Archives of Razi Institute, Vol. 71, No. 2 (2016) 87-96

DOI: 10.22034/ari.2016.106446

Original Article

S1 gene sequence analysis of infectious bronchitis virus vaccinal strains (H120 & H52) and their embryo-passaged derivatives

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Received 20 June 2015; accepted 13 September 2015
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ABSTRACT

Avian infectious bronchitis is an acute and highly contagious disease that mainly causes respiratory symptoms in poultry. A number of serotypes and variants of the viral agent with poor cross-protection are the major problem to achieve desired immunity from vaccination. The S1 subunit of S glycoprotein (spike) is the major determinant of IBV so that a minor change in amino acid sequence of this protein, alters the virus strain. Therefore, characterization of the sequence of S1 gene is necessary to identify virus strains and their similarities with the vaccinal strains. In this research, the S1 sequence of H52 and H120 vaccinal strains of Razi Institute was fully characterized, and also the effect of serial passages in embryonated - eggs (5 passages beyond the master seed) on the S1 gene was investigated. The results showed that H120 and H52 strains of Razi Institute are 100% identical to the reference vaccine strains available in the GenBank. In addition, the H52 strain showed one amino acid substitution from the 3rd passage in which Glycine (G) was replaced by Valine (V) at position 118 making these passages exactly identical to the H120 strain while no change occurred for the H120 strain during these passages. Analysis of the original vaccinal strains which are widely administered in Iran, is definitely useful for prevention and control strategies against the circulating viruses. To identify the genetic change(s) responsible for attenuation of these strains during passages in embryonated-egg, characterization of other genes, especially those involved in replication is recommended.

Keywords: IBV, Vaccinal strain, S1 gene characterization, egg- passaged

INTRODUCTION

Avian infectious bronchitis is an acute, highly contagious and economically important disease of poultry with worldwide distribution. The viral agent mainly affects respiratory epithelium, making the birds susceptible to other infectious agents (Cavanagh, 2007). Infectious bronchitis virus (IBV) is classified in the genus *Gammacoronavirus* within the *Coronaviridae*. The virus, almost 120 nm in diameter, is an enveloped spherical to pleomorphic particle, which contains 20 nm spikes projecting from the outer surface of the particle (Masters and Perlman, 2013). Structural proteins are comprised of the Spike (S), membrane

(M), nucleoprotein (N) glycoproteins and tiny amounts of small membrane protein (E) (Cavanagh, 1981; Sutou et al., 1988). The S glycoprotein consists of S1 and S2 proteins, S1 is anchored to the membrane by S2 and is responsible for attachment, entry and inducing hemagglutination inhibition and neutralizing antibodies. The S1 is about 520 amino acids comprising the hypervariable region (amino acids 38-387); minor changes in this area induces remarkable decline in protective immunity against heterologous serotypes and strains (Koch et al., 1990; Cavanagh et al., 1992; Kant et al., 1992). The variation in the S1 sequences of vaccinal strains has also been found within the same vaccine serotype produced by different companies, and even different batches by a single manufacturer (McKinley et al., 2008). Passages of vaccinal strains in embryonated eggs can also result in mutations in the IBV genome, for this reason only a limited number passages (up to 5) beyond the master seed is allowed for vaccine production by the World Organization for Animal Health (OIE) (http://www.oie.int/en/international-standard-setting/terrestrial-manual). This study was aimed to characterize the S1 gene of H52 and H120 vaccinal strains from Razi Institute and also evaluate the effect of five serial passages in emryonated-eggs on the entire length of S1 sequences.

MATERIALS AND METHODS

Virus preparation. The Master seeds of both strains (H120 and H52) were inoculated into the allantoic cavities of specific-pathogene free (SPF) chicken embryos. After two days of incubation at 37 °C, the allantoic fluid was harvested and inoculated into the new SPF eggs. The Subsequent passages were serially carried out until the 5th passage as above.

Viral RNA extraction. Viral RNA was extracted by using commercial viral High pure Viral Nucleic Acid kit (Roche; Germany) according to the manufacturer's instruction.

Reverse transcription reaction. Reverse transcription (RT) was carried out using RevertAid™ First Strand cDNA synthesis Kit (Fermentas; Canada) as follows: 6 µl RNA and 1 µl Random hexamer primer with 5 µl DEPC-treated water heated at 65 °C for 5 minutes and cooled on ice. Then, 4 µl 5X Reaction buffer, 1 µl Ribolock™ Rnase inhibitor (20u/µl), 2 µl of 10 mM dNTP Mix, 1 µl RevertAid™ M-MulV Reverse Transcriptase (200 u/µl) were added to the solution to final volume of 20 µl. The mixture was put in a thermocycler at 25 °C for 5 minutes, followed by 42 °C for 60 minutes and 72 °C for 5 minutes.

Polymerase chain reaction (PCR). The PCR mixture contained 5 µl of 10 X buffers, 2 µl dNTP (0.2 mM of each), 6 µl MgSo4 (3mM), 7µl cDNA, 1 µl (20 pmol) of each primer, Forward (5'-TGAAAACTGAACAAAAGACA-3') and Reverse (5'-CATAACTAACATAAGGGCAA-3') flanking the whole S1 glycoprotein gene (known et al., 1993) and 2.75 units (1.1 µl) of *pfu* DNA polymerase (Fermentas; Canada) in a total volume of 50 µl. The PCR cycling program was started with initial denaturation at 95 °C for 2 minutes then followed by 37 cycles of 94 °C for 50 seconds, 47 °C for 60 seconds, 72 °C for 2 minutes and 40 seconds and, a final extension at 72 °C for 10 minutes. PCR products were run on agarose gel (1%). Samples were identified as positive based on the presence of the expected amplicon (1720 bp) with ethidium bromide staining.

Sequence analyzing. PCR products were purified using High Pure PCR Purification (Roche; Germany). Samples (50 µl) containing 40 ng/ml were sent to Bioneer company (South Korea) for sequencing. The PCR products were sequenced in both directions using the above mentioned forward and reverse primers.

The obtained sequences were assembled by Geneious (4.8.3) software. The whole sequences from H52 and H120 passages were compared to the sequences available in the GenBank (http//Blast.ncbi.nlm.nih.gov/Blast.cgi) and were aligned together using Clustal W program (Thompson et al., 1994) and MEGA.5 software. The sequence of the first passage of H52 and H120 were deposited in the GenBank with accession number of KR605488 and KR605489, respectively.

Viral neutralization (VN) Test. Egg infectious dose (EID_{50}) was calculated using Kärber method (1931) for the H52 and H120 vaccinal strains and their embryo – passaged strains. Ten SPF chickens were inoculated by 1 dose ($10^{3.5}$ EID_{50} viral particles) of each vaccinal strain via eye-drops. The chickens were monitored for 21 days and were bled 3 weeks after vaccination. The antibodies against IBV were measured by VN test according to the OIE protocol., 2001).

RESULTS

PCR amplification and sequence analyzing. The PCR product (1720 bp) was successfully amplified for all passages of both (H120 and H52) strains and observed on agarose gel.

Alignment of the entire length of the S1 gene from H52 strain during serial passages (1 to 5) in embryonated-egg showed that they are highly identical (99.8% nucleotide identity) with minor changes occurred from the third passage as follows and illustrated in Figure 1: Nucleotide No. 345 (T) in passages 1 and 2 changed to C in passages 3, 4 and 5, with no amino acid alteration.

1-Nucleotides No. 353 (G) in passage 1 and 2 changed to T in passage 3, 4 and 5. This change led to amino acid substitution of Glycine (118) to Valine.

2-Nucleotide No. 426 (G) in passage 1 and 2 changed to T in passages 3, 4 and 5, with no amino acid alteration.

Alignment of the all serial passages (1 to 5) of H120 strain revealed that they are absolutely identical (100 % nucleotide identity). Strikingly, the sequences of H120 passages were 100% identical to the 3rd to 5th passages of the H52 strain. Alignment of the full length of the S1 gene sequences of the H52 and H120 strains (1st passage) are depicted in Figure 2. The two S1 sequences and the encoded amino acids found in this study: (H52 passages 1 to 2) and [H52 (passages 3 to 5) - H120 (passages 1 to 5)] were also compared with the sequences available in the GenBank. The results showed that both of the sequences are 100% identical to the reference vaccinal strains of IBV submitted in the GenBank (Table 1 and 2).

Viral neutralization. The neutralizing antibodies elicited against the all serial passages of H52 and H120 strains efficiently and consistently neutralized the homologous IBV Massachusetts serotype.

DISCUSSION

IBV, as a member of the *Coronaviridae*, has high capacity for genetic change occurring through point mutation, insertion, deletion and genetic recombination (Thor et al., 2011; Jackwood et al., 2012). These mechanisms of genetic diversities lead to emergence of new IBV serotypes and variants which complicate designing appropriate control strategies using the most homologous vaccine (Lee and Jackwood, 2001; Jackwood et al., 2010). The evolution of IBV is also influenced by the application of multiple vaccinal strains, population density and host immune status (Lee and Jackwood, 2001). It is, therefore, an obvious requirement for monitoring and characterization of circulating viruses in the field as well as assessment of the effectiveness of vaccines used against these viruses (Cavanagh, 2007). Furthermore, molecular characterization is now considered as an essential component of vaccine evaluation for vaccine manufacturers in the world (www.oie.int/en/international-standard- setting/terrestrial-manual). The H52 and H120 vaccinal strains have been produced and administered in Iran for a long time while molecular characterization of these strain was largely expected. In the first step, we attempted to characterize the full length of the S1 gene of these vaccinal strains as a major determinant of the IBV. This aim was successfully achieved, emphasizing that the H52 and H120 strains manufactured in Razi institute are absolutely identical to the reference vaccinal strains submitted to the GenBank. These results, together with the VN test findings approve the certainty of these strains and also justify their application against the homologous serotype and variant in the field.

Table 1. Ten sequences in the GenBank most identical the H52 Strain of Razi Institute

Isolate name	Accession number	Isolated from
Infectious bronchitis virus isolate MassD/Cvial1 S1 protein (S1) gene	EU283085.1	Vaccine vial
Infectious bronchitis virus strain H120 spike glycoprotein (S) gene	KF188436.1	Vaccine vial
Infectious bronchitis virus isolate UFMG/PM1 spike glycoprotein S1 subunit gene	JX182773.1	Chicken
Infectious bronchitis virus isolate Mass/Avial1 S1 protein (S1) gene	EU283073.1	Vaccine vial
Avian infectious bronchitis virus strain Ma5 spike glycoprotein S1 subunit precursor (S1) gene	AY561713.1	Vaccine vial
Infectious bronchitis virus strain ck/CH/LSD/110726	KJ425512.1	Chicken
Infectious bronchitis virus strain ck/CH/LJL/121059	KJ425509.1	Chicken
Infectious bronchitis virus strain ck/CH/LHLJ/110310	KJ425505.1	Chicken
Infectious bronchitis virus strain ck/CH/LHB/131142	KJ425501.1	Chicken
Infectious bronchitis virus strain ck/CH/LHB/131118	KJ425499.1	Chicken

Table 2. Ten sequences in the GenBank most identical the H120 Strain of Razi Institute

Isolate name	Accession number	Isolated from
Avian infectious bronchitis virus (strain H120) peplomeric protein gene encoding the S1 and S2 subunits	M21970.1	Vaccine vial
Infectious bronchitis virus strain ck/CH/LSD/121228	KJ435285.1	Chicken
Infectious bronchitis virus strain ck/CH/LHLJ/131216	KJ425507.1	Chicken
Infectious bronchitis virus strain ck/CH/LDL/101212	JF828981.1	Chicken
Infectious bronchitis virus isolate IR/17/00 S1 spike glycoprotein gene	HQ 842709.1	Chicken
Infectious bronchitis virus strain ck/CH/LDL/101212 S1 protein (S1) gene	JF330848.1	Chicken
Infectious bronchitis virus isolate THA320352 spike glycoprotein S1 subunit (S1) gene	GQ885138.1	Chicken
Infectious bronchitis virus strain ck/CH/LHLJ/111050	KJ425506.1	Chicken
Infectious bronchitis virus strain ck/CH/LHB/131142	KJ425501.1	Chicken
Infectious bronchitis virus strain ck/CH/LHLJ/111050 S1 protein (S1) gene	JQ739315.1	Chicken

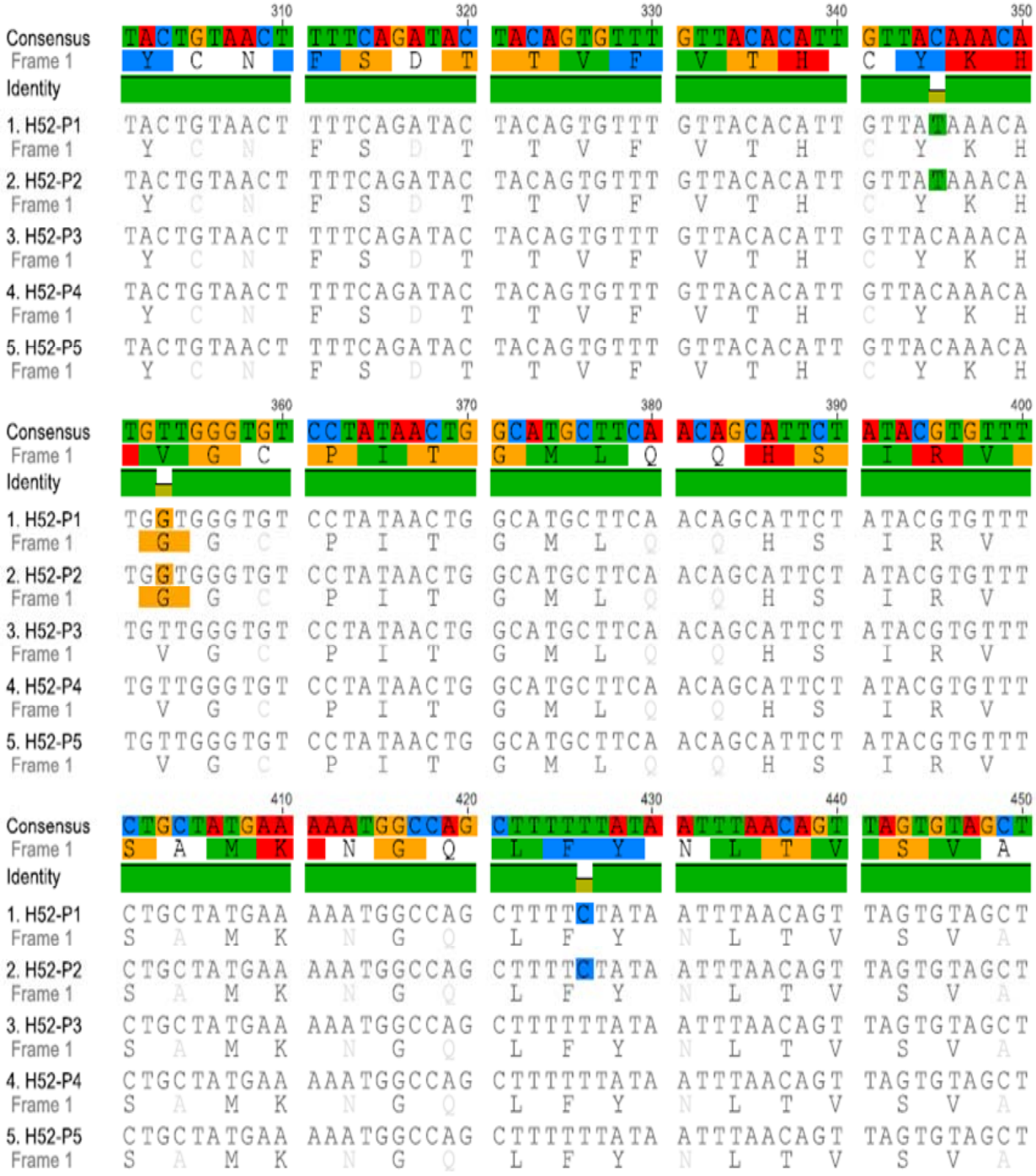
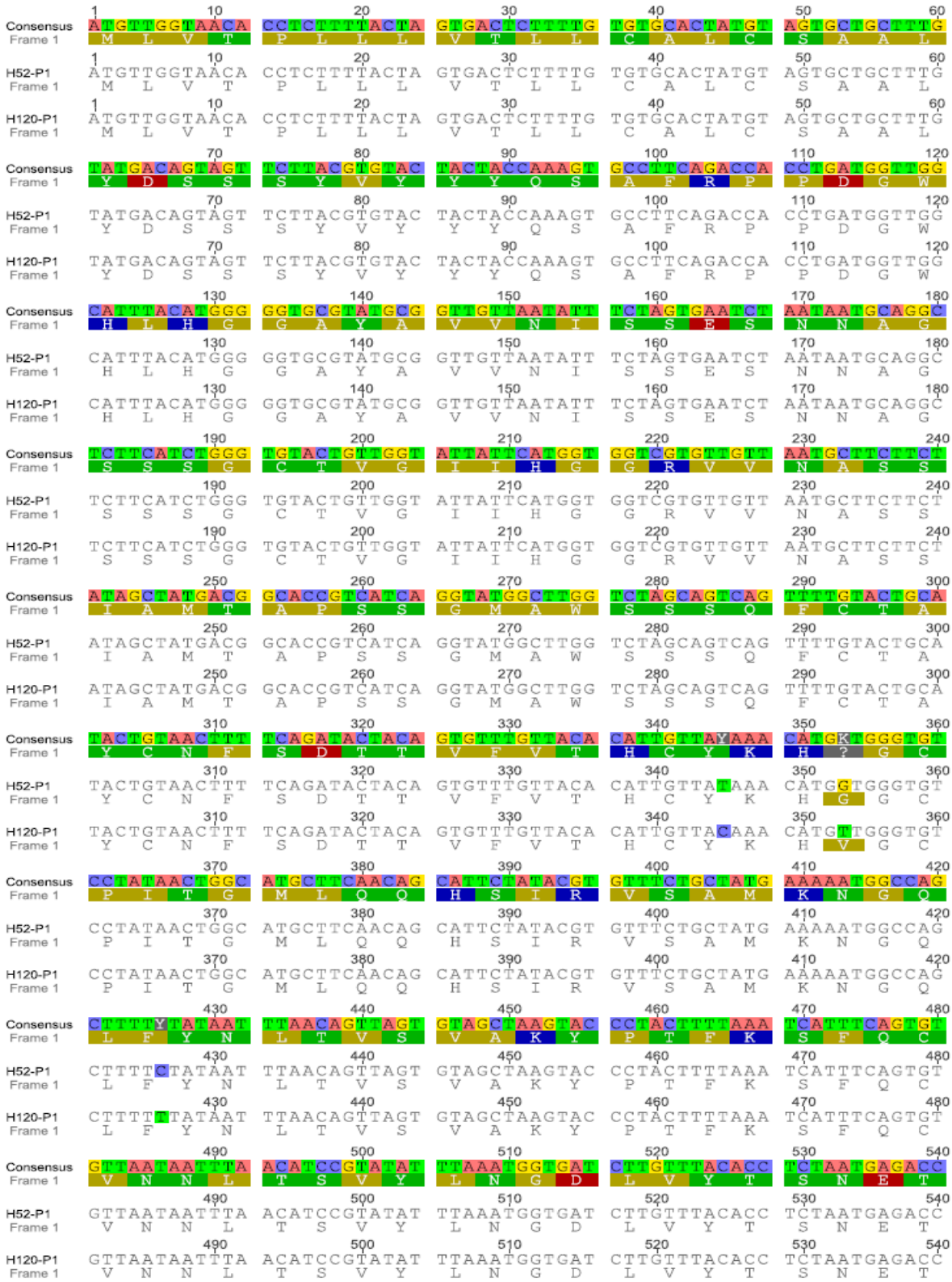


Figure 1. The region of the H52 strain illustrating the nucleotide and amino acid changes occurred from the third passages.


Consensus
Frame 1
H52-P1
Frame 1
H120-P1
Frame 1


Consensus
Frame 1
H52-P1
Frame 1
H120-P1
Frame 1

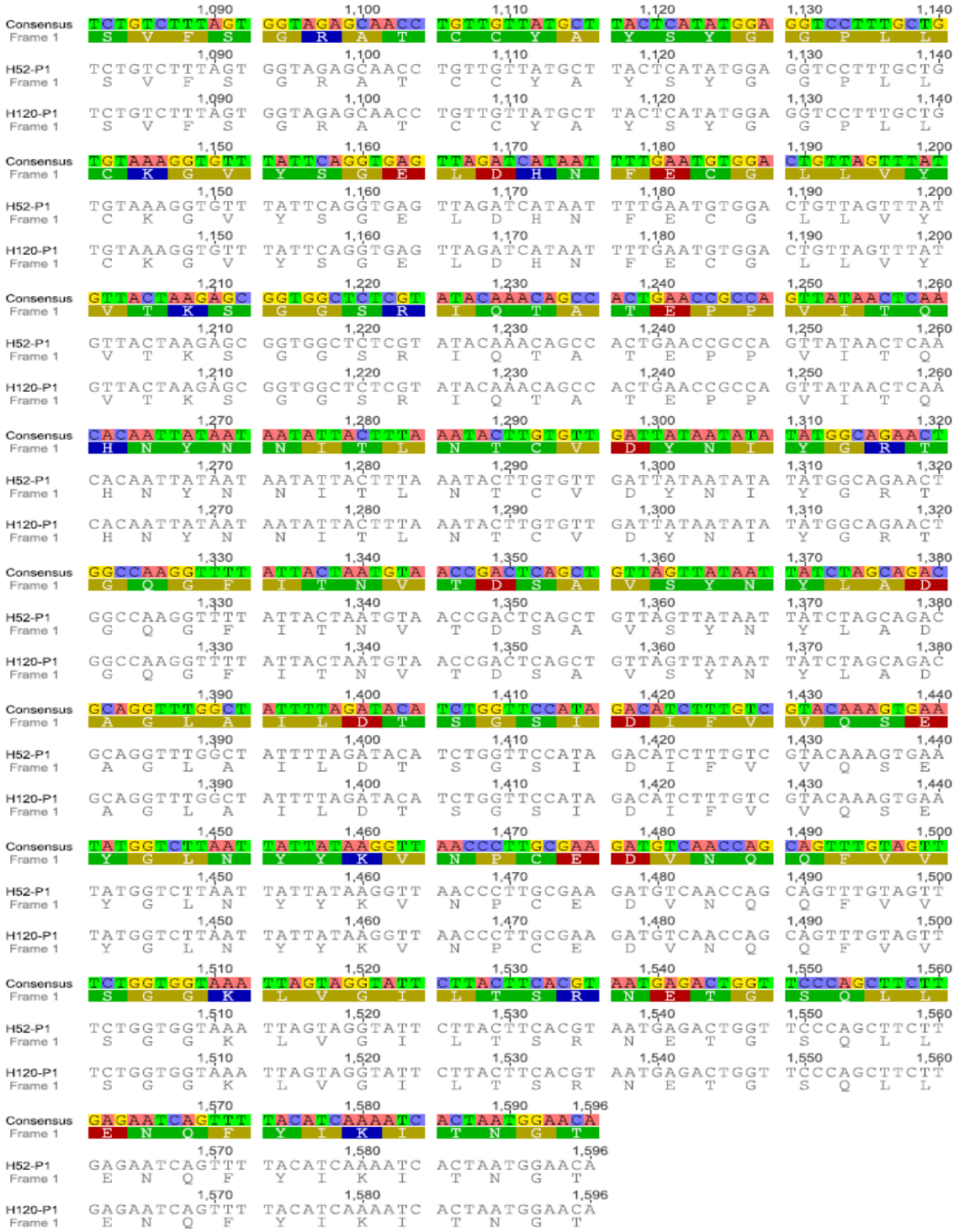


Figure 2. Full-length characterization and alignment of the S1 gene of H52 and H120 strains from Razi Institute at both nucleotide and amino acid level.

Comparison of these S1 sequences with those available in the GenBank also revealed that they are 100% identical to some sequences isolated in the field which can be resulted from either reisolation of vaccinal viruses circulating in the susceptible hosts (Jackwood et al., 2012) or highly frequent recombination of the IBV genome during co-circulation of the vaccinal and field strains (Capua et al., 1999; Lee and Jackwood, 2001; Jackwood et al., 2010; Jackwood et al., 2012).

The changes of nucleotide and amino acid sequences observed from the 3rd passage of H52 strain made these three passages exactly identical to the H120 strain. As these passages are 52nd and 120th passages of the Massachusetts serotype in embryonated-egg (Bijlenga et al., 2004), it can be assumed that the changes established in the 3rd passage of H52 strain remained constant until the 120th passages with no effects on immunogenicity and pathogencity of these strains. The amino acid G to V substitution, at position 118 within the hypervariable region is reminiscent of G to V substitution at position 110 reported by Cavanagh et al. (1988). It would be of interest to determine whether this specific substitution within the hypervariable region resulted from host adaptation during serially embryonated-egg passages. In this respect, it has been reported that alternate passage of the field strain 793/B in chick and embryonated eggs favored selection of serine and alanine at position 95, respectively,. This change is speculated to be a consequence of host-driven selection due to internal host factors (Cavanagh et al., 2005).

These data emphasize that the S1 gene of H52 and H120 strains from Razi institute did not show a substantial change during serial passages in embryonated eggs. To determine the genetic change responsible for the distinct virulence of these strains, characterization of the full genome of these strains is recommended. In this point of view, it has been reported that the attenuation of IBV in egg is associated with nonstructural (nsp3) protein (Ammayappan et al., 2009; Phillips et al., 2012).

Ethics

I 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.

Grant Support

This study was supported by Razi Vaccine & Serum Research Institute, project number 2-18-18-87044.

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