our laboratory are shown by black dots.)

Spain/99/327 (amino acid 392), as well as Spain/98/315 (amino acid 393), which were reported in 2006, 2008, and 2013. There are no data regarding the onset of 793/B vaccination in Spain. If there was a long gap between the prevalence of 793/B serotype and application of 793/B vaccine in this country, the similar amino acid substitutions would be identical to what happened in Iran. Amino acid 349 (L) has remained unique to the Iranian 793/B isolates; therefore, it could be still used to differentiate the Iranian isolates from the foreign 793/B isolates. This amino acid was located at the HVR III (amino acid 274-387), and also along with amino acids 392 and 393, located at one of the regions containing antigenic sites that induce neutralizing antibodies (amino acids 291-398) (Ignjatovic and Sapats, 2005). Amino acid 349 was located at the region containing antigenic sites capable of inducing neutralizing antibodies and in the region introduced as HVRIII. Regarding this, it would be so valuable to perform further studies by site-directed mutagenesis to assess the role of this amino acid, as well as those of amino acids 392 and 393, in the antigenic and immunogenic characteristics of the virus. These amino acid changes which occurred in the hypervariable and variable regions of S1 gene could change some characteristics of the virus, compared with those of the foreign 793/B isolates. Therefore, it might be advisable to develop a native live attenuated vaccine (LAV) of 793/B serotype by means of the Iranian isolates. There is a possibility that native LAV produces better protective immune responses against the Iranian field 793/B isolates. Presence of alanine amino acid instead of serine at position 95 is a criterion for 793/B attenuated vaccines. Based on this amino acid, it was found that IR/491/08 has a vaccine origin among the other Iranian field 793/B isolates. However, it is advisable to use this criterion, along with the observed changes in the three mentioned amino acid markers. Deletions in IR-3654-VM, IR-1062-GA, IR-1061-PH IR/512/99, IR/525/99, and Spain/99/327, which resulted in the deletion of amino acids at regions within positions 53-61, were located at HVRI. There are no data regarding the effect of these deletions on antigenic epitopes in HVRI; accordingly, the implementation of studies investigating the role of these deletions in virus characteristics would be valuable. Old Iranian 793/B viruses were located in proximity to one of the French 793/B isolates (i.e., FR-94047-94) in nucleotide and amino acid phylogenetic trees. Considering the vast importation of frozen chicken meat from France to Iran and the high percent identity observed between the Iranian 793/B viruses and this virus, it is possible that this serotype has entered Iran by means of frozen chicken meat. This possibility has also been emphasized by Bijanzad et al. (2013). In nucleotide and especially amino acid phylogenetic trees, the H120 vaccine virus was located far from the 793/B viruses. This explains why Massachusetts vaccines cannot produce suitable immune response against 793/B serotype and demonstrates the necessity of using 793/B attenuated vaccines to combat 793/B serotype. A high amino acid difference in the S1 gene between the Iranian 793/B viruses and H120 vaccine virus (26%) was the reason for IB outbreaks in Iran before 2006 in spite of H120 mass vaccination. Therefore, it was rational to start using 793/B vaccine in Iran in 2006. The results also demonstrated the proximity of QX strain to 793/B viruses. It has been shown that QX strain is controllable by using a combination of Ma5 and 793/B vaccines (Terregino et al., 2008). The proximity of QX strain to 793/B viruses in the amino acid phylogenetic tree might explain the efficacy of the combination of 793/B and Ma5 vaccines against this serotype. The IR/680/2002 and IR/801/2001 were placed in the same group with the H120 vaccine virus. There was no difference between IR/680/2002 and H120 vaccine viruses. However, IR/801/2001 was different from H120 vaccine virus at positions 64 and 95, while it was very similar to the H120 vaccine strain. It seems that the virus was an H120 vaccine virus which has been passaged in chicken and undergone amino acid changes. The virus had a different amino

acid at position 95 (S to R) in comparison with H120 vaccine strain. This amino acid also underwent a mutation in the 793/B vaccine (S to A). It was suggested that changes in amino acid 95 in 793/B serotype might be related to the adaptation of the virus to its host (i.e., serine in chicken and alanine in chicken embryo) (Callison et al., 2001; Cavanagh, 2007). It is not clear whether amino acid changes at position 95 (S to R) in IR/801/2001 (Massachusetts serotype) plays a key role in host adaptation similar to 793/B serotype.

It can be concluded that the presence of three mentioned amino acid markers in a 793/B virus is a reliable criterion for the differentiation of the Iranian field 793/B viruses. However, it seems that amino acid 349 has remained the only specific amino acid for the Iranian field 793/B viruses. Moreover, because these amino acid changes have occurred in HVRs, it is advisable to develop a LAV based on the native 793/B isolates in Iran. It is predicted that this type of vaccine can produce better immunity in our field condition.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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