Iranian Journal of Virology 2017;11(4): 16-24
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Original Article

Sequence Analysis of the Spike Glycoprotein, Nucleocapsid Gene and 3' Untranslated Region of Razi Institute H-120 & H-52 Vaccine Strains of Infectious Bronchitis Virus

Khalesi B, Masoudei Sh, Bakhshesh M, Shoshtari A H

Department of Research and Production of Poultry Viral Vaccine, Razi Vaccine and Serum Research institute, Agricultural Research Education and Extension Organization (AREEO), Karaj, Iran.

Abstract

Background and Aims: Infectious bronchitis (IB) is an economically important disease of chickens. The existence of very large number of IBV serotypes and variants which insufficiently induce cross protection against each other is the major problem to control the disease.

Materials and Methods: This study was performed to characterize S1, N and 3'UTR region of the genome of H-120 and H-52 vaccine strains of Razi Vaccine Institute. The S1, N and 3'UTR region were sequenced and compared with standard strain in gene bank.

Results: Based on nucleotide identity, the S1, N and 3'UTR region of the genome of Iranian IBV vaccine strain showed 100% similarity to the commonly standard IBV strains. To better characterization of these strains analysis of other genes involved in virulence and pathogenesis of the virus and performing protective tests against field strains are recommended.

Conclusions: The results revealed that H-52 and H-120 strains of Razi institute were identical to the standard of strains in the Gene Bank.

Keywords: IBV, vaccine, S1 gene, nucleocapsid gene, 3' untranslated region.

Introduction

Infectious bronchitis (IB) is an economically important poultry disease that has detrimental effects on egg quality and production in layers, while in broiler chickens it leads to reductions in weight gain and feed efficiency (1). The viral agent mainly affects respiratory epithelium, making the birds susceptible to other infectious agents (2). The causative agent of IB, the infectious bronchitis virus (IBV), is a member of the genus Coronavirus of the Coronaviridae family. The IBV genome is a single-stranded linear RNA molecule 27.6 kb (3).

The virus, is an enveloped spherical to pleomorphic particle, 120 nm in diameter, which contains 20 nm spikes projecting from the outer surface of the particle (4). Its virion consists of four structural proteins, namely a spike glycoprotein (S), an integral membrane glycoprotein (M), a nucleocapsid protein (N), and a small membrane envelope protein (E) (5, 6) .The genes in the IBV genome are arranged in the following order: 5'-polymerase-SE-M-N-3', with a few non-structural genes interspersed among these genes (7).

The high frequency of new IBV variants is a distinguished characteristic of this virus among other corona viruses.3 According to Ignjatovic et al. (2000more than 50 serotypes of IBV have been identified and new variants continue to emerge despite the use of live attenuated and killed IBV vaccines (2, 9). However, as these

*Corresponding author: Bahman khalesi, Razi Vaccine and Serum Research institute, Tel: 02634570038.
Email: khalesi20022002@yahoo.com

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vaccines offer little cross-protection, outbreaks of IB can still occur between serologically distinct viruses (10, 11). Therefore, it is very important to identify the field strains of IBV in circulation in order to select suitable vaccine strains for use in different geographical regions (11).

Traditional methods for of the identification of IBV serotypes include hemaglutination-inhibition and virus neutralization tests, but both of these procedures are labor-intensive and time consuming. Since the early 1990s, reverse transcription polymerase chain reaction (RT-PCR) technology has been used successfully to identify the IBV genome rapidly (3, 12). Molecular analyses and detailed sequencing have enabled the precise fingerprinting of IBVs, which means that molecular epidemiology can be used to trace the origins of novel viruses and track virus dissemination across the world (13). Nevertheless, the means of spread by these viruses are seldom well understood (13).

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 (17, 18). 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 (16). Most previous studies have focused on the molecular analysis of the S1 gene. However, like the S1 protein, the N protein has also been shown to play an important role in the induction of immune responses against IBV (17, 19). Therefore, characterization of the S1 and N gene sequences is important for selecting the most useful and effective vaccine (13, 19). Furthermore, the N protein plays a role in viral replication and assembly and it binds to the viral RNA forming a helical nucleocapsid (20). Immediately downstream of the N gene is the 3´untranslated region (UTR), which is presumably important in the initiation of negative-strand RNA synthesis. Sequence analyses of the 3' UTR of several IBV strains have revealed that this region has two hypervariable and conserved regions (21) making this region worthy of further investigation as a method for differentiating IBV strains (22, 23). In the present study, the entire S1, N and partial 3' UTR genes of two Razi institute IBV vaccine strain were sequenced and compared with sequences of standard strain if IB virus in gene bank.

Methods

Virus Preparation. The Master seeds of both strains (H-120 and H-52) were propagated by inoculated into the allantoic cavities of specific-pathogen free (SPF) chicken embryos. After two days of incubation at 37 °C, the allantoic fluid was harvested and sorted at -80 °C untill RNA extraction.

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. For each RNA extraction, 50 µl allantoic fluids were used and RNA was eluted in 30 µl elution buffer. Extracted RNA was used immediately for cDNA synthesis or stored at -70°C for later use.

Reverse transcriptase-polymerase chain reaction (RT-PCR). 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,

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Forward (5'TGAAAACTGAACAAAAGACA-3') and Reverse (5'- CATAACTAACATAA-GGGCAA-3') flanking the whole S1 glycoprotein gene (29) and 2.75 units (1.1µl) of pfu DNA polymerase (Fermentas;Canada) in a total volume of 50µl. 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. Also PCR was performed to amplify a fragment of 1.8 kb containing whole N gene and partial 3' UTR of the IBVs.

The amplification reactions used a designed forward primer, 5b-F2 (5' CCTTTTCGCGG-AGCAATAG 3') that binds to the 3' end of gene 5 of the IBV genome (complementary to bases 25703 to 25721 of the H-120 strain; accession number FJ888351), and the previously described reverse primer, UTR-R1 (5'CTGTACCCTCGATCGTACTC 3') (23) that binds to the 3' UTR. The PCR involved an initial denaturation for 2 min at 94°C, followed by 35 cycles of incubation at 94°C for 45 s, 55°C for 40 s and 72°C for 2 min, then a final extension at 72°C for 5 min.

Gel electrophoresis. The PCR products were separated in 1% agarose gel and observed using ultraviolet transillumination.

Purification of PCR products. PCR (24) 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.

Sequence analysis. 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 sequences from H-52 and H-120 virus were compared to the sequences available in the GenBank (Table 1) and were aligned together using Clustal W program (25) and MEGA.5 software.

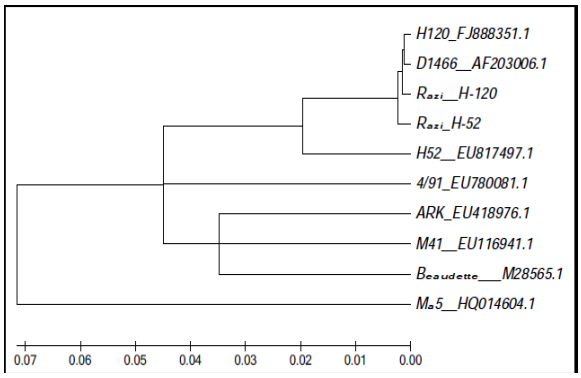

Fig. 2. Phylogenetic tree generated based on the N sequences of 11 IBV strains using the UPGMA method of the MEGA5 software.

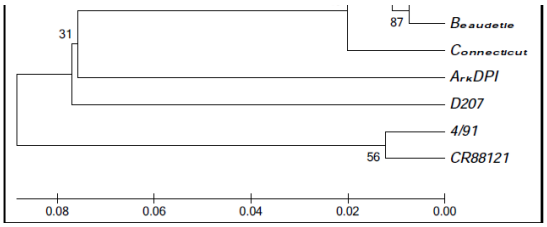

Fig. 1. Phylogenetic tree generated based on the S1 sequences of 9 IBV strains using the UPGMA method of the MEGA5 software.

Results

S1 sequence analysis. The S1 gene of H-120 and H-52 strain of Razi IBV (29) virus were aligned and compared with 9 other S1 gene sequences of IBV viruses. The alignment showed 99.8% nucleotide identity (figure1) also 81 – 99% was in the blast of S1 of H-52 and H-120 strains of the Razi Institute (http//Blast.ncbi.nlm.nih.gov/ Blast.cgi).

The N sequence analysis. The N gene sequence showed 99% identity with other standard IB virus. Phylogenetic analysis of the N genes showed that Razi H-120 and H-52 IBV were clustered together and separate from all other IBV (Figure 2). In addition the Blast of N gene of H-52 and H-120 strains of the Razi Institute with numbered strains in table one in the gene bank revealed that these strains are closely related to the H-120 and H-52 standard strain with 88 – 99%.

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Table1. Accession numbers from the GenBank database of IBV sequences used in this study.

Numbers	IBV strain	Accession numbers	Numbers	IBV strain	Accession numbers	Numbers	IBV strain	Accession numbers	Numbers	IBV strain	Accession numbers
1	H-120 Razi (3' UTR)	Being registered at the Gene Bank	**8**	4/91 (3' UTR)	HM210804.1	**15**	Beaudette (N)	M28565.1	**22**	Ma5 (S1)	AY561713.1
2	H-52 Razi (3' UTR)	Being registered at the Gene Bank	**9**	D207 (3' UTR)	AJ278335.1	**16**	Ma5(N)	HQ014604.1	**23**	4/91 (complete gene)	KF377577.1
3	H-120 (complete genome)	FJ888351.1	**10**	D1466 (3' UTR)	AF203007	**17**	4/91 (N)	4/91 EU780081.1	**24**	Beaudette (complete genome)	NC_001451.1
4	H-52 (complete genome)	EU817497.1	**11**	ARK (complete genome)	EU418976.1	**18**	D1466 (N)	AF203006.1	**25**	D207 (S1)	M21969.1
5	M41 (complete genome)	AY851295	**12**	Razi H-120 (N)	Being registered at the Gene Bank	**19**	H-120 Razi (S1)	KR605489	**26**	D1466 (S1)	X58001.1
6	Ma5 (3' UTR)	HM210831.1	**13**	Razi H-52 (N)	Being registered at the Gene Bank	**20**	H-52 Razi (S1)	KR605488.1			
7	Beaudette (complete genome)	Z30541.1	**14**	M41 (N)	EU116941.1	**21**	M41 (S1)	AY561711.1			

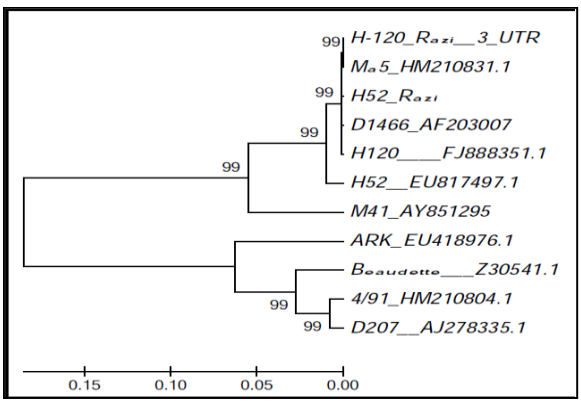

Fig. 3. Phylogenetic tree generated based on the 3'UTR sequences of 11 IBV strains using the UPGM A method of the MEGA5 software.

The 3'UTR sequence analysis. The 3'UTR of Razi H-120 and H-52 vaccine strain were aligned and compared with other vaccine strains of IBV and 7 other important strain submitted in the GenBank (H-120: FJ888351; H-52 : EU817497.1) and showed more than 99% sequence similarity (figure 3) Our results showed that there is similar genetic orientation between S1, N, 3´UTR with standard IBV vaccine. In addition the Blast of 3´UTR of H-52 and H-120 strains of the Razi Institute with numbered strains in table one in the gene bank revealed that these strains were closely related to the H-120 and H-52 52 standard strain with 95 – 99%.

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Discussion

IBV, as a member of the Coronaviridae, has high capacity for genetic change occurring through point mutation, insertion, deletion and genetic recombination (24, 25). 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 (26). The evolution of IBV is also influenced by the application of multiple vaccinal strains, population density and host immune status (26). 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 (2).

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 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.

Among viral proteins, the S1 (Spike) protein plays the principle role in inducing neutralizing antibodies which defines serotypes and attachment to the host cells. A few amino acid differences in the S1 result in the change of strains and serotypes. Therefore, analysis of sequences of S1 is very important to characterize field isolates and to define their degree of identity with vaccine strains. Also analysis of the original vaccine strains is definitely useful for prevention and control of the circulating viruses.

Comparison of these S1 sequences with those available in the GenBank 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 which is we find in our results (25) or highly frequent recombination of the IBV genome during co-circulation of the vaccine and field strains (25, 26) Molecular analyses of the S1 and N genes of IBVs together has demonstrated that it could be useful for detecting recombination events in different IBV strains (27, 28). The S1 gene of Iranian IBVs shared almost 74% similarity with the H-120 vaccine strain used widely in Iran also it was shown that these isolates belonged to the 793/B serotype and shared more than 94% sequence similarity with IBVs of the 793/B serotype isolated in the United Kingdom (29).

Most previous studies have focused on the molecular analysis of the S1 gene. However, like the S1 protein, the N protein has also been shown to play an important role in the induction of immune responses against IBV and characterization of the S1 and N gene sequences is important for selecting the most useful and effective vaccine. Molecular analyses of the S1 and N genes of IBVs together has demonstrated that it could be useful for detecting recombination events in different IBV strains (27, 28).

Furthermore, the N protein plays a role in viral replication and assembly and it binds to the viral RNA forming a helical nucleocapsid. Immediately downstream of the N gene is the 3' untranslated regions (UTR), which is presumably important in the initiation of negative-strand RNA Synthesis.

In addition other authors emphasized that although the typing of IBV strains is usually based on features of the S protein (30, 31, 32), recent evidence indicates that, like the S protein, the N protein is also a major inducer of immune responses against IBV and therefore may be another important target in preventing IB outbreaks (33, 34).

Moreover, deletions in the N genes (38) and 3' UTRs of different IBV strains have been reported previously (23, 38, 39).

Some molecular analysis based on N gene and also 3'UTR has accomplished in a few studies.

But there is not enough information about N gene and 3'UTR of IBV stains circulating in Iran However the nucleocapsid gene (N) and 3' untranslated region (UTR) of two IBVs isolated from Iranian poultry farms were sequenced and compared with other IBV strains by Majdani, et al 2010 (40). Their results showed that nucleotide identity of the N gene and 3' UTR sequences of Iranian IBVs revealed 90%

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similarity to the commonly used IBV H-52 and H-120 vaccine strains.

There are different reports about differentiating IBV strains based on N gene. In another study, Brazilian isolates clustered together in a distinct group based, on N gene RFLP analysis but some other surveys reported different cluster assignments for some Italian and Korean strains based on N gene and S1 molecular analysis.

In an analysis of N gene sequences from Korean IBVs, it was shown that these shared almost 90% similarity with non-Korean IBVs (40), which is similar to the results of Majdani, et al 2010 (40), where N gene sequences for Iranian IBVs were compared with non-Iranian IBV strains.

Furthermore, Shanker (2007) (35) isolated an IBV strain in the United States and showed that its N gene is not closely related to any other published N gene sequences.

Based on N gene and 3' UTR sequences, the Iranian isolates were most closely related to strains from the United States and Europe, which confirms previous findings obtained using the S1 gene of Iranian IBVs (32). The similar clustering of strains based on S1 and N gene sequences has been reported elsewhere (29). Previously, Sapats (1996) (38) showed that the S1 and N genes of IBV strains have evolved in parallel.

Comparing 3' UTR of H-52 and H120 Razi vaccine strain with other IBV 3' UTR sequences revealed that the of H-52 and H120 Razi vaccine strain showed greatest similarity with H-120 and H-52 standard strain (23 , 38, 39).

Therefore, it is crucial to monitor IBVs circulating in the field to facilitate a relevant vaccination program against IB.

Our data suggests that no point mutations, including insertion and deletion, have occurred in the N genes and 3' UTRs of IBV vaccine H-120, H-52 strains. IBV evolution is a continual process and the use of vaccines based on geographically relevant strains, is needed to enable effective disease control. The molecular evolution of Iranian IBV strains is not well understood and little genomic data is available. Therefore, a detailed molecular analysis of Iranian IBV strains isolated during the last few decades is needed to aid the understanding of evolutionary relationships between strains.

In addition, continuous molecular surveillance of recent IBV outbreaks is necessary for the rapid identification of new isolates circulating in the field and to enable the successful introduction of new vaccination and control programs.

Therefore characterization of the sequence of S1, N gene and 3' UTR genes sequences is necessary to identify virus strains and their similarities with the vaccine strains. The H-52 and H-120 vaccine strains have been produced and administered in Iran for a long time while molecular characterization of these strain was largely expected.

In this study, we attempted to characterize the full length of the S1, N, and 3' UTR genes of these vaccine strains as a major determinant of the IBV. This aim was successfully achieved, emphasizing that the H-52 and H-120 vaccine manufactured in Razi institute are absolutely identical to the reference vaccine strains submitted to the GenBank. The results of this study showed that the Blast of H-52 and H-120 strains of the Razi Institute with strains found in the gene bank revealed that these strains are closely related to the standard ones.

Given the fact that the IBV isolates identified in recent years have been identified and registered in the gene bank, we can state two hypotheses about this subject: 1- The vaccine strains have been isolated from the field and have been recorded in the gene bank, or, new isolated strain have been made over the years by recombination of vaccine strain with circulating field strain. 2- The possibility of hybridization between them and the production of new viruses have been proven (27, 29).

In addition, the probability of having different types of quasispecies in the products of companies that producing a vaccine has also been reported, and this is due to the high volume of vaccines used to dominate the type of virus that is less immunized against it in vaccinated chickens or by recombining the virus, new viruses that grow despite the lack of adequate protection against them in unvaccinated chickens (11, 16).

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The present study is the first time that the N genes and 3' UTRs of H-120 and H-52 strains of Razi Institute belonging to the Massachusetts serotype have been sequenced and compared with IBVs of standard ones.

In summing up the results of this research, the following points can be considered: Molecular sequence of S1, 3' UTR and N genes of the Razi H-120, H-52 Infectious Bronchitis vaccine seeds complies with standard strains of Massachusetts Serotype and the observed differences do not affect the properties of the proteins studied in this study.

Therefore, vaccination is the most effective way of preventing infectious bronchitis in poultry and the maximum protection achieved by the accurate vaccination program. Also, to complete this project and to more fully identify the Razi Institute's vaccine strains, the following studies on these strains are recommended:

To determine the immunity level of vaccinate chicks with H-52 and H-120 strains against field strains, challenge testing with isolated viruses and determining the degree of immunity of these viruses is recommended. Also, sequencing of other genes of these two strains can help to complete the identification of these two virus (40).

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 2-18-18-87060.

Acknowledgments

The authors sincerely thank the Razi vaccine and serum research Institute for Bioscience and Biotechnology facilities at department of research and production of poultry viral vaccines where this investigation was carried out.

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