Molecular analysis of the nucleocapsid gene and 3' untranslated region of two infectious Bronchitis Virus field isolates from Iranian poultry farms

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Infectious bronchitis virus; polymerase chain reaction; sequencing; nucleocapsid gene; 3' untranslated region.

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Abstract

Infectious bronchitis (IB) is an economically important disease of chickens. Due to the emergence of new variants of infectious bronchitis virus (IBV), the control of IB has become a serious problem for the poultry industry worldwide. In the present study, 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. Based on nucleotide identity, the N gene and 3' UTR sequences of Iranian IBVs showed 90% similarity to the commonly used IBV vaccine strains, H52 and H120. However, based on phylogenetic analyses, Iranian IBVs were found to cluster separately from the IBV vaccine strains used in Iran as well as other IBVs isolated in China, Australia and the United States. It was concluded that IBVs circulating in Iran are genetically distinct from IBV vaccine strains that have been used in Iran for many years. Therefore, it is important to develop a new vaccine based on these newly identified strains for controlling IB in Iranian poultry farms.

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 (Cavanagh and Naqi, 2003; Van Roekel *et al*., 1951). The causative agent of IB, the infectious bronchitis virus (IBV), is a member of the genus *Coronavirus* of the family *Coronaviridae*. The IBV genome is a single-stranded linear RNA molecule of 27.6 kb (Bochkov *et al.*, 2006; Boursnell *et al.*, 1987). 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) (Cavanagh, 1981; Sutou *et al.*, 1988). The genes in the IBV genome are arranged in the following order: 5'-polymerase-S-E-M-N-3', with a few non-structural genes interspersed among these genes (Lai and Cavanagh, 1997).

The high frequency of new IBV variants is a distinguished characteristic of this virus among other corona viruses (Bochkov *et al.*, 2006). According to Ignjatovic *et al*. (2000) more than 50 serotypes of IBV have been identified and new variants continue to emerge despite the use of live attenuated and killed IBV vaccines (Cavanagh, 2007; Cook *et al*., 1996; Liu and Kong, 2004). However, as these vaccines offer little cross-protection, outbreaks of IB can still occur between serologically distinct viruses (Hofstad, 1981; Liu *et al.*, 2006). 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 (Liu *et al.*, 2006).

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 (Lin *et al*., 1991; Zwaagstra *et al*., 1992; Adzhar *et al*., 1996; De Wit, 2000; Liu *et al*., 2003; Bochkov *et al*., 2006). 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 (Jones *et al.*, 2009). Nevertheless, the means of spread by these viruses are seldom well understood (Jones *et al.*, 2009).

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. Therefore, characterization of the S1 and N gene sequences is important for selecting the most useful and effective vaccine (Cavanagh *et al.*, 1997; Ignjatovic *et al.*, 1997; Seo *et al.*, 1997; Wang *et al.*, 1994). Furthermore, the N protein plays a role in viral replication and assembly and it binds to the viral RNA forming a helical nucleocapsid (Lai and Holmes, 2001). 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 (Williams *et al.*, 1993) making this region worthy of further investigation as a method for differentiating IBV strains (Hewson *et al.*, 2009; Mardani *et al.*, 2006).

Viruses that belong to the 793/B serotype were first reported in France in 1988 (Picault *et al.*, 2003) and this serotype is now found in many countries, with the exception of the United States (Jones *et al.*, 2009). In Iran, viruses that belong to the 793/B serotype were isolated from broiler and layer poultry farms during 1997 to 2004.

In the present study, the N gene- and 3' UTR genome fragment of two IBV field isolates from Iranian poultry farms were sequenced and compared with sequences from non-Iranian origin. The results reveal that, based on N gene and 3' UTR sequences, Iranian IBV isolates cluster together but these are phylogenetically distanced from the common vaccine strains used in Iran.

Materials and Methods

Viruses

Two Iranian IBV field isolates (MNS-7862-1 and MNS-7862-2), that were confirmed by serotyping and molecular analysis of the S1 gene to belong to the 793/B serotype, were obtained from the Faculty of Veterinary Medicine at Tehran University. These isolates were circulating in Iranian poultry farms during 1998 to 2003. They were isolated from broiler flocks (5 to 6 weeks of age) that showed signs of respiratory disease characterized by coughing, sneezing, tracheal rale, nasal discharges and elevated mortalities. An IBV isolate from broiler farms in Urmia, West Azerbaijan in 2009 (designated UR1/09) was also used in this study. Chickens in the flocks infected with UR1/09 were aged between 26 and 35 days and had received the H120 vaccine at 1 day of age. Coughing, sneezing, nasal discharges and elevated mortalities were the main clinical signs. At necropsy, the chickens had pulmonary and tracheal congestion, high to moderate exudates in trachea and pale, swollen kidneys.

RNA extraction

IBVs underwent RNA extraction using the RNeasy Mini kit (Qiagen, Hilden, Germany). RNA extraction was carried out according to the manufacturer's instructions. For each RNA extraction, 50 µl allantoic fluid was 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.

cDNA synthesis

cDNAs were synthesized in a 25 µl reaction mixture, according to a previous report (Mardani *et al.*, 2006). For each cDNA synthesis reaction, 5 µl of extracted RNA was mixed with 1 µl of 25 µM oligo(dT) (Fermentas, Cinnagen, Iran). This mixture was incubated at 100˚C for 1 min and cooled down by placing on ice for 5 min. Then, to this was added 19 µl of premix that contained 24 U of RNA Guard (Fermentas, Cinnagen, Iran), 50 µM each of dATP, dTTP, dGTP and dCTP, 5 µl of 5X polymerase chain reaction (PCR) buffer and 200 U of Moloney murine leukemia virus reverse transcriptase (Fermentas, Cinnagen, Iran). The reaction was incubated at 42˚C for 1 h followed by inactivation of the reverse transcriptase enzyme at 100˚C for 5 min. The resultant cDNAs were used immediately for PCR or stored at -20˚C for later use.

PCR

PCR was performed to amplify a fragment of ca. 1.8 kb containing the whole N gene and partial 3' UTR of the IBVs. The amplification reactions used a designed forward primer, 5b-F2 (5' CCTTTTCGCGGAGCAATAG 3') that binds to the 3' end of gene 5 of the IBV genome (complementary to bases 25703 to 25721 of the H120 strain; accession number FJ888351), and the previously described reverse primer, UTR-R1 (5' CTGTACCCTCGATCGTACTC 3') (Mardani *et al.*, 2006) that binds to the 3' UTR. PCR was carried out in a 25 µl reaction volume containing 50 µM each of dATP, dCTP, dGTP and dTTP, 0.5 µM of each primer, 2.5 µl of 10X High Fidelity PCR buffer (Fermentas,Cinnagen, Iran), 2 mM of magnesium chloride, 1.0 U of High Fidelity DNA polymerase (Fermentas, Cinnagen, Iran) and 3 µl of cDNA template. 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. The PCR products were separated in 1% agarose gel and observed using ultraviolet transillumination.

Purification of PCR products

PCR products were excised from the 0.8% agarose gel and purified using a DNA extraction kit (Fermentas, Cinnagen, Iran), according to the instructions of the manufacturer.

DNA sequencing

PCR products were cloned in pGEM-T using the pGEM-T vector system cloning kit (Promega, Madison, WI). Different primers were designed for the N gene and 3' UTR cloned PCR products were sent for sequencing to Genfanavarn Company (Tehran, Iran).

Sequence analyses

Sequences from the Iranian IBVs were aligned with sequences of geographically diverse IBVs, which were obtained from the GenBank database. Accession numbers for the sequences from the GenBank are listed in Table 1. Evolutionary relationships between IBV sequences were examined by phylogenetic analyses using Maximum Composite Likelihood in MEGA4 (Tamura *et al.*, 2004; Tamura *et al.*, 2007).

Table 1: Accession numbers from the GenBank database of IBV sequences used in this study.

	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	D1466	AF203006 (N) AF203007 (UTR)	11	LX4	AY338732	19	N1-62	DQ490206
4	D41	AF321275 (N) AF322368 (UTR)	12	A2	EU526388	20	UK/918/68	AJ278334 (UTR
5	Cal99	AY514485	13	BJ	AY319651	21	D207	AJ278335 (UTR
6	CU-T2	U04805 (N) U04804 (UTR)	14	LTD3	AY702975	22	DE072	AF203001 (N) AF203002 (UTR
7	Ark	EU418976	15	QX	AF199412			
8	Mass 41	AY851295	16	SAIBK	DQ288927			

Results

N gene sequence analysis

The N gene sequences from the IBV Iranian field isolates were aligned and this showed that the two isolates, MNS-7862-1 and MNS-7862-2, shared more than 99% sequence similarity. As a result, only the N gene sequence of MNS-7862-1 was compared with 19 other N gene sequences from different countries deposited in the GenBank database (Table 2). MNS-7862-1 showed greatest similarity to the other Iranian field isolate, UR1/09 (96.7% sequence identity). The Iranian field isolates showed 84.6 to 91.5% sequence similarity with N genes from IBVs in other countries. The N gene sequences of the Iranian field isolates showed least similarity (84.6 to 87.0%) with N genes of IBVs isolated from China (LX4, A2, BJ, LTD3, QX and SAIBK), while sharing greatest sequence similarity (90.9 to 91.4%) with the N genes of IBVs isolated from the United States (Ark, Cal99 and CT-U). Phylogenetic analysis of the N genes showed that Iranian IBVs were clustered together and were separate from all other IBVs (Figure 1). The Iranian

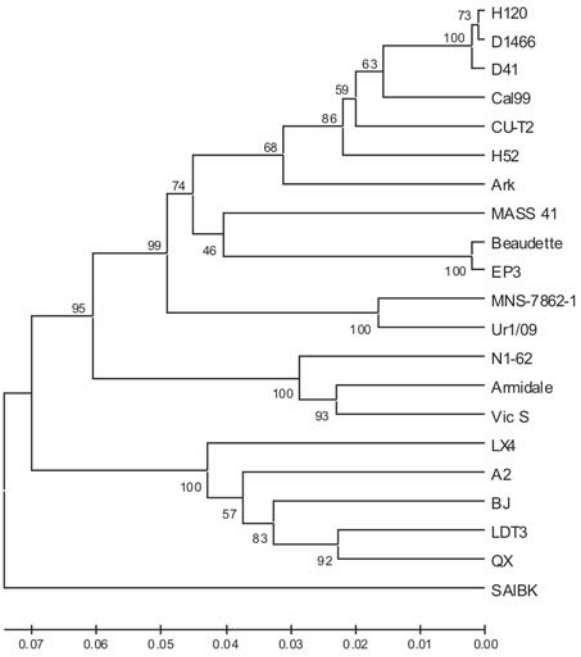

Figure 1: Phylogenetic tree based on the N gene sequences of 21 IBV strains using the UPGMA method of the MEGA 4 software.

Table 2: Estimates of evolutionary similarity based on the pairwise analysis of 21 N gene sequences from different IBV strains.

	H120	D1466	D41	Cal99	CU-T2	H52	Ark	Mass 41	Beaudette	EP3	MNS-7862-1	Ur1/09	N1-62	Armidale	Vic S	LX4	A2	BJ	LTD3	QX
D1466	99.8																			
D41	99.6	99.5																		
Cal99	96.8	96.8	96.9																	
CU-T2	95.7	95.8	95.8	96.6																
H52	95.8	95.7	95.6	96.0	94.9															
Ark	93.1	93.0	93.2	95.0	95.2	93.1														
Mass 41	89.9	89.8	90.0	91.0	91.2	94.1	93.3													
Beaudette	90.1	90.0	90.2	90.7	90.9	90.1	92.9	91.9												
EP3	90.4	90.3	90.5	90.8	91.0	90.2	93.0	92.0	99.6											
MNS-7862-1	89.4	89.3	89.5	91.4	90.9	89.4	91.4	89.2	89.2	89.3										
Ur1/09	90.3	90.2	90.4	91.5	91.4	89.6	92.0	89.4	90.1	90.2	96.7									
N1-62	87.7	87.6	87.8	88.5	88.8	88.7	89.3	89.2	88.9	89.0	89.8	89.9								
Armidale	86.0	85.9	86.1	86.5	87.5	86.1	88.2	88.1	87.9	87.8	87.2	88.3	94.3							
Vic S	86.7	86.6	86.8	87.2	87.6	87.8	88.6	89.2	88.3	88.4	87.8	88.2	94.2	95.4						
LX4	88.0	87.9	88.1	88.3	86.9	87.5	87.7	85.3	86.1	86.2	85.2	86.3	86.9	86.3	85.7					
A2	84.5	84.4	84.7	85.6	85.3	84.1	86.1	85.6	85.5	85.4	84.6	85.3	86.2	86.4	85.2	92.5				
BJ	85.7	85.6	85.8	86.9	86.8	86.5	87.9	87.1	86.9	87.0	86.7	88.0	88.7	87.8	87.6	90.9	92.1			
LTD3	84.3	84.2	84.4	85.8	85.8	84.7	86.6	85.6	85.2	85.3	84.8	85.8	87.0	86.7	85.9	91.9	94.0	94.2		
QX	84.7	84.6	84.8	86.3	85.9	85.3	86.3	85.2	85.3	85.4	85.1	85.8	86.8	86.0	85.7	90.4	91.5	92.7	95.5	
SAIBK	84.5	84.4	84.6	85.4	84.9	84.3	86.0	84.7	84.9	85.0	85.1	85.5	85.6	84.8	84.9	85.9	85.9	85.5	86.5	85.(

Table3: Estimates of evolutionary similarity based on the pairwise analysis of 21 3' UTR sequences from different IBV strains.

	Ark	CU-T2	D207	DE072	MNS-7862-1	Ur1/09	UK/918/68	Beaudette	EP3	Cal99	Vis S	Armidale	N1-62	Mass 41	SAIBK	A2	BJ	D41	H120	D1466
CU-T2	100.0																			
D207	99.3	99.3																		
DE072	99.3	99.3	98.7																	
MNS-7862-1	98.0	98.0	98.7	97.3																
Ur1/09	98.7	98.7	99.3	98.0	99.3															
UK/918/68	97.3	97.3	98.0	96.6	96.6	97.3														
Beaudette	98.7	98.7	99.3	98.0	98.0	98.7	98.7													
EP3	98.7	98.7	99.3	98.0	98.0	98.7	98.7	100.0												
Cal99	97.3	97.3	98.0	96.6	96.6	97.3	95.9	97.3	97.3											
Vic S	96.6	96.6	97.3	95.9	95.9	96.6	95.2	96.6	96.6	95.2										
Armidale	95.9	95.9	96.6	95.2	95.2	95.9	96.0	95.9	95.9	94.5	96.6									
N1-62	97.3	97.3	98.0	96.6	96.6	97.3	95.9	97.3	97.3	95.9	98.0	98.7								
Mass 41	95.9	95.9	96.6	95.2	95.2	95.9	95.9	97.3	97.3	94.5	95.9	95.2	96.6							
SAIBK	95.9	95.9	96.6	95.2	95.2	95.9	94.5	95.9	95.9	95.1	95.2	93.0	94.5	93.0						
A2	96.6	96.6	97.3	95.9	95.9	96.6	95.3	96.6	96.6	95.9	94.5	93.8	95.2	93.8	98.7					
BJ	96.6	96.6	97.3	95.9	95.9	96.6	95.3	96.6	96.6	95.9	94.5	93.8	95.2	93.8	98.7	100.0				
D41	95.2	95.2	95.9	94.5	94.5	95.2	95.9	95.9	95.9	95.2	93.0	93.8	93.8	93.0	92.3	93.1	93.1			
H120	95.2	95.2	95.9	94.5	94.5	95.2	95.9	95.9	95.9	95.2	93.0	93.8	93.8	93.0	92.3	93.1	93.1	100.0		
D1466	95.2	95.2	95.9	94.5	94.5	95.2	95.9	95.9	95.9	95.2	93.0	93.8	93.8	93.0	92.3	93.1	93.1	100.0	100.0	
H52	95.2	95.2	95.9	94.5	94.5	95.2	95.9	95.9	95.9	95.2	93.0	93.8	93.8	93.0	92.3	93.1	93.1	100.0	100.0	100.0

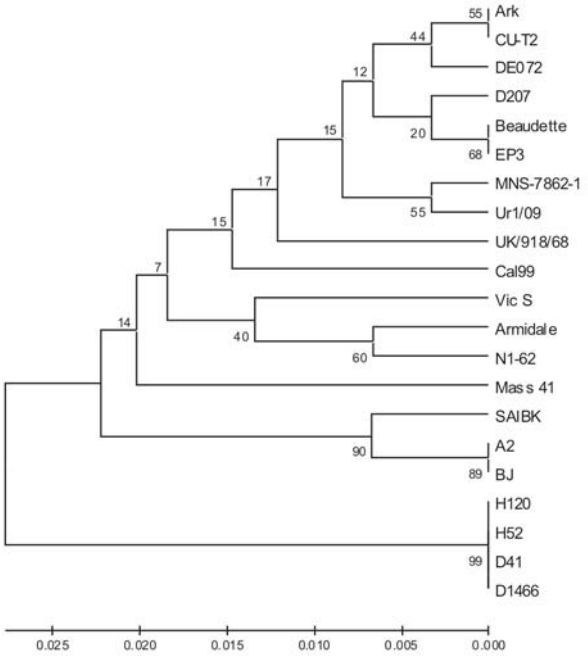

Figure 2: Phylogenetic tree generated based on the 3' UTR sequences of 21 IBV strains using the UPGMA method of the MEGA4 software.

IBV N gene sequences were located between IBVs from the United States and Australia and were distanced from the most regular vaccine strains used in Iran (H52 and H120).

3' UTR sequence analysis

The variable region of the 3' UTR (300 bp) of the Iranian IBV field isolates were aligned and this showed that the two Iranian isolates shared 99.3% sequence similarity (Table 3). Comparing 3' UTR of Iranian IBVs with other IBV 3' UTR sequences revealed that the Iranian IBVs showed greatest similarity with isolates from the United States but, interestingly, least sequence similarity with the H52 and H120 vaccine strains. According to phylogenetic analysis, the two Iranian IBVs clustered together and were located in a same cluster with the strains from the United States (Ark, CU-T2, Beaudette, EP3, DE072) and Europe (D207, UK/918/68). The vaccine strains (H52 and H120) clustered with strains D1466 and D41 in the cluster farthest from the Iranian IBVs (Figure 2).

Discussion

IBV strains of the 793/B serotype were first identified in France in 1985, and then in Great Britain in 1991. Subsequently, these strains spread to other countries in Europe, Asia and North America and became one of the most common IBV serotypes in certain countries (Cavanagh *et al.*, 2005; Cook *et al.*, 1996; Gough *et al.*, 1992). The existence of a 793/B serotype of IBV in Iran (namely, isolate 4/91) was first reported by Vasfi Marandi and Bozorgmehri Fard (Vasfi Marandi and Bozorgmehri Fard, 2000). Since 1998, it has been the 793/B and Massachusetts serotypes that are the predominant serotypes circulating in Iranian poultry farms. One of the most sensitive methods for analyzing IBVs is the determination of nucleotide sequences from the genome, especially the S1 and N genes, which are thought to contribute to IBV selection and evolution (Abreu *et al.*, 2006; Huang *et al.*, 2004; Shieh *et al.*, 2004). Although the typing of IBV strains is usually based on features of the S protein (Cavanagh, 1995; Cavanagh *et al*., 1992; Shankar and Carol, 2007), 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 (Ignjatovic and Galli, 1993; Ignjatovic and Sapats, 2005; Park *et al*., 2005). Moreover, deletions in the N genes (Sapats *et al.*, 1996) and 3' UTRs of different IBV strains have been reported

previously (Boursnell *et al.*, 1985; Mardani *et al.*, 2006; Sapats *et al.*, 1996; Williams *et al.*, 1993).

The present study is the first time that the N genes and 3' UTRs of Iranian field isolates belonging to the 793/B serotype have been sequenced and compared with IBVs of non-Iranian origin. Sequence alignments of the N genes and 3' UTRs from Iranian IBV isolates with sequences from non-Iranian IBV strains revealed that there is high similarity between these sequences (generally 90 to 95%). In a previous study on the S1 gene from Iranian IBV field isolates, it was shown that 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. The S1 gene of Iranian IBVs shared almost 74% similarity with the H120 vaccine strain used widely in Iran (Akbari Azad *et al.*, 2007). In an analysis of N gene sequences from Korean IBVs, it was shown that these shared almost 90% similarity with non-Korean IBVs (Park *et al.*, 2005), which is similar to the results in the present study where N gene sequences for Iranian IBVs were compared with non-Iranian IBV strains. Furthermore, Shanker *et al*. (2007) 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 (Akbari Azad *et al.*, 2007). The similar clustering of strains based on S1 and N gene sequences has been reported elsewhere (Ignjatovic *et al.*, 2006). Previously, Sapats *et al*. (1996) showed that the S1 and N genes of IBV strains have evolved in parallel. 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 (Ignjatovic *et al.*, 2006; Shieh *et al.*, 2004). Based on N gene and 3' UTR sequence analyses, the present study shows that field isolates of Iranian IBVs share low similarity with the vaccine strains used widely in Iranian poultry farms, namely H120 and H52. This indicates the need to develop improved vaccines that are based on IBVs circulating in Iran. Therefore, it is crucial to monitor IBVs circulating in the field to facilitate a relevant vaccination program against IB. Our data suggests that point mutations, including insertion and deletion, have occurred in the N genes and 3' UTRs of Iranian IBV 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.

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