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Molecular Analysis of Infectious Bronchitis Viruses Isolated in Iran from 1998-2008

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Abstract: Ten isolates of Infectious Bronchitis Virus (IBV) were detected from the trachea and lung of IB suspected chickens from 1998-2008 in Iran by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). For further analysis, the entire *S1* gene of the IBV isolates amplified by other RT-PCR. Phylogenetic analysis based on both *S1* gene nucleotide and amino acid deduced sequences showed that isolates of Iran were classified into two genotypes Massachusetts (3 isolates) and 4/91 (7 isolates). Three isolates of Massachusetts serotype had very strong genetic relatedness to Massachusetts-type vaccines (particularly H120) indicating the probability of the detection and isolation of vaccine strains. The present study demonstrates that 4/91 type has been circulating in the country since 1998. Comparison of 4/91 isolates of Iran and almost all foreign isolates of 4/91 showed that Iranian 4/91 isolates (except for IR/491/08) had maximum average nucleotide and amino acid identities with French isolate FR-94047-94, supporting this probability that 4/91 isolates of Iran originated from France. The isolate IR/491/08 recovered from flock vaccinated with 4/91 vaccine strain and had high sequence similarity of the *S1* gene with 4/91 vaccine strain. Therefore, it is likely that this isolate was re-isolation vaccine strain. The results also revealed that the application of Massachusetts vaccines may not contain the replication of the 4/91 type.

Key words: Infectious bronchitis virus, *S1* gene, phylogenetic analysis, replication, foreign, Iran

INTRODUCTION

Infectious bronchitis is an acute highly contagious viral disease of poultry with worldwide distribution (Cavanagh and Naqi, 2003; Cavanagh, 2005). The most characteristic clinical sings are those derived from the respiratory disease. However, infectious bronchitis can also show renal (Liu and Kong, 2004), enteric and reproductive clinical sings (Dhinakar and Jones, 1997). This disease causes major economic losses not only because of poor performance or decreased egg production and quality but also because of secondary infections (Cavanagh and Naqi, 2003).

Infectious Bronchitis Virus (IBV), the aetiological agent of infectious bronchitis belongs to the family Coronaviridae (Cavanagh, 2005). It is a pleomorphic enveloped virus with club-shaped surface projections (spikes) on the surface of the virion and its genome consists of the single stranded positive sense RNA molecule of approximately 27 kilobases (Boursnell *et al*., 1987). The virion contains four major structural proteins: the Nucleocapsid protein (N), the surface Spike glycoprotein (S), the small integral Membrane glycoprotein (M) and the Envelope protein (E). The S protein comprises of two glycolpolypeptides: S1 and S2 (Stern and Sefton, 1982). The S protein is responsible for the attachment of the virion to the host cells and the S1 subunit is involved in the induction of neutralizing, serotype specific and haemagglutination inhibiting antibodies (Cavanagh *et al*., 1988; Koch *et al*., 1990). The S1 subunit is the most variable protein including three Hypervariable Regions (HVRs), located within amino acids 38-67, 91-141 and 274-387 (Cavanagh *et al*., 1988; Moore *et al*., 1998).

A number of IBV serotypes and subtypes have been reported worldwide (Jackwood *et al*., 2005; Mondal and

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Cardona, 2007). Various serotypes, subtypes or variants of IBV are thought to develop by nucleotide insertions, deletions, point mutations and by RNA recombination in the S1 subunit (Cavanagh *et al*., 1988; Wang *et al*., 1993) which are responsible for outbreaks of IB in the vaccinated chicken flocks. Thus, the molecular characterization of IBV has been focused on analysis of the S1 protein gene.

In Iran, IBV was first demonstrated with serological and virus isolation methods in 1994 and reported presence of Massachusetts serotype as major circulating IBV type in the country (Aghakhan *et al*., 1994). Introducing of new serotype was reported by following investigations which showed the existence of a new serotype, 793/B (4/91) (Vasfi Marandi and Bozorgmehri Fard, 2000; Momayez *et al*., 2002). A recent study revealed that 4/91 type has been dominant type of IBV between 1999-2004 in Iran (Shoushtari *et al*., 2008).

The purpose of this study was to obtain IBV field isolates from different outbreaks in commercial poultry flocks of Iran from 1998-2008 and to analyse them phylogenetically to determine the relationships between them and reference strains.

MATERIALS AND METHODS

Samples: Tissue samples (trachea and lung) were obtained from different outbreaks in commercial broilers and layers flocks showing respiratory clinical sings suspected to be related to IB infection between 1998-2008 (Table 1).

For virus isolation, organs (trachea and lung) from at least five birds of each flock were pooled and 10% w/v tissue suspensions were made in 0.1 M phosphate-buffered saline containing 100 U penicillin, 100 μg streptomycin and 80 μg gentamycin per mL. After 2 h incubation at room temperature 200 μL supernatant of the suspensions was inoculated into the allantoic cavity of 9-11 days old embryonated chicken specific pathogen free eggs (Lohman, Germany). Five eggs were used for each sample. After 48-72 h incubation at 37°C, the allantoic fluids were harvested and tested for presence of IBV using the RT-PCR technique.

RNA extraction, RT-PCR and nested PCR: Viral RNA was extracted from 200 μL allantoic fluids using the High Pure Viral RNA kit (Roche, Germany) and dissolved in 50 μL water. The RT-PCR reaction was performed using the Titan one-tube RT-PCR system (Roche). Primers S1Uni2+(5'-CCCAATTTGAAAACTGAACA-3') (Binns *et al*., 1985) and XCE2-(5'-CCTCTATAAAC-ACCCTTGCA-3') (Adzhar *et al*., 1997), both from *S1* gene, with 1 μL RNA were used for amplification of a segment approximately 1200 base pairs (bp), common to all IBVs. For the RT reaction, the mixture was incubated at 45°C for 45 min and was then heated at 94°C for 2 min. The PCR reaction was performed using the following conditions; denaturation (94°C, 30 sec), annealing (48°C, 2 min) and extension (68°C, 2 min), 35 cycles followed by a final extension step (68°C, 10 min). The amplified fragment was used in three specific nested PCRs with oligonucleotide XCE3-(5'-CAGATTGCTTAC-AACCACC-3') which is common for all three strains and oligonucleotides DCE1+(5'-TTCCAATTATATC-AAACCAGC-3'), MCE1+(5'-AATACTACTTTTACG-TTACAC-3')andB1+(5'-AAGTGCCTTTAGGCCTGG-3') that are specific for types D274, Massachusetts and 793/B, respectively and generating fragments of 217, 295 and 972 bp length, respectively (Adzhar *et al*., 1997). The nested PCRs were performed with 1 μL of a 1/10 dilution of the first PCR reaction in a final volume of 100 μL using following conditions; denaturation (94°C, 1 min), annealing (48°C, 2 min) and extension (72°C, 90 sec), 35 cycles followed by a final extension step (72°C, 10 min).

Table 1: Epidemiologic information of Iranian field IBV isolates included in the study

Isolates	Years	Breed	Age	Genotype	Accession No.
IR/573/98	1998	Broiler	39 days	4/91	HQ842706
IR/512/99	1999	Broiler	42 days	4/91	HQ842707
IR/525/99	1999	Broiler	40 days	4/91	HQ842708
IR/17/00	2000	Layer	38 days	Mass.	HQ842709
IR/70/02	2002	Broiler	N/A	Mass.	HQ842710
IR/803/03	2003	Broiler	26 days	4/91	HQ842711
IR/14/07	2007	Broiler	35 days	4/91	HQ842712
IR/43/08	2008	Broiler	45 days	Mass.	HQ842713
IR/19/08	2008	Broiler	35 days	4/91	HQ842714
IR/491/08	2008	Broiler	N/A	4/91	HQ842715

N/A = Data not available Isolates have been named as proposed by Cavanagh (2001). Country/isolate number/year of isolation (I.R. Iran)

The final amplification product was analysed by electrophoresis in a 1% agarose gel, stained with ethidium bromide and visualized by UV transillumination.

***S1* gene sequencing:** Ten IBV isolates were sequenced after identified as IBV by RT-PCR and typed by nested PCR. For sequencing, the entire *S1* gene amplified by other RT-PCR. Viral RNA was extracted and purified as described before.

The RT-PCR reaction was carried out using the Titan one-tube RT-PCR system (Roche). In this RT-PCR the S1 OLIGO3' (5'-CATAACTAACAT-AAGGGCAA-3') and S1 OLIGO5' (5'-TGAAAACTG-AACAAAAGACA-3') primers were used (Kwon *et al*., 1993). For the RT reaction, the mixture was incubated at 45°C for 45 min and was then heated at 94°C for 2 min. PCR was performed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 2 min and polymerization at 68°C for 150 sec. The final polymerization step was conducted at 68°C for 10 min. The PCR products were analysed on a 1% agarose gel. The predicted size of the PCR product was about

1720 bp. PCR products were purified using the high pure PCR product purification kit (Roche) according to the manufacturer's recommendations. Purified PCR products were sequenced bidirectional by automated sequencing (MWG Co., Germany).

Sequence analysis: DNA sequences were compiled and edited using the DNAStar package Version 5.2. Nucleotide and amino acid deduced sequences were aligned using Clustal W software. Phylogenetic analysis was performed with the MegAlign program version 2.8. Complete *S1* gene nucleotide sequences of the ten field viruses were deposited in GenBank (Table 1). Reference sequences used in this study were; Ark-DPI (AF006624); Ark-99 (L10384); B1648 (X87238); Beaudette (X02342); Connecticut (EU283057); D274 (X15832); D1466 (M21971); Florida (AF027512); FR-85131-85 (AJ618985); FR-88061-88 (AJ618986); FR-94047-94 (AJ618987); Gray (L14069); Holte (L18988); H120 (M21970); H52 (AF352315); IR-1061-PH (AY544778); IR-1062-GA (AY544777); IR-3654-VM (AY544776); Italy 02 (AJ457137); JMK (L14070); M41 (X04722); Ma5 (AY561713); Spain/92/35 (DQ386091); Spain/95/194 (DQ064802); Spain/99/327 (DQ386097); UK/7/91 (Z83975); UK/5/91 (Z83978); UK/7/93 (Z83979); 4/91 attenuated (AF093793) and 4/91 pathogenic (AF093794).

RESULTS AND DISCUSSION

Detection and molecular differentiation: Ten IBV isolates that were detected as IBV by RT-PCR using S1Uni 2+ and XCE2-primers classified into 2 groups by nested PCR. Seven isolates (IR/573/98, IR/512/99, IR/525/99, IR/803/03, IR/14/07, IR/19/08 and IR/491/08) produced 972 bp fragments and identified as 793/B (4/91) type and three isolates (IR/17/00, IR/70/02 and IR/43/08) produced 295 bp fragments and identified as Massachusetts type in nested PCRs. No band was identified as D274 type.

Sequencing and sequence analysis: The whole *S1* gene of ten IBV isolates was sequenced to further characterize the isolates. The nucleotide and deduced amino acid sequences of these IBV isolates were determined and compared with the published sequences. The analysis showed that the isolates of Iran had a close genetic relationship with strains of the 2 following genotypes; 793/B (4/91) and Massachusetts. Phylogenetic analysis based on both *S1* gene nucleotide and amino acid deduced sequences of the ten Iranian isolates and reference IBV strains showed that Iranian isolates were classified into two separate genetic groups or genotypes (Fig. 1). Genotype I (4/91 genotype) comprised seven field isolates that were grouped with 4/91 reference strains

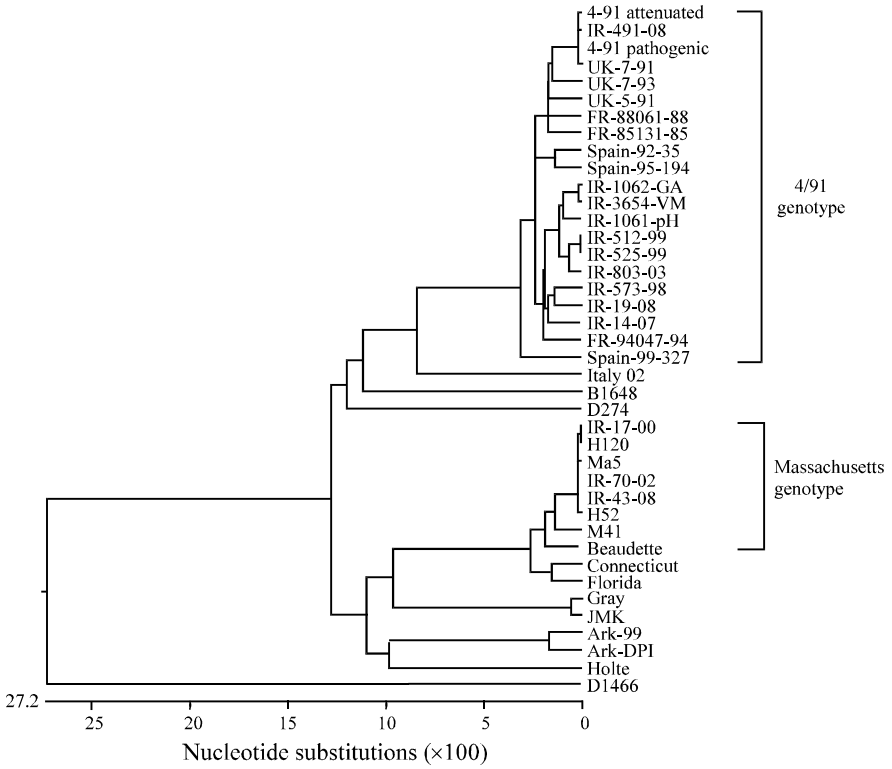

Fig. 1: Phylogenetic relationships of the Iranian IBV isolates and selected reference strains based on the entire *S1* gene sequences using the MegAlign program with the Clustal W method (DNAStar. USA)

Table 2: Nucleotide and amino acid identities of IBV *S1* gene sequences: comparison of the ten Iranian field isolates and 20 reference IBV strains of different serotypes obtained from GenBank

Amino acid identity (%)	Nucleotide identity (%)																												
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
4/91 pathogenic	-	99.8	99.8	97.2	97.2	96.5	95.5	95.7	93.0	84.5	94.8	94.9	94.8	94.7	94.9	95.0	94.6	94.1	94.4	99.9	79.0	78.9	78.9	79.0	78.9	78.9	79.2	78.1	78.6	78.9
4/91 attenuated	99.4	-	99.6	97.0	97.0	96.4	95.3	95.5	92.8	84.4	94.6	94.7	94.7	94.7	94.7	94.8	94.4	94.1	94.2	99.8	78.9	78.8	78.8	78.9	78.8	78.9	79.1	77.9	78.5	78.9
UK/7/91	99.4	98.9	-	97.3	97.2	96.5	95.3	95.5	92.8	84.3	94.6	94.7	94.6	94.5	94.6	94.7	94.3	93.9	94.2	99.6	78.8	78.8	78.8	78.8	78.8	78.9	79.2	77.9	78.4	78.7
UK/5/91	94.1	93.5	94.2	-	96.9	96.8	95.7	95.2	93.5	84.5	94.9	95.2	95.1	94.9	95.3	95.3	94.6	94.1	94.4	97.0	78.9	79.0	79.0	78.9	79.0	79.0	79.5	78.1	78.8	79.2
UK/7/93	94.8	94.2	95.0	93.9	-	96.0	94.7	94.9	92.2	84.5	94.3	94.5	94.5	94.1	94.3	94.3	93.9	93.4	93.3	97.0	79.2	79.2	79.2	79.2	79.2	79.2	79.6	78.1	78.5	79.3
FR-85131-85	93.3	92.8	93.5	93.3	92.0	-	96.3	96.0	93.9	84.1	95.4	95.7	95.6	95.8	95.6	95.7	95.4	95.1	94.9	96.4	78.5	78.6	78.6	78.5	78.6	78.4	78.6	77.6	78.1	79.2
FR-94047-94	92.4	91.8	91.8	92.2	90.5	93.3	-	96.5	95.9	84.3	96.9	97.2	97.1	97.0	97.3	97.3	96.6	96.0	95.9	95.4	78.3	78.5	78.5	78.3	78.5	78.3	78.8	78.0	78.2	79.2
Spain/92/35	93.9	93.3	93.3	92.2	92.4	93.9	94.2	-	93.7	84.5	95.3	95.7	95.8	95.7	95.5	95.5	95.4	95.2	94.9	95.5	78.5	78.6	78.6	78.5	78.6	78.5	78.9	78.2	78.4	79.3
Spain/99/327	91.6	91.0	91.0	92.0	89.6	92.4	95.0	92.4	-	86.8	94.5	94.7	94.7	94.4	94.7	94.8	94.2	93.9	93.7	92.8	78.0	78.1	78.1	78.0	78.1	77.9	78.3	77.8	78.5	78.6
Italy 02	83.1	82.9	82.7	81.8	82.2	81.8	81.4	82.3	83.8	-	84.7	85.1	85.0	84.2	84.9	84.9	84.2	84.0	83.5	84.5	78.5	78.6	78.6	78.5	78.6	78.6	78.7	79.0	79.6	80.5
IR-1061-PH	91.2	90.6	90.6	91.2	90.6	92.1	94.0	93.1	92.9	81.6	-	98.1	98.1	97.8	98.2	98.3	97.8	96.7	96.8	94.6	78.4	78.3	78.3	78.4	78.3	78.3	78.8	78.1	78.3	78.5
IR-1062-GA	91.6	91.1	91.1	92.0	91.4	92.6	94.4	93.1	93.3	81.9	97.2	-	99.8	97.5	98.1	98.1	97.5	96.5	96.5	94.7	77.9	78.0	78.0	77.9	78.0	77.9	78.3	78.1	77.9	79.0
IR-3654-VM	91.2	90.7	90.7	91.6	91.4	92.2	94.0	93.1	92.9	81.8	96.8	99.6	-	97.5	98.1	98.0	97.4	96.5	96.4	94.7	78.0	78.1	78.1	78.0	78.1	78.0	78.4	78.2	78.0	79.0
IR/573/98	91.5	91.3	90.9	91.7	90.0	92.4	94.1	93.3	92.9	81.6	95.3	95.3	95.2	-	98.2	98.3	97.5	97.3	97.3	94.7	78.2	78.3	78.3	78.2	78.3	78.2	78.6	77.9	78.0	79.1
IR/512/99	92.9	92.3	92.3	93.1	91.2	93.8	95.5	94.2	94.2	82.4	97.0	97.4	97.0	97.0	-	99.9	98.7	96.8	97.1	94.8	78.1	78.2	78.2	78.1	78.2	78.0	78.7	78.2	78.3	79.2
IR/525/99	93.1	92.5	92.5	93.3	91.4	94.0	95.3	94.4	94.4	82.4	97.2	97.2	96.8	97.2	99.8	-	98.8	96.8	97.2	94.8	78.1	78.3	78.3	78.1	78.3	78.1	78.8	78.1	78.3	79.3
IR/803/03	92.4	91.8	91.8	92.2	90.7	93.1	94.4	93.7	92.9	81.8	96.1	96.1	95.7	95.9	97.9	97.9	-	96.6	96.4	94.4	78.2	78.3	78.3	78.2	78.3	78.1	78.8	77.9	78.0	79.1
IR/14/07	91.1	90.7	90.5	90.9	89.1	91.8	93.1	93.1	92.7	81.4	94.2	94.4	94.0	95.2	95.5	95.5	95.0	-	96.0	94.1	78.1	78.2	78.2	78.1	78.2	78.1	78.4	77.9	77.6	78.9
IR/19/08	92.0	91.5	91.5	91.8	89.8	92.8	93.3	92.9	92.4	81.3	94.8	94.6	94.2	95.4	97.0	97.2	95.2	94.4	-	94.2	78.5	78.5	78.4	78.5	78.5	78.4	78.8	78.0	78.1	79.1
IR/491/08	99.6	99.4	99.1	93.7	94.4	92.9	92.0	93.5	90.7	82.9	90.8	91.2	90.9	91.5	92.3	92.5	92.0	90.9	91.7	-	79.0	78.9	78.9	79.0	78.9	78.9	79.2	78.0	78.5	78.9
IR/17/00	75.0	74.7	74.7	74.7	75.2	74.3	73.9	76.2	74.8	75.2	75.1	74.7	74.7	74.7	75.5	75.7	75.4	74.9	75.2	74.7	-	99.9	99.8	100.0	99.6	99.8	97.6	79.8	83.9	81.2
IR/70/02	75.0	74.7	74.7	74.7	75.0	74.3	73.9	76.2	74.8	75.2	75.1	74.7	74.7	74.7	75.5	75.7	75.4	74.9	75.2	74.7	99.8	-	99.9	99.9	99.8	99.8	97.6	79.9	84.0	81.3
IR/43/08	75.0	74.7	74.7	74.5	75.0	74.3	73.9	76.2	74.8	75.2	75.1	74.7	74.7	74.5	75.3	75.5	75.4	74.9	75.2	74.7	99.4	99.6	-	99.8	99.6	99.8	97.6	79.9	84.0	81.3
H120	75.0	74.7	74.7	74.7	75.2	74.3	73.9	76.2	74.8	75.2	75.1	74.7	74.7	74.7	75.5	75.7	75.4	74.9	75.2	74.7	100.0	99.8	99.4	-	99.6	99.8	97.6	79.8	83.9	81.2
H52	74.9	74.5	74.5	74.5	74.9	74.1	73.7	76.0	74.6	75.0	75.1	74.5	74.5	74.5	75.3	75.5	75.2	74.7	75.2	74.5	99.1	99.3	98.9	99.1	-	99.5	97.4	79.8	83.9	81.3
Ma5	75.0	74.7	74.7	74.5	75.2	74.3	73.9	76.2	74.8	75.2	75.1	74.7	74.7	74.5	75.3	75.5	75.4	74.9	75.4	74.7	99.6	99.4	99.4	99.6	98.7	-	97.7	79.8	83.9	81.2
M41	74.5	74.1	74.1	74.5	75.2	73.7	73.7	75.8	74.7	74.7	74.3	74.7	74.7	74.1	75.1	75.1	75.0	74.3	74.7	74.1	96.3	96.1	95.9	96.3	95.3	96.1	-	80.1	84.0	80.9
Ark-99	76.6	76.3	76.3	76.1	76.4	76.3	75.9	76.4	77.2	77.8	75.5	76.2	76.4	75.0	76.1	76.1	76.1	74.6	75.7	76.3	77.3	77.3	77.3	77.3	76.9	77.5	76.9	-	82.4	79.7
Gray	77.0	76.8	76.6	76.1	76.8	76.4	75.7	77.1	77.1	78.5	76.0	75.8	76.0	75.7	76.4	76.6	76.1	75.1	75.9	76.8	79.9	79.9	79.9	79.9	79.5	79.9	79.3	80.3	-	81.0
D274	78.8	78.6	78.3	78.1	78.6	79.9	78.3	80.3	78.2	80.1	77.0	78.0	77.8	78.1	78.7	78.9	78.8	78.3	78.6	78.6	78.4	78.4	78.4	78.4	78.2	78.4	77.7	79.7	79.2	-

and genotype II (Mass. genotype) included three field viruses that were grouped with isolates of the Massachusetts serotype.

4/91 genotype: The nucleotide and amino acid identities of 7 isolates assigned into the Iranian genotype I group ranged from 94.1% (between IR/14/07 and IR/491/08) to 99.9% (between IR/512/99 and IR/525/99) and from 90.9% (between IR/14/07 and IR/491/08) to 99.8% (between IR/512/99 and IR/525/99), respectively (Table 2). Comparison of the 7 Iranian field isolates *S1* gene sequences and other 4/91 *S1* gene sequences previously published in the GenBank database including British, French, Spanish and Iranian isolates showed that 6 Iranian isolates including IR/573/98, IR/512/99, IR/525/99, IR/803/03, IR/14/07 and IR/19/08 had maximum average nucleotide and amino acid identities with previous 4/91 isolates of Iran (IR/1061/PH, IR/1062/GA and IR/3654/VM) and French isolate FR-94047-94 (data not shown). Isolate IR/491/08 showed maximum nucleotide and amino acid identities with UK/4/91 (4/91 pathogenic) as the origin of vaccine strain and 4/91 vaccine strain (4/91 attenuated). Its nucleotide and amino acid similarities to the 4/91 vaccine strain were 99.8 and 99.4%, respectively. This isolate had minimum nucleotide and amino acid identities with other isolates of genotype I (94.1-94.8 and 90.9-92.5%, respectively). The similarities of the other isolates of genotype I to the vaccine strain were somewhat low and identities of the nucleotide and amino acid sequences of the *S1* gene were 94.1-94.8 and 90.7-92.5%, respectively. Isolates of genotype I showed 74.1-75.9% amino acid similarities with Massachusetts-type vaccines (H120, H52, Ma5 and M41) (Table 2). Comparison of 4/91 Iranian field isolates sequences revealed four amino acid deletions at positions 55, 56, 57 and 58 (located in HVR1) in two isolates from the same year (IR/512/99 and IR/525/99). Previous 4/91 isolates of Iran and some of the Spanish 4/91 isolates showed amino acid deletions at positions 54, 55, 56, 57 and 58 (located in HVR1). No insertions were observed within *S1* gene sequences of the Iranian 4/91 isolates.

Mass. genotype: The S1 sequences of three isolates of genotype II were very similar and the nucleotide and amino acid similarities were 99.8-99.9 and 99.4-99.8%, respectively (Table 2). The S1 sequence of isolate IR/17/00 was completely identical to that of the H120 vaccine strain (commonly used in vaccination programs of flocks in Iran). Isolates IR/70/02 and IR/43/08 showed 99.8 and 99.4% amino acid identities with H120 vaccine strain. Comparison of the *S1* gene sequences of genotype II Isolates with Massachusetts-type vaccines (H120, H52, Ma5 and M41) revealed that 3 isolates of Mass. genotype had maximum average nucleotide and amino acid identities with H120 strain. These 3 isolates had minimum nucleotide (97.6%) and amino acid (95.9-96.3%) identities with M41 strain (Table 2). No deletions or insertions were observed within S1 gene sequences of isolates of Mass. genotype. The spike glycoprotein of IBV is translated as a precursor protein (S_0) and then cleaved into two sub-units S1 and

S2. The S_1-S_2 cleavage site consists of five basic amino acid residues (Cavanagh *et al*., 1986; Jackwood *et al*., 2001). The six isolates of 4/91 genotype including IR/512/99, IR/525/99, IR/803/03, IR/14/07, IR/19/08 and IR/491/08 had the sequence Arg-Arg-Ser-Arg-Arg at cleavage recognition site like most of the 4/91 isolates, Ark-DPI, Gray and D274 strains whereas three isolates of Mass. genotype had the sequence Arg-Arg-Phe-Arg-Arg like Beaudette, H120, H52, Ma5 and M41 strains. Isolate IR/573/98 had sequence at this site distinct from that in isolates of genotype I. The cleavage site sequence in IR/573/98 isolate was His-Arg-Ser-Arg-Arg like Ark-99 strain.

Infectious bronchitis is currently one of the major diseases in poultry flocks all over the world. The major problem in immunization against IBV is the presence of various serotypes which do not induce a proper immunity against each other and the frequent emergence of new variants. Natural outbreaks of IBV often are the result of infections with strains that differ serologically from the vaccine strains (Xu *et al*., 2007). Therefore, it is imperative to know the prevalent status of IBVs and characterize the IBVs circulating in Iranian flocks in order to select the appropriate vaccines to prevent the disease.

In this study, the researchers detected and characterized ten IBV isolates in poultry flocks of Iran from 1998-2008. RT-PCR technology was used for detection followed by sequencing. Traditionally, IB is diagnosed using the virus isolation in embryonating eggs followed by immunological identification of the isolates. This procedure is time consuming and requires the use of specific polyclonal or monoclonal antibodies. Moreover, some isolates could be mixtures of different types of IBV that can confuse the interpretation of serotyping results (Meulemans *et al*., 2001). RT-PCR using IBV RNAs extracted from allantoic fluid has been shown to be very efficient for the detection of IBV and for the identification of IBV types (Adzhar *et al*., 1996; Jackwood *et al*., 1997; Handberg *et al*., 1999). Using specific nested primers, IBV isolates of Iran separated into 2 types; Mass. and 793/B (4/91). S1 sequencing of this isolates confirmed the results of typing by nested PCRs. Nucleotide sequencing of the *S1* gene region using appropriate primers is recognized as the most useful technique for the differentiation of IBV strains (Bochkov *et al*., 2006).

Sequence analysis of Iranian IBV isolates showed that two serotypes Mass. and 4/91 existed in Iranian poultry flocks. This study is the most comprehensive sequence analysis to date of IBV isolates in Iran. The Mass. type, the first type of IBV detected in the 1930s in the USA has the widest geographic distribution in the world and has been currently identified in North and South America, Europe and Asia due to long time use of attenuated vaccine strains of this serotype in many countries with the only exception being the Australian continent where IBV has evolved independently from the rest of the world since the early 1930s (Bochkov *et al*., 2006). In Iran, Massachusetts-type vaccines (particularly H120) are widely used in commercial flocks against IBV. Three isolates of Mass. serotype showed very strong genetic relatedness to the vaccine strains particularly H120. These isolates recovered from flocks vaccinated with live Massachusetts-type vaccines. Unfortunately, the researchers have no information about the vaccination program of poultry flock from where isolate IR/43/08 was isolated. There are many reports represent that live IBV vaccines have been found to persist in chicks for many weeks after administration (Worthington *et al*., 2008). Therefore, it is likely that researchers detected and isolated vaccine strains.

The 793/B (4/91) type was first isolated in France in 1985. It spread to the UK in 1990/1991 (Capua *et al*., 1999; Meulemans *et al*., 2001) and other countries in Europe, Asia and North America and it was one of the major IBV types in some countries (Bochkov *et al*., 2006). The present study shows 4/91 as one of the major types also in Iran and demonstrates it has been circulating in the country since 1998. All of the Iranian 4/91 isolates recovered from flocks vaccinated with Massachusetts-type vaccines which produced insufficient protection against these isolates. The poor genetic relatedness between these isolates and Massachusetts vaccine strains (74.1-75.9% amino acid identities) could explain occurrence of IBV in these flocks. Cross-protection tends to diminish as the degree of amino acid identity between the S1 proteins of two IBV strains decreases (Gelb *et al*., 2005; Cavanagh, 2007). The observations also support previous reports that the application of Massachusetts vaccines may not contain the replication of the 4/91 type (Parsons *et al*., 1992; Cavanagh *et al*., 1999). Comparison of 10 Iranian field isolates of 4/91 genotype, seven from the present study and 3 previously described (Akbari Azad, 2003) and almost all foreign isolates of 4/91 genotype revealed that field isolates of Iran except for IR/491/08 had maximum average nucleotide and amino acid identities with French isolate FR-94047-94. This finding supports this probability that 4/91 isolates of Iran originated from 4/91 isolates of France. Among 7 Iranian isolates of 4/91 genotype only isolate IR/491/08 recovered from flock vaccinated with 4/91 vaccine strain. Comparison of isolates of the genotype I showed that isolate IR/491/08 had minimum nucleotide and amino acid similarities with 6 other isolates. This isolate showed high sequence similarity of

S1 gene with 4/91 vaccine strain whereas *S1* gene similarities of 6 other isolates with 4/91 vaccine strain were somewhat low. These findings indicate the probability of the detection and isolation of vaccine strain. However, it is necessary more studies to support this conclusion.

CONCLUSION

The present study shows existence of 2 serotypes Mass. and 4/91 in commercial poultry flocks of Iran. In Iran, Massachusetts-type vaccines are widely used in commercial flocks against IBV whereas 4/91 type vaccines used in some flocks. The results showed that the application of Massachusetts vaccines may not contain the replication of the 4/91 type. Therefore, researchers propose using of combinations of two vaccine types (Massachusetts and 4/91) in vaccination programs of poultry flocks in Iran. The combinations of live vaccines containing two different virus serotypes (Gelb *et al*., 1991) or administering Massachusetts type vaccine at 1 day old with subsequent revaccination with heterologous 4/91 strain at 2 weeks of age (Cook *et al*., 1999) produced broader protection than single strains.

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