Original Article

Molecular Analysis of Nucleocapsid Gene and 3' Untranslated Regions of an Infectious Bronchitis Virus Isolate Originated from Broilers in Maragheh

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ABSTRACT

Avian Infectious Bronchitis Virus, has become one of the most problematic causes of economic losses in poultry farms. To effectively control the virus, monitoring and surveillance of circulating virus strains in poultry farms is inevitable. Internal organ samples of broilers with clinical signs of infectious bronchitis and two samples of the commonly used vaccine strains (4/91 and H120) in Iranian poultry flocks were used for amplification of a 1.8 kbp fragment including nucleocapsid (N) gene and 3′ untranslated region (UTR) by reverse transcription polymerase chain reaction (RT-PCR) method. The amplified fragments were digested with the restriction endonuclease enzyme, *Alu*I. The sequence similarity of the field isolate (Ma1/16) with previous isolates and reference strains of IBV was then investigated. Also, the phylogenetic relationship of Ma1/16 with viruses from other regions was determined based on the sequence of two 600 bp partial sequences of the N gene using Mega7 software. Seven IBVs were classified into two groups based on restriction fragment length polymorphism (RFLP) patterns of the N-3´UTR fragment; all of five field isolates and vaccine strain 4/91 were clustered together. Ma1/16 had the highest similarity with two other Iranian IBV isolates, Ur1/09 and MNS-7861-1 (91.7 % and 90 %, respectively), based on the 600 nucleotides of 5´ end of the N-3' UTR fragment of the isolate. The nucleotide sequence of 600 nucleotides at the 3´ end of the amplified fragment in the Ma1/16 isolate (N-3'UTR) had the highest similarity to the BJ strain (86.4%). Regarding the induction of humoral and cellular immune responses using a vaccine candidate based on T-cell epitope peptides in IBV nucleocapsid protein, the gene sequence data of N-3'UTR fragment can be helpful in monitoring of circulating strains of IBV, designing effective IBV vaccines, and successfully controlling IB disease in Iran.

Keywords: Infectious Bronchitis Virus, N gene, 3´untranslated region, RFLP

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1. Introduction

Infectious bronchitis (IB), which causes with major health problems and significant economic losses in poultry farms, is caused by a Gammacoronavirus called infectious bronchitis virus (IBV). Avian infectious bronchitis virus, the IB pathogen has become one of the most problematic causes of economic losses in poultry farms, due to its highly contagious nature (1). In spite of primarily replication of IBV strains in the respiratory tract of birds, some strains can replicate in other epithelial cells; furthermore, and the pathogenesis of different IBV strains has varied from mild respiratory symptoms to severe kidney and oviduct disease. New knowledge about IBV isolates and their epidemiology has helped to develop control strategies (2). The causative agent of infectious bronchitis, IBV, with a single-stranded genome size of 27.6 kb, has a positive-sense genomic RNA. The 3′ end of the genomic RNA encodes four structural proteins, including spike glycoprotein (S), envelope (E), membrane (M), and nucleocapsid (N) proteins, and four non-structural accessory proteins, 3a, 3b, 5a and 5b. There is a region at the 3′ end of IBV genome of that is not translated (UTR) but plays an important role in the synthesis of negative-strand RNA in the virus. Similar to other coronaviruses, genetic diversity in IBV is generated by both recombination events and by mutations, including substitutions, deletions and insertions, that occur during replication of the viral genome. The S1 function includes viral neutralization, cell attachment and the presence of serotype-specific epitopes (4). The selective force on the regions of the IBV genome with antigenic features is mainly exerted by the immune response (5). Both S and N proteins are the major inducers of the immune response to IBV infection. Also, N protein plays a critical role in viral replication, especially in the transcription and translation of viral RNA (4). Also, the formation of a helical ribonucleoprotein complex with interaction between M and N proteins is thought to play role in RNA packaging (5). To protect their flocks, many poultry farms have adopted vaccination programs with one, or a combination of live attenuated and inactivated vaccines, which are routinely used (6). Regular vaccination and appropriate biosecurity have not been effective in preventing the emergence of new strains of IBV; moreover, because of the high mutation frequency of the virus strains, the protection provided by vaccines is not complete (7). Due to the role of S1 and N proteins as major inducers of immune responses in IBV, molecular analysis of these genes in new IBVs could be very helpful in designing effective vaccines against the disease (8). Most of the conventional methods are limited to the identification of IBVs based on S1 gene analysis. Then, reverse transcription and polymerase chain reaction (RT-PCR) accompanied by molecular analysis of S1 gene have been used for rapid and sensitive identification of IBV isolates. However, because of the high rate of diversity and mutation in the S1 gene of the IBV genome, rapid molecular analysis based on the S1 gene is difficult in IBV outbreaks (9). A high degree of identity among different IBV strains has been observed based on the sequence of the N protein as a highly conserved structural protein. Also, the N protein is the most abundantly expressed protein in IBV infection, and it causes the release of high titers of antibodies by cross-reactions and increases a strong T-cell response. In addition, cytotoxic T lymphocyte (CTL) epitopes were found in the 120-amino acid polypeptide of the C-terminal of IBV N protein, which induces protective immunity. A vaccine candidate based on T-cell epitope peptides in the N of various IBV; induced a high rate of humoral and cellular immune responses in this study, leading to protection against QX-like and TW-like IBV strains. According to their results, a multi-epitope vaccine was proposed as an attractive alternative for the development of an efficient and safer IBV vaccine (10). The detection of important mutations (deletion of 58 bases) in the 3′ UTR of the IBV genome has been used to differentiate IBV strains in recent studies (10). The purpose of the present study was to characterize Iranian IBV field isolate and commonly used vaccine strains based on the N gene and 3′ UTR of their genome using the RT-PCR and RFLP and also molecular typing.

2. Materials and Methods

2.1. Viruses

30 tissue samples from trachea, lungs and cecum tonsils were collected from four broiler farms (age 5- 8 weeks) with clinical signs of IB. The samples were obtained from four different poultry flocks around Maragheh in the northwestern Iran. These flocks received live attenuated vaccine, H120, during the first week of their life. Tissue samples were homogenized and prepared for molecular assays to detect the circulating field IBVs in this region. Two vaccine strains of IBV including H120 (Razi Vaccine and Serum Research Institute) and 4/91 (Intervet Institute Netherlands), common IB vaccines used in regional use, were also obtained.

2.2. Viral RNA Extraction

Extraction and purification of IBV vaccine strains and viral RNA from field isolates were carried out using the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For each RNA extraction approximately 100 µl of prepared homogeneous tissue suspension in PBS buffer (for field isolates) or vaccine suspension was used, and finally extracted RNA was eluted in 30 µl of elution buffer. The resulting RNA was used immediately for cDNA synthesis or stored at −70°C.

2.3. Synthesis of cDNA

Reaction of cDNA synthesis, was performed according to a previous study (11). For each reaction, the extracted RNA was denatured at 100°C for 1 min. It was then immediately placed on ice and mixed with 20µl of prepared premix. The premix solution contained 24U RNAguard (Fermentas, Cinnagen, Iran), 0.5 µM oligo (dT)(Fermentas), 25 µM each of dATP, dTTP, dGTP and dCTP, 5µl of 5X reaction buffer (Fermentas), 200 U Moloneymurine leukemia virus reverse transcriptase (Fermentas),and 10 µl diethyl

pyrocarbonate-treated water. Each reaction was then incubated at 42°C was carried out for 1 h. Reverse transcriptase enzyme inactivation was performed at 100°C for 5 min. The cDNA products were immediately used in a PCR reaction or transferred to −70°C.

2.4. Polymerase Chain Reaction

2.4.1. Amplification of the N Gene and 3′ UTR

The entire N gene and a partial fragment of the 3′ UTR of IBVs were amplified using primer pairs, 5b-F2 (5′ CCTTTTCGCGGAGCAATAG 3′), which binds within the 5b gene and UTR-R1 (5′ CTGTACCCTCGATCGTACTC 3′), which binds within 3′ UTR region. Amplification of 1.8 Kb genomic fragment of N-3′ UTR was performed in a 25-µl mixture of PCR reaction containing 2.5 µl of 10X smart PCR buffer (Cinnagen, Iran), 1.0 U smart DNA polymerase (Cinnagen, Iran), 25 µM each of dATP, dTTP, dGTP and dCTP, 0.25 µM each of primers, 2 mM MgCl2, and 5 µl cDNA as template. The PCR reaction was an initial denaturation at 94°C for 2 min, incubations at 94°C for 45 s (35 cycles), 55°C for 40 s, and 72°C for 2 min with a final extension step at 72°C for 5 min. The resulting PCR products were separated on a 1.0% agarose gel and observed by ultraviolet transillumination. PBS was used as negative control and vaccine strain H120 (Razi Vaccine and Serum Research Institute) was used as positive control.

2.4.2. PCR product purification and enzyme digestion

The PCR products were excised from 1.0% agarose gels and purified using a DNA extraction kit (Purification Kit, Bioneer, AccuPrep Gel, Takapouzist, Iran) to remove non-specific bindings, according to the manufacturer's instructions.

2.5. RFLP

Molecular analysis of amplified fragment was done by restriction fragment length polymorphism (RFLP) technique and also partial sequencing. In RFLP, digestion of purified PCR products was subjected to restriction endonuclease enzyme *Alu*I (Bioscience Jenna, Iran). Each digestion reaction was performed in a 15µl mixture containing 5 µl of purified PCR product and 10 U *Alu*I with an incubation time of 3h at 37°C. Digested PCR products were separated on a 2% agarose gel and visualized by ultraviolet transillumination.

2.6. DNA Sequencing

To determine the sequence of the field isolate of IBV, the PCR product was cloned into pGEM-T using the pGEM-T vector system cloning kit (Promega, Madison, WI). Different primers were designed for the N gene, and the 3' UTR. Cloned PCR products were sent to Macrogen Korea Company for sequencing (Takapouzist, Tehran, Iran).

2.7. Sequence Analysis

The sequence of IBVs isolated from Iranian poultry farms was aligned with sequences of geographically diverse IBVs, which were obtained from the GenBank database. The accession numbers the the GenBank sequences used in this study, are listed in Table 1. Phylogenetic analyses among IBV sequences were examined using Maximum Composite Likelihood in MEGA7 to define the evolutionary relationships.

3. Results

3.1. Amplification of the N-3UTR Region of IBV Strains

1.8 kb fragment including the N genes and 3 'UTR regions of the two vaccine strains commonly used in broiler flocks (4/91 and H120) and five IBV isolates obtained from 30 samples suspected of causing infectious bronchitis in poultry farms around Maragheh were successfully amplified (Figure 1(a)).

3.2. Restriction endonuclease digestion of the N gene and 3′ UTR

The restriction fragment length polymorphism patterns obtained as a result of enzymatic digestion of the N-3'UTR gene fragment by application of *Alu*I enzyme in seven IBVs (vaccine strains and field isolates) are shown in Figure 1(b). Based on the obtained results, two different patterns were created and H120 vaccine strain, by creating a specific pattern, was placed in one group and the five farm isolates of infectious bronchitis virus and 4/91 vaccine strain, having similar patterns, were classified together in another group.

Table 1. Accession numbers of the IBV strains used in this study, obtained from the gen bank

	IBV Strain	Accession number		IBV strain	Accession number		IBV strain	Accession number
1	H120	FJ888351	9	Armidale	DQ490205	17	Beaudette	NC_001451
2	H52	EU817497	10	Vic S	DQ490221	18	EP3	DQ001338
3	Ck,CH,LHB,121010	KP036503	11	Ur1/09	HQ607366	19	N1-62	DQ490206
4	Conn	FJ904716	12	4/91	KF377577			
5	Cal99	AY514485	13	BJ	AY319651			
6	CU-T2	U49858	14	MNS-1	HQ607365			
7	Ark	EU418976	15	QX	AF199412			
8	Mass 41	AY851295	16	SAIBK	DQ288927			

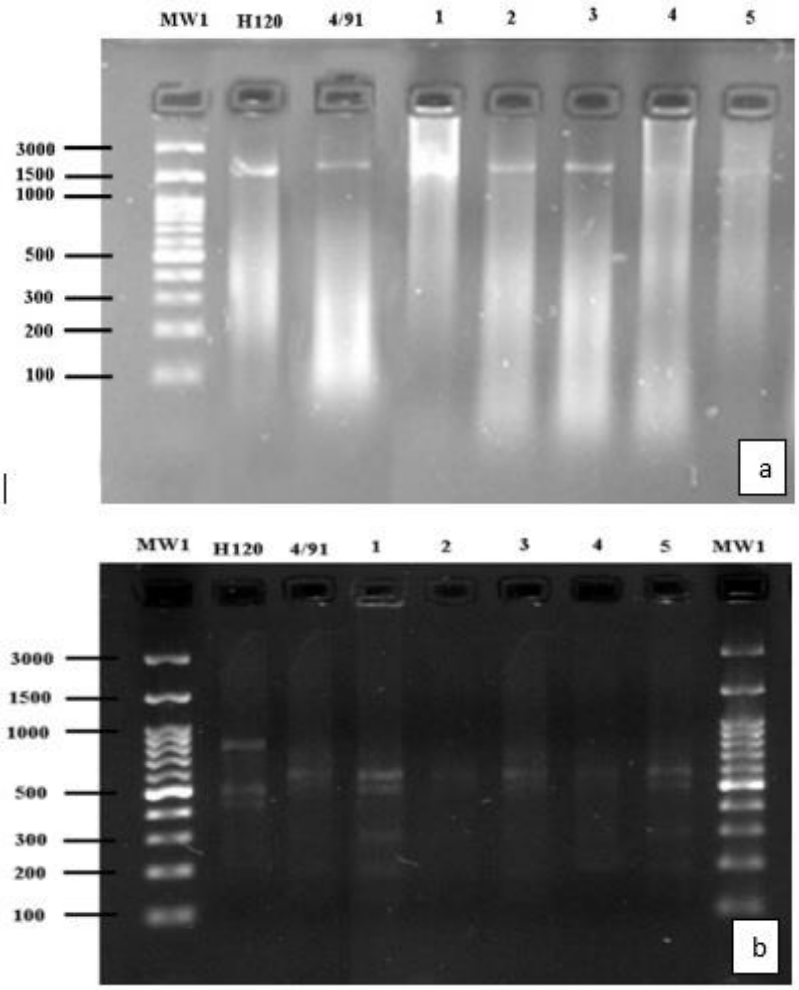

Figure 1. The 1.8 kb amplified fragment bands of the N-3'UTR in seven IBVs used in this study (a); RFLP patterns of the 1.8 kb N-3'UTR in seven IBVs digested with *Alu*I (b).

3.3. Analysis of the Obtained Nucleotide Sequences

3.3.1. The Sequence Analysis of the 5'-end of the N-3'UTR Fragment

600 nucleotide sequences of the 5' end of N gene between IBVs isolated from poultry farms around Maragheh (Ma1/16) were determined and deposited in the Gene bank with accession number of KY523471. Then, it was aligned. It showed that Ma1/16 had the most similarity with two other IBVs isolated from Iran, Ur1/09 and MNS-7861-1 (91.7 % and 90 % , respectively). Then, the partial 5-N gene sequence of Ma1/16 (600 N from the 5 end of N gene) was compared with 19 other N gene sequences from Iran and other countries obtained from GenBank database. Ma1/16 showed the highest similarity with Ur1/09 (91.7%) from Iran and the lowest similarity with BJ strain from China (79.5%).The rate of its similarity with the other IBV isolate from Iran (MNS-7861-1) was 90%. Also, the nucleotide similarity between Ma1/16 with 4/91 vaccine strain and H120 vaccine strain was 85.4 and 83.9%, respectively. The IBVs isolated from Iran showed 79.5% (BJ) to 92.5% (California 99) sequence similarity with the partial 5-N genes of IBVs from other countries. The partial 5'-N gene sequences of the field isolates from Iran showed a similarity of 85.4 to 89.6% with the 5-N genes of vaccine strain 4/91, while they shared sequence similarity of 83.9 to 90.3% with H120 vaccine strain (Figure 2). Phylogenetic analysis of the N genes showed that the IBV isolated in this study were clustered together with other two IBVs isolated from Iran and were separated from all other IBVs (Figure 3). Vaccine strain 4/91 was located alone in a separate group. Most of the Chinese strains were clustered together and the Australian strains were classified together.

3.3.2. Analysis of the Nucleotide Sequence of 3' End of N-3'UTR Fragment

Nucleotide sequence of another region in the 3´end of the 1.8kb genomic fragment (600 bp) including the variable region of the 3'UTR (300bp) and 300 nucleotides of the 3'end of the N gene of the field isolate from Iran, Ma1/16, was compared with the similar other IBV sequences obtained from the Gene Bank. The accession numbers of the nucleotide sequences used are shown in table 1.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1. Ma1/16																			
2. MNS-7862-1	90																		
3. Ur1/09	91.7	96.2																	
4. 4/91 vaccine	85.4	89.6	89.4																
5. H120	83.9	89.6	90.3	88.5															
6. H52	81.9	89.6	88.9	87.2	91.5														
7. Vic	79.8	85.9	88.7	86	85.8	87.9													
8. CU-T2	84.5	91.2	91	88.5	94.8	93.1	87.1												
9. SAIBK2	82.8	88.4	88.4	88.2	89.1	90	86.6	91.4											
10. Mass41	0.81	87.5	87.1	86.6	88.7	96.9	87.5	90.3	90.9										
11. California99	85.7	92.5	91.6	89	95.5	93.8	86.6	97	91.8	91.1									
12. Beaudette	81.1	88.1	88.9	87.3	89.1	89.1	85.9	90	89.7	90.4	90								
13. B17	83.9	89.6	90.3	88.5	100	91.5	85.8	94.8	89.1	88.7	95.5	89.1							
14. ARkDPI11	85.9	92.1	91.8	88.6	93.3	93.3	87.3	96.2	93.4	93.3	96.5	92.4	93.3						
15. Conn 46 1996	85.9	92.1	91.8	88.6	93.3	93.3	87.3	96.2	93.4	93.3	96.5	92.4	93.3	100					
16. Ck/CH/LH/B121010	84.2	90.4	90.5	87.6	90.7	90.4	87	92.3	91	91.1	93.2	90.4	90.7	95.5	95.5				
17. N1-62	82.2	89.2	88.5	86.9	87	89	93	88.6	88	88.5	88.6	87.3	87	88.8	88.8	88.2			
18. EP3	81.3	88.3	89	87.5	89.7	89.3	86.1	90.2	89.9	90.6	90.2	99.5	89.7	92.6	92.6	90.6	87.5		
19. BJ	79.5	84.6	85.8	85	85	86.5	86.6	85.7	90.7	86.1	86.3	86.3	85	87.8	87.8	88.2	88.1	86.5	
20. Armidale	78.6	84.9	85.7	85.2	85.8	86	95	86.9	85.8	85.9	86.5	86.4	85.8	86.9	86.9	92.3	92.3	86.3	85.8

Figure 2. Estimated evolutionary similarities between different IBV strains based on 20 nucleotide sequence analysis of the 5′ end of the N gene (600N).

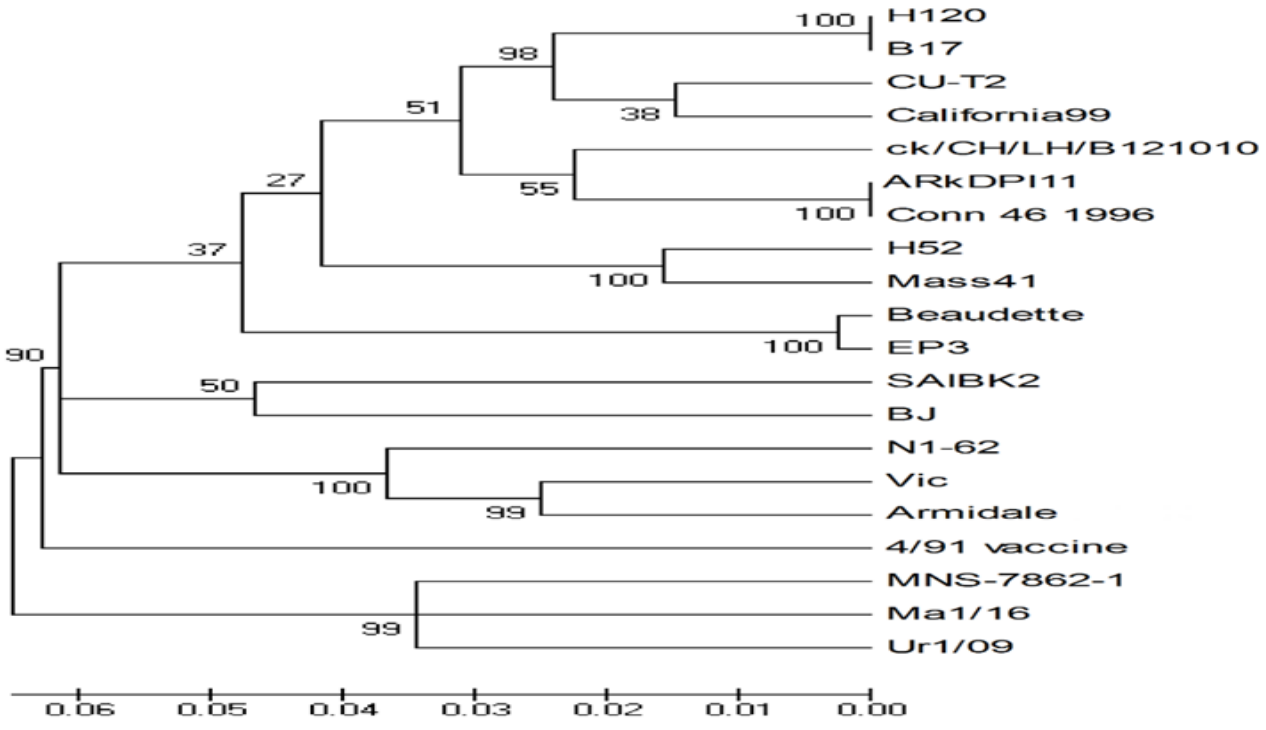

Figure 3. The phylogenetic tree based on the sequence of the 5' end of the N gene in 20 strains of IBV using MEGA7 software

3.3.2. Analysis of the Nucleotide Sequence of 3' End of N-3'UTR Fragment

Nucleotide sequence of another region in the 3´end of the 1.8kb genomic fragment (600 bp) including the variable region of the 3'UTR (300bp) and 300 nucleotides of the 3'end of the N gene of the field isolate from Iran, Ma1/16, was compared with the similar other IBV sequences obtained from the Gene Bank. The accession numbers of the nucleotide sequences used are shown in table 1. The nucleotide sequence of this region in Ma1/16 isolate had the highest similarity to the BJ strain (86.4%). Vaccine strain 4/91 and N1-62 strain showed a high similarity rate of 78.4%, while 4/91 had the least similarity with sequences of Mass41 isolate strains with a rate of 79.2%. The similarity rate of Ma1/16 to H120 was 81.6% (Figure 4). According to the phylogenetic tree based on N-3'UTR of different strains of IBV, isolate Ma1/16 was classified in a different group from other strains. Strains from China were grouped together. Vaccine strain 4/91 clustered in a group with strains H120, California 99 and H52 (Figure 5).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1. Ma1/16																	
2. 4/91 vaccine	84.7																
3. Armidale	83.7	87.5															
4. BJ	86.4	88.9	92														
5. EP3	83.2	89.3	88.7	88.6													
6. N1-62	84.7	89.8	94.7	92.2	89.8												
7. Ck/CH/LHB121010	81.8	84.7	85	89.1	86	85.6											
8. Conn 46 1996	84.1	89.3	90.1	90.9	93.2	90.1	85.7										
9. ARkDPI11	83.5	88.7	89.8	90.3	92.9	89.8	85.1	99.5									
10. B17	81.6	90.9	84.8	86.9	88.2	87.2	82.7	90.7	90.7								
11. Beaudette	83.2	89.3	88.7	88.6	99.5	89.8	86	93.2	92.9	88.2							
12. California99	82.8	91.5	85.4	88.1	83	87.5	83.9	91.2	91.2	97.5	89.3						
13. H52	81.6	90.9	84.8	86.9	88.2	87.2	82.7	90.7	90.7	100	88.2	97.5					
14. H120	81.6	90.9	84.8	86.9	88.2	87.2	82.7	90.7	90.7	100	88.2	97.5	100				
15. Mass41	79.2	85.1	88	86.8	88.9	87.1	81.6	88.6	88.4	85.7	88.9	85.4	85.7	85.7			
16. SAIBK2	81.4	87.7	88	93.9	86.6	88.9	85.8	88.6	88	86	86.6	86.6	86	86	86.1		
17. CU-T2	83.4	90.9	88	89.4	90.7	88.9	84.1	94.4	93.9	93.1	90.7	93.6	83.1	83.1	87.5	88	
18. Vic	83.8	88.9	95.5	95.5	90.9	93.6	85.9	90.7	90.4	86.6	90.9	87.5	86.6	86.6	89.7	89.5	88.1

Figure 4. Estimated evolutionary similarities between different IBVs based on analysis of N-3'UTR of 18 nucleotide sequences

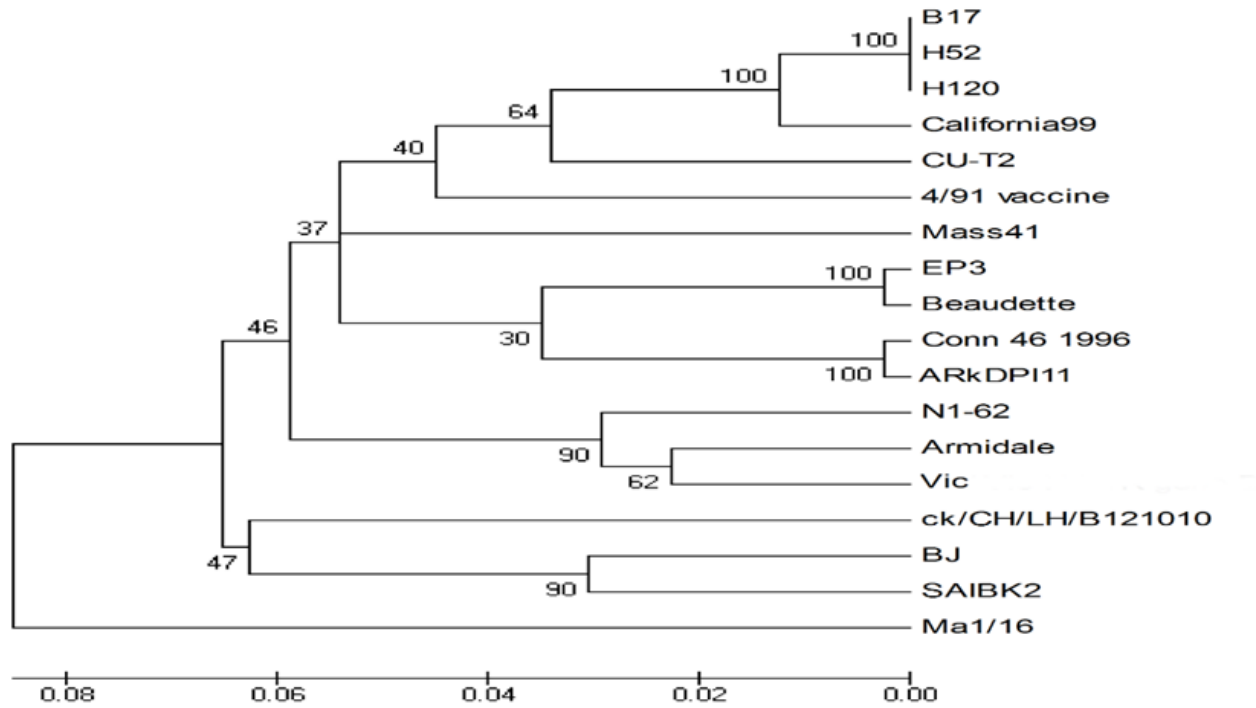

Figure 5. Phylogenetic tree based on the sequence of the N-3'UTR in 18 IBV strains using MEGA7 software

4. Discussion

Due to the increasing rate of evolution of IBVs and the significance of IB as a serious and contagious disease, the identification and characterization of IBV circulating strains in different regions are critical for the designing and selecting appropriate vaccination programs (13). Despite previous studies based on the significance of the S protein in the typing of IBV strains, some reports have introduced the N protein as an important inducer of immune responses against IBVs, which could be a critical target in studies to control and prevent IB outbreaks (3, 11,14). In this study, five isolates of infectious bronchitis virus were detected by RT-PCR method in samples collected from broilers with clinical signs of infectious bronchitis. Two molecular assays were used to characterize field isolates of IBV. Enzyme digestion of N-3'UTR fragment and nucleotide sequence determination of two 600 nucleotide fragments in the 3' end and 5' end of the 1.8 Kb fragment were used. Vaccine strains, H120 and 4/91 were also subjected to enzyme digestion. Restriction fragment length polymorphism patterns showed identical patterns for five isolated IBVs and vaccine strain 4/91, and vaccine strain H120 clearly classified. Seven IBVs were then located in two groups. In the previous study by Majdani et al, 2010, two field isolates from Iran (4/91 field isolate and MNS-78621) were clustered into two distinct groups. The isolate, MNS-78621, was grouped separately from the vaccine strains used based on 3'-UTR nucleotide analysis (H52, H120 and 4/91). Similar differentiation potential of two genomic fragments of S1 gene and N-3'UTR of IBVs was also observed in the study (15). In a study in Italy, after amplification of the S1 and N genes of IBVs, five field isolates were clustered together based on S1 molecular analysis. In contrast, according to molecular analysis of the more conserved partial N gene sequences, showed different clustering. For example, three variants of IBVs, which had 96.7-99.2% S1 gene nucleotide identity with each other, belonged to three separate subgroups based on their N gene sequences (16). In the sequence analysis of 600 nucleotides from the 5' end of the N gene, Ma1/16 had the highest similarity with other field isolates from Iran and lower similarity with vaccine strains, H120 and 4/91 and the lowest similarity was shown with Chinese strain BJ. In the phylogenetic analysis of the 5'end of the N-3'UTR fragment, Ma1/16 was classified with other Iranian isolates and clearly separated from 4/91 and H120 strains. Regarding the sequence analysis of 600 N of the 3' end of the N-3'UTR fragment, unfortunately the sequence of this genomic fragment of IBV isolates from Iran wasn't found in GenBank. Then we aligned the sequence with IBV isolates from other countries; Ma1/16 showed the highest similarity with the strain of BJ from China and the lowest similarity with the strain Mass41. In the phylogenetic tree based on the sequence of the 3' end of the N-3'UTR fragment, Ma1/16 was placed in a different group from other strains and also from vaccine strains. Overall, the similarity rate of 600 nucleotides of the 5'end of the amplified fragment in this study among different strains was higher than the similarity rate of 600 nucleotides of the 3' end of the N-3'UTR among different strains of IBV which could happen due to more variety of nucleotide sequence of 3'UTR in different strains of IBV. Recent reports have demonstrated the existence of new circulating strains of IBV. The co-circulation of several different IBV serotypes/strains with various recombination has been reported in Mexican chickens, which may lead to the generation of new IBV variants with the potential to spread in poultry farms in the country (17). In addition, the emergence of some new types of IBV strains that are genetically different from the predominant vaccine strain H120, has increased in southern China , and the authors emphasized the importance of renewing vaccination strategies based on the current circulating strains in the region (18). Other studies have also introduced the monitoring of circulating IBVs in wild birds as an important factor in updating the vaccination strategies and developing a vaccine with a high rate of cross-protection against the majority of the IBV circulating genotypes (19). In a study on IBVs in Poland during 2008-2011, samples obtained from wild animals suspected to IB were analyzed based on the sequence of the N gene and the 3' UTR. 10 samples were positive for the presence of IBV, and the isolates showed high relationship with Massachusette, QX and 793/B strains (20). In another study in 2011 on N gene analysis of five isolates of IBV showed that all of these isolates were clustered with strain CK/CH/LDL/97I from China and Taiwan (21). In 2012, an isolate of IBV belonging to strain ck/CH/LZJ/111113 was isolated from a chicken vaccinated with H120 vaccine strain and molecular analysis of S1 and N genes, showed that the isolate was a derivative of two IBV strains ck/CH/LDL/091022 and 4/91(22). In the USA, some IBVs have been isolated without any significant relationship to other IBV strains based on N gene analysis (23). In a study in Italy, eight IBV strains were similarly clustered into five groups using RT-PCR- RFLP with the enzymes *Alu*I and *Mnl*I (16). Most previous studies on circulating strains of IBV in Iran have been based on S1 gene analysis, but few studies on IBVs have been based on N gene sequence analysis. In a previous study by Majdani et al, based on molecular typing of N gene, the similarity of two IBV isolates from Iran, MNS-7862-1 and MNS-7862-2 with vaccine strain

H120 was 90% and the rate of their similarity based on 3' UTR sequences was 94.5% and 94.2% ,respectively which was not consistent with our results (24). Nosrati et al, 2012, showed a high degree of relatedness between isolated IBVs from Iran and Ur1/09 and also the American strainaccording to molecular analysis of S, M and N genes (25). In a study by Ghalyanchi langeroudi et al, 2015, the percentages of variant 2 (IS-1494 like), 793/B, and QX genotypes were reported as 66.7%, 26.7%, and 6.6%, respectively and they reported that QX as the first time and variant2 was the dominant genotype in eastern Iran (26). In 2016, Najafi et al, reported the presence of seven genotypes of IBV (Massachusetts (Mass), 793/B, IS-1494, IS-720, QX, IR-1, and IR-2) are reported in Iran (27). In a study by Mousavi et al, they reported IS-1494 like IBVs as the dominant genotype circulating in Iran; they determined the full-length genome of this Iranian IS-1494 like IBV was (Mahed) to study its evolutionary relationships. It was found that the sequence similarity between the isolate and other reference strains of IBV was about 88-91% according to N gene analysis, while it was estimated to be 73-81% based on S1 gene analysis (28). Ghalyanchi langeroudi et al, 2019, reported the presence of D274 in Iranian poultry farms for the first time. IS/1494/06 was revealed dominant IBV type in broiler flocks. Other types reported in the study included 793/B (7%), QX, and Mass with prevalent rate of 5% and 2%, respectively, and D274 with the incidence of 1% (29). In addition, in the Khorasan Razavi province of Iran in 2019, QX and variant 2-like strains were reported as the main IBVs circulating in broiler and layer flocks in northeastern Iran (30). In another study in this province, sequencing of the S1 partial gene and phylogenetic analysis classified eight IBVs in GI-23 lineage (Is-Variant2), two viruses in GI-1 lineage (Mass), and one IBV in GI-12 lineage (793B) (31). Rezaei et al, 2020, revealed the presence of QX and IS-1494 strains in live birds in Gilan province based on S1 gene sequence analysis (32). According to our results, molecular analysis of the N-3'UTR had the power to differentiate IBV strains. No differentiation was observed among the farm isolates, and the RFLP pattern of these isolates was similar to 4/91 vaccine strain, which could be due to the vaccination of poultry flocks in the region with the mentioned vaccine strain. Despite the similarity of RFLP pattern in 4/91 vaccine strain with isolates from the farm, there is a relatively high nucleotide sequence difference between Ma1/16 isolate and the mentioned vaccine, which can suggest the emergence of a different strain of the virus derived from the farm isolates and the vaccine strain in a process of genetic variations including mutations. The comparison of the nucleotide sequence of two regions of the amplificated fragment of the N-3'UTR nucleotide sequences with existing reference sequences in the gene bank revealed its similarity to other IBVs from Iran, but the rate of this similarity was not very high even in the regions previously accepted as conservative regions. Rapid and accurate diagnosis of pathogens is very important for timely control of IB using geographically relevant vaccine strains based on IBVs circulating in Iran. Therefore, continuous monitoring of the circulating IBVs in the field is an urgent need. Based on our results, mutations, including insertions and deletions, may have occurred in the N genes and 3' UTRs of IBV strains originating from Iran. Unfortunately, there are few genomic data available on the evolution of IBV strains in Iran. Therefore, detailed molecular analysis of IBV isolates from Iran plays a crucial role in understanding their evolutionary relatedness in recent years. Our data suggest that the continuous molecular surveillance of IB outbreaks and accurate and rapid identification of new circulating field isolates circulating in the field could be effective in the successful implementation of new controlprograms.

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Authors' Contribution
Study concept and design: R. Majdani, Acquisition of data: A. Shaki Masuleh, Analysis and interpretation of data: R. Majdani, Drafting of the manuscript: R. Majdani & A. Shaki Masuleh, Critical revision of the manuscript for important intellectual content: R. Majdani.

Ethics
Not applicable.

Conflict of Interest
The authors declare that there is no conflict of interest.

Data Availability
The data that support the findings of this study are available on request from the corresponding author.

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