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REGULAR ARTICLES

Detection and identification of infectious bronchitis virus by RT-PCR in Iran

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Abstract Infectious bronchitis virus (IBV) causes severe diseases in poultry with significant economic consequences to the poultry industry in Iran. The aim of this study was the detection and identification of IBV by reverse transcription(RT)-PCR in Iran. Ten IB virus strains were detected by testing trachea, cecal tonsil, and kidney tissues collected from broiler and layer farms in Iran. In order to detect infectious bronchitis virus, an optimized RT-PCR was used. Primers targeting the conserved region of known IBV serotypes were used in the RT-PCR assay. Primers selectively detecting Massachusetts and 793/B type IB viruses were designed to amplify the S1 gene of the virus and used in the nested PCR test. Our findings indicate the circulation of at least three genotypes of IB viruses (Massachusetts, 793/B, and variant 2) among poultry flocks.

Keywords IBV in Iran · RT-PCR · 793/B · Massachusetts

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Introduction

Infectious bronchitis virus (IBV) is a major poultry pathogen with significant economic consequences worldwide. Molecular biology techniques such as type-specific reverse transcription (RT)-PCR and sequencing of S1 gene (De Wit et al. 2011; Jackwood et al. 2013) are useful for the rapid diagnosis and identification of IBV serotypes. IBV genome is approximately 27 kb in length (Xu et al. 2007) and codes four main structural proteins. Spike glycoprotein (S) is the major protein of IBV. RT-PCR amplifying the S1 gene followed by sequencing allows differentiation of IBV strains (Jackwood et al. 2013). The first isolation of IBV in Iran was reported by Aghakhan et al. (1994). The isolate showed an antigenic relationship to the Mass serotype. Later the presence of IBV variants identified as 4/91 type was reported in Iran (Seyfi-abad Shapouri et al. 2004). Both live (Mass and 793/B) and inactivated vaccines have been used in Iran through different vaccination regimes. However, the virus strains causing IBV outbreaks are often antigenically different from the vaccine strains (Cavanagh et al. 1997). Therefore accurate serotype identification is essential in order to select the most relevant vaccines and vaccination program. The aim of this study was to detect and identify the IBV strains circulating in poultry farms of Iran.

Materials and methods

During the period from October 2011 to March 2012, organs including the trachea, kidney, and cecal tonsils were obtained from 20 farms representing broiler and layer flocks demonstrating respiratory, kidney, and reproductive disorders. All the samples were homogenized and processed as described by

others (Hosseini et al. 2015; Jackwood et al. 2013; Katarzyna et al. 2007).

RNA extraction

Viral RNA was extracted from homogenized tissues using High pure Viral Nucleic Acid Kit (Roche, Germany) according to the manufacturer instruction.

RT-PCR and nested PCR

The RT-PCR reaction was performed using Titan one Tube RT-PCR kit (Roche-Germany) according to the product manual. The oligonucleotide primers used and details of RT-PCR and nested RT-PCR test were performed as described previously by others (Adzhar et al. 1997; Katarzyna et al. 2007). The data are shown in Table 1.

Sequencing of the S1 gene fragment

For sequencing, ten RT-PCR products (464 bp) obtained with the XCE1/XCE2 primers were purified using commercial kit (High Pure PCR Product Purification Kit—Roche, Germany) according to the manufacturer instruction. Then RT-PCR products were sequenced (MWG-Biotech, Germany). The sequences were processed and phylogenetic analysis was performed by using DNA SIS MAX 3 (Hitachi, Pharmacia, Hitachi software Engineering company, Yokohama, Japan). Reference sequences used in this study are as follows:

793/B; UK/7/93 (Z83979), 4/91 attenuated (AF093793), UK/7/91 (Z83975), IR-1062-GA (AY544777), IR-1061-PH (AY544778), IR-3654-VM (AY544776), Massachusetts; H120 (M21970), H52 (352315), Massachusetts (GQ504724), ITA/90254/2005 (FN430414), MA5 (AY561713), ARK99 (L10384), variant 2; Israel/720/99 (AY091552), variant 2 (AF093796), IS/885 (AY279533), Sul/01/09 (GQ281656), Egypt/Beni-Suef/01 (JX174183), Eg/CLEVB-2/IBV/012 (JX173488). Comparison of the nucleotide identity of these strains against the main types of IBV was done (Table 2).

GenBank accession number

The 464-bp fragment of S1 gene of ten Iranian isolates were sequenced and submitted to the GenBank database. GenBank accession numbers are JX909280, JX909281, JX909282, JX909283, JX909284, JX909285, JX909286, JX909287, JX909288, and JX909289, respectively.

Results

To evaluate sequences variation among the Iranian IBV isolates, the sequences of ten field isolates were compared with each other and with the reference sequences (Table 2). This survey showed that the Iranian isolates obtained during this study have close relationship with the following serotypes: Massachusetts, variant 2, and 793/B. Molecular differentiation of 793/B (4/91) and Mass IBV types was achieved using specific primers in nested PCR which amplified a154- and 295-bp fragment, respectively. Nested PCR technique performed on the RT-PCR products of the isolates using specific primers to Massachusetts (Mass) type and 793/B type variant IBV revealed that two isolates belonged to Mass type (IR/10/2011, IR/11/2011) and eight isolates belong to 793/B type variant IBV (IR/7/2011, IR/8/2011, IR/9/2011, IR/12/2011, IR/14/2011, IR/15/2011, IR/17/2011, and IR/21/2011) (Figs. 1, 2, and 3)

. Phylogenetic analysis of the sequences of ten Iranian isolates revealed that five isolates belonged to the 793/B type (IR/12/2011, IR/14/2011, IR/15/2011, IR/17/2011, and IR/21/2011), two isolates (IR/10/2011 and IR/11/2011) were included in the Mass type of IBV, while three strains (IR/7/2011, IR/8/2011, and IR/9/2011) were classified into separate branches. These isolates have unclear relationship with the other isolates (Fig. 4). This could suggest that IR/7/2011, IR/8/2011, and IR/9/21 isolates belong to variant 2 type; however, this should be confirmed by more survey (Fig. 4). The amino acid identities

Table 1 Sequences of oligonucleotide used in RT-PCR and nested PCR

Oligonucleotide	Sequences	Specificity	Band size (bp)
RT-PCR			
XCE1+	5′-CACTGGTAATTTTTCAGATGG-3′	Universal	464
XCE2-	5′-CTCTATAAACACCCTTACA-3′		
Nested PCR			
MCE1+	5′-AATACTACTTTTACGTTACAC-3′	Massachusetts	295
BCE1+	5′-AGTAGTTTTGTGTATAAACCA-3′	793/B	154
Xce3−	5′-CAGATTGCTTACAACCACC-3′		

Table 2 Amino acid identity of the partial S1 gene sequence

	1	2	3	4	5	6	7	8	9	10	11	12	13
1. IR/21/11	0	93.4	94.5	93.7	96.2	72.6	72.4	72.3	71.7	72.1	90.1	58.1	60.1
2. IR/17/11	93.4	0	94.7	94.1	93.6	71.8	72.8	73.4	71	70.2	90.4	61.1	57.3
3. IR/15/11	94.5	94.7	0	93.8	94.1	72.1	73.5	72.2	75.5	73.9	91.1	59.4	61.8
4. IR/14/11	93.7	94.1	93.8	0	94.7	71.9	72.1	72.6	62.1	60.5	90.8	60.1	58.2
5. IR/12/11	96.2	93.6	94.1	94.7	0	72	74.3	69.2	64.5	61.1	89.9	61.5	57.2
6. IR/7/11	72.6	71.8	72.1	71.9	72	0	97.1	95.3	66.4	63.2	71.7	91.5	51.2
7. IR/8/11	72.4	72.8	73.5	72.1	74.3	97.1	0	94.1	67.7	65.1	73.2	92.7	53.3
8. IR/9/11	72.3	73.4	72.2	72.6	69.2	95.3	94.1	0	65.3	64.8	72.4	90.1	56.5
9. IR/10/11	71.7	71	75.5	62.1	64.5	66.4	67.7	65.3	0	90.2	64.1	59.2	88.9
10. IR/11/11	72.1	70.2	73.9	60.5	61.1	63.2	65.1	64.8	90.2	0	62.2	57.1	90.4
11. UK/7/93	90.1	90.4	91.1	90.8	89.9	71.7	73.2	72.4	64.1	62.2	0	83.2	79
12. Variant2	58.1	61.1	59.4	60.1	61.5	91.5	92.7	90.1	59.2	57.1	83.2	0	77.8
13. Ma5	60.1	57.3	61.8	58.2	57.2	51.2	53.3	56.5	88.9	90.4	79	77.8	0

of 5 isolates assigned into the 793/B genotype ranged from 93.4 to 96.2. The amino acid identities among Massachusetts genotype ranged from 90.2 to 90.2 and 94.1 to 97.1 in Vvriant 2 genotype. To analyze and compare the sequences of S1 gene fragment of the Iranian IBV isolates, the sequences of 18 other IBV strains from the GenBank were included in the study (Fig. 4).

Discussion

Infectious bronchitis (IB) is currently a major disease concern for the Iranian poultry industry. It is imperative to know the prevalent strain(s) of infectious bronchitis virus in a region or country, to select the best vaccine strain and vaccination program for controlling the disease. The major problem in the immunization against IBV is the presence of various IBV serotypes in the field against which the available vaccines do not induce proper immunity. This situation justifies the necessity of monitoring field situation. The aim of the present study was to detect and identify the type of prevailing infectious bronchitis virus strains in Iran. Current diagnosis of IB is commonly based on virus isolation in embryonated eggs, followed by serological identification of the isolates. This procedure is time-consuming and requires the use of specific polyclonal or monoclonal antibodies. In the reported study, we used RT-PCR for the detection of infectious bronchitis virus from chicken flocks with a history of IB disease. This method had been shown to be very efficient for detection and identification of IBV. The published oligonucleotides of XCE1+ and XCE2− as primers which can detect all types of IBV were used to detect IBV (Adzhar et al. 1997). The amplicons obtained by RT-PCR were subjected to specific

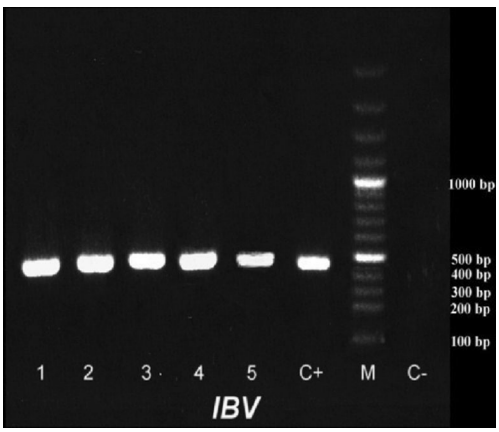

Fig. 1 RT-PCR for general infectious bronchitis virus (IBV) detection. *Positive* band at 464 bp, *C+* positive control, *C−* negative control, *M* 100-bp DNA ladder marker

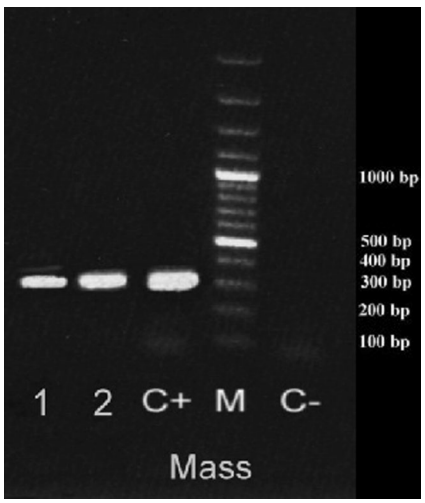

Fig. 2 Nested PCR for mass types of IBV. *Positive* band at 295 and 154 bp, *C+* positive control, *C−* negative control, *M* 100-bp DNA ladder marker

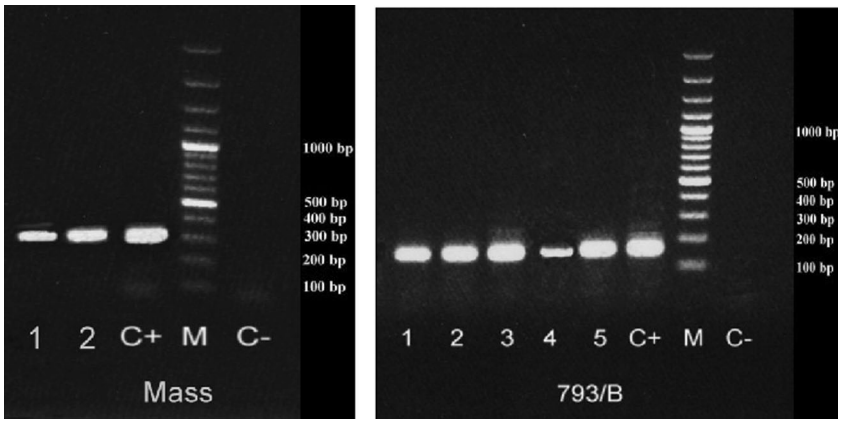

Fig. 3 Nested PCR for 793/B types of IBV. *Positive* band at 295 and 154 bp, *C+* positive control, *C−* negative control, *M* 100-bp DNA ladder marker

nested PCR to differentiate Massachusetts and 793/B type strains. Previous study comparing conventional and nested RT-PCR methods had indicated that nested RT-PCR is more sensitive for detection of IBV (Adzhar et al. 1997; Cavanagh et al. 1997; Domanska et al. 2007; Katarzyna et al 2007).

793/B type IBV was first isolated in France in 1985. Then it spread to the UK in 1990–1991 (Meulemans et al. 2001) and other countries in Europe, North America, and Asia (Roussan et al. 2008). Subsequently, 793/B type IBV was detected as the dominant serotype in Iran (Seyfi Abad Shapori et al. 2004; Shoushtari et al. 2008). This study, in agreement with previous investigations, showed that three major genotypes of IBV, the Mass, 793/B, and variant 2 have been present in Iran (Ghahremani et al. 2011; Hosseini et al. 2015; Nouri et al. 2003; Seify abad Shapouri et al. 2002). Shoushtari et al. (2008) presented two hypotheses about the probable introduction of 793/B into Iran. According to the first theory ,793/B and H9N2 AIV were concurrently introduced in 1999 causing extensive respiratory problem. The second probability is that 793/B-type IBV might had been around already before the

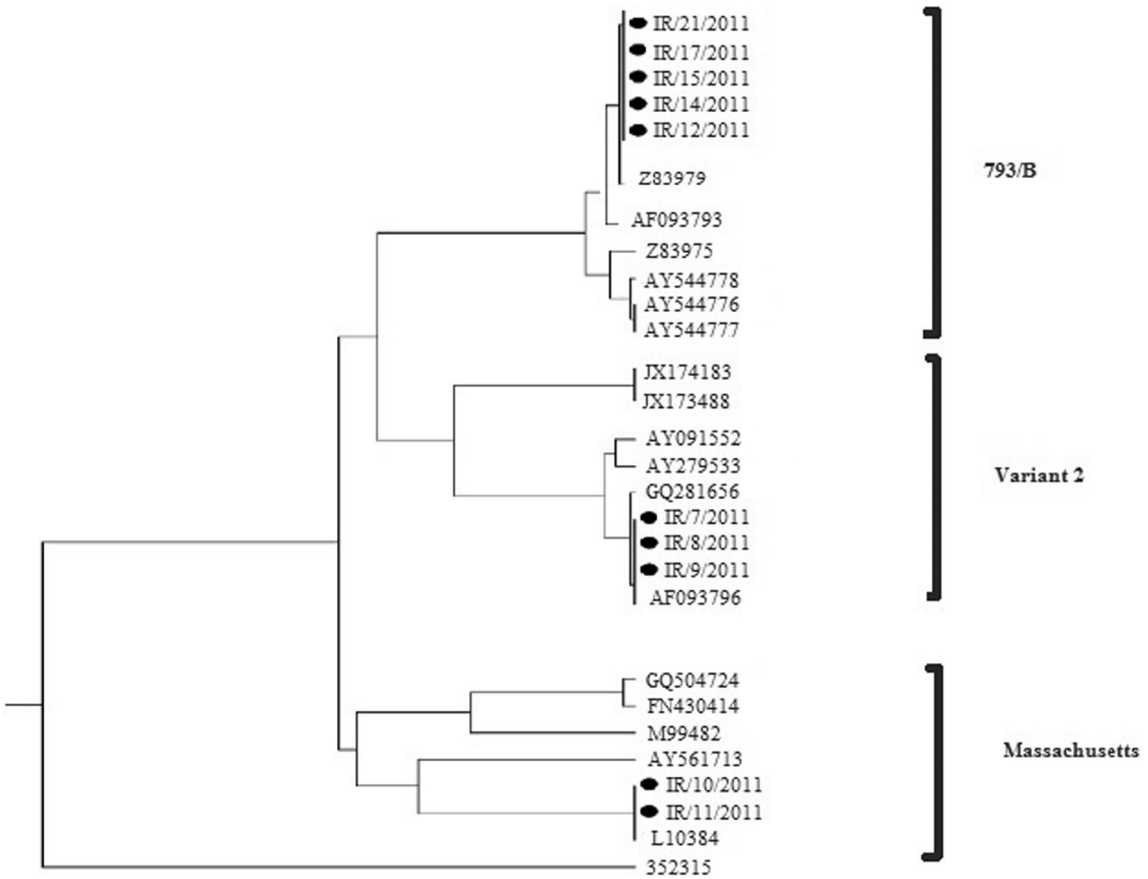

Fig. 4 Phylogenetic tree of the 464 bp fragments of the S1 sequence for ten isolates of this study and other IBV strains of GenBank

introduction of H9N2 AIV in 1999 and the interaction of the two viruses led to a severe respiratory problem in poultry flocks after 1999. In another study, Hosseini et al. (2015) reported that seven distinct genotypes, including Mass, 793/B, IS720, variant 2, QX, IR-I and IR-II had been circulating in Iran between 2010 and 2014. Our results which show that 793/B, Massachusetts, and variant 2 are three major dominant genotypes in Iran are in good agreement with these previous reports.

Isolates of IR/7/2011, IR/8/2011, and IR/9/2011 appeared different from the mentioned IBV types and formed separate branches in the phylogenetic tree (variant 2). In the cases these three isolates were obtained, we observed visceral gout and severe kidney damage on postmortem examination. Mahmoud et al. (2011) reported a new IBV isolate in the Kurdistan region from 2008 to 2010 that caused kidney lesions. Their results indicated the circulation of 793/B with variant 2 in poultry flocks in accordance with our results. From 2004 to 2014, variant 2 was reported from Turkey, Jordan, Israel, and Libya (Ababneh et al. 2012; Awad et al. 2014; Hosseini et al. 2015; Kahya et al. 2013; Meir et al. 2004). In some parts of Iran, only Massachusetts-type vaccines are used, and despite their use, diagnosis of IB in the vaccinated chickens is common. The results of this study may partially explain the failure of Massachusetts-type vaccines and necessitate revising the Iranian vaccination regime against infectious bronchitis. Since Mass and 793/B types of IBV are prevalent in some provinces of Iran, the methods used in this study will be valuable for rapid identification, differential diagnosis, and epidemiological investigation. However, because the primers were selected specifically for these 2 types of IBV, other types might have been present but not detected. In some provinces of Iran, Massachusetts-type and 793/B vaccines are widely use in commercial chicken flocks; however, the low identity between most of Iranian isolates with Mass-type vaccine strain, the presence of variant 2, and other new genotypes like QX may be the cause of vaccination failure. Moreover, secondary infections and immunosuppressive agents like: IBDV and CAV may also lead to vaccination failure and consequently IBV outbreaks among poultry flocks. A combination of two different serotypes (Mass type and 793/B) at day old (Jackwood et al. 2013) suggested to produce better protection than just using a vaccine belonging to one serotype. Molecular characterization of IBV in the different regions could be recommended as necessary to adopt a suitable vaccination program to control the common field serotypes for vaccination.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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