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BRIEF REPORT

Co-circulation of three clusters of 793/B-like avian infectious bronchitis virus genotypes in Iranian chicken flocks

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Abstract Avian infectious bronchitis (IB) is an acute and highly contagious viral disease causing severe economic losses in the poultry industry. The 793/B IB virus is an important infectious bronchitis virus (IBV) genotype currently circulating in several countries, including Iran. One hundred confirmed IBV samples (between 2014 and 2015; from 15 provinces in Iran) were selected for genotyping based on S1 sequencing. After phylogenetic analysis, it was found that 30% of the IBV isolates belonged to the 793/B genotype. Results showed that the Iranian 793/B-like IBV isolates could be divided in to three clusters: 4/91-like (50%), 1/96-like (40%), and IB88-like (10%). The sequence similarity between Iranian 793/B-like IBV isolates is 87.69%–100%. The highest identity is between the 4/91 and IB88 clusters (96.38%), and the lowest similarity is between the 1/96 and IB88 clusters (87.62%). This study provides a comprehensive analysis of 793/B-type IBV in Iran and characterization of IBV molecular epidemiology in the country.

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Introduction

Avian infectious bronchitis virus (IBV) is a gamma-coronavirus which causes a highly contagious respiratory disease of economic importance in chickens that is prevalent throughout the world [13]. A variety of different IBV strains have been reported in chickens worldwide, with pathology ranging from mild respiratory symptoms to severe kidney and oviduct diseases [31]. The coronaviruses are enveloped and contain a single-stranded, positive-sense RNA genome of 28–32 kb, which is un-segmented, 5‘-capped and 3‘-polyadenylated [5]. The genome encodes multiple proteins including structural proteins (E), nucleoprotein (N), spike (S), and membrane (M) [8]. The first known strain of the 793/B serotype, also known as 4/91 and CR88 was isolated in France in 1985 [11]. This serotype may also have entered the United Kingdom in the winter of 1990/91, where it was sometimes associated with deep pectoral muscle myopathy in addition to the more usual manifestations of IB [11]. Subsequently, it was discovered that this serotype has been present in most European countries. Following this, 793/B-type viruses were detected in several Asian countries, including Japan [21], India [30], Iran [32], and Iraq [26]. The first report of IBV isolation from Iranian chicken flocks was in 1994 [29]. IBV strains isolated in Iran were classified into seven distinct phylogenetic groups: Massachusetts, 793/B, IS/1494, IS/720, QX, IR-1, and IR-2 [19, 22]. Currently, the major control measures for IB in Iran are vaccination with live attenuated IB vaccines (Massachusetts serotypes), such as H120, Ma5 and 793/B strains, including 4/91 (Intervet; from 2006),

IB88 (Merial; from 2007), and iBird (Ceva; from 2016). As several types of 793-like IBV vaccines are used in Iran and the 793/B is a prevalent genotype in Iran the aim of this study was to generate more detailed information on the distribution of 793/B-like IBVs in Iran.

One hundred confirmed IBV samples (Ghalyanchilab, Department of Microbiology and Immunology, Faculty of Veterinary Medicine, University of Tehran), which were collected from chickens (broiler) suspected of disease between 2014 and 2015 (from 15 provinces) were selected for the study. Also, several 793/B-type IBV vaccines (iBird (1/96), IB88, and 4/91) were selected as positive controls. The presence of IBV was confirmed using a 5' UTR real-time reverse-transcription polymerase chain reaction [RT-PCR] [6]. Viral RNA was extracted from samples using the Cinapure RNA extraction kit (Sinaclon Co., Iran), following the manufacturer's instructions. For the reverse transcription (RT) reaction, random hexamers were used as described previously [24]. Nested PCR was then performed using spike gene primers that were designed to amplify a 390-bp fragment of the gene (a partial segment), as described previously [24, 33]. Sequencing was performed with the primers (both directions) used in the second step of nested PCR (Bioneer Co., Korea). Sequences were initially analyzed in Chromas PRO to confirm good quality read data had been obtained. We then performed NCBI Blast on the results and only 793/B-positive samples were subsequently considered for continued bioinformatics analysis. To determine phylogenetic relationships between the isolates, the deduced amino acid sequence for the hyper-variable region of the S gene sequence obtained in this study was compared with sequences from our lab and with corresponding regions from representative sequences available in GenBank. Alignment and comparison of amino acid sequences were performed using Clustal W in MEGA 7.0 [14]. Phylogenetic trees were constructed using MEGA7.0 with the neighbor-joining algorithm (bootstrap values of 1000) and the Kimura2 parameter model [20]. (Nucleotide sequences used in this study were submitted to the NCBI database with the accession numbers: KX702136-KX702181.) We detected 793/B-like IBV isolates in 30% of our IBV samples. The results showed that Iranian IBV 793/B-like genotypes could be divided in to three clusters: 4/91-like, 1/96-like, and IB88-like (Figure 1). Fifty percent of the IBV 793/B-like viruses belonged to the 4/91-like cluster, 40% were placed in the 1/96-like cluster and 10% belonged to the IB88-like cluster. The average sequence similarity of the Iranian isolates was 87.62 –100%. The highest homology was between 4/91-like and IB88-like clusters (96.38%), and the lowest similarity was found between the 1/96-like and IB88-like clusters (87.62%). The sequence similarities within each cluster (4/91-like, 1/96-like, and IB88-like) were 98.87–100%, 95.3–100% and 96.48–100%, respectively (Table 1). Phylogenetic analysis revealed that the first 793/B IBV genotype submitted to GenBank (VM/113654) in 2000 is located within the 1/96-like cluster (Figure 1). Considering the presence of our isolates within this cluster and the fact that the 1/96 vaccine has only recently been imported into the country (after this study), it can be concluded that the Iranian 1/96–like isolates are likely field viruses. Viruses that grouped in the 1/96-like cluster were close to isolated made in France, Brazil, Poland and Spain. Viruses grouped in the 4/91 cluster have a close relationship with viruses isolated in the Ukraine, India, and Poland.

The 4/91 IBV type was first identified in the UK in 1990/1991 but was retrospectively found to have been present in France since 1985 [9]. Antibodies reactive with the 4/91 type have been detected in chickens in Europe, Asia and Africa [12]. The first isolation of IBV in Iranian chicken flocks was reported by Aghakhan *et al* (1994), which was confirmed to be the Massachusetts serotype [1]. Vasfi Marandi and Bozorgmehri Fard (2001) subsequently performed serotyping on other Iranian IBV isolates between 1997 and 2000. They found a strain (isolated in 1998) that was antigenically different from the Massachusetts strain and predicted the presence of a new IBV genotype (793/B) [32]. Akbari Azad *et al* (2004) worked on Iranian IBV isolates through RFLP analysis. Based on the resulting patterns, eight of the 12 strains showed a 793/B-like pattern and the rest (4/12) showed a Massachusetts pattern [2]. Vasfi Marandi *et al* (2007) also performed molecular analysis of three Iranian 793/B IBVs. These three Iranian IBVs belonged to the 793/B genotype with nucleotide differences of 5.64–6.07% to UK/793/B (a prototype 793/B strain) [3]. Vasfi Marandi *et al* (1998) also isolated IBVs that grouped within a 1/96 like cluster [32]. This detection of 1/96-like isolates from 1988 onwards in Iran and as well as the fact that the 1/96 vaccine was not used in Iranian farms until recently (2016), allows us to conclude that these 1/96-like isolates are wild type field viruses, and do not originate from vaccines. Furthermore, Cavangh *et al* studied Iranian 793/B IBVs that were isolated in 2000 (IR-13-2000 & IR-10-2000). These two Iranian isolates shared 97.5% identity with each other, and had 94.7% and 96.4% nucleotide identity, respectively, with UK/7/91. The Iranian isolates had approximately 95% identity with the 4/91 and IB88 live vaccine strains. Thus, these Iranian isolates also differ from the two vaccine strains. At eight of the nucleotide positions where either, Iran 10/2000 or, 13/000 differed from UK7/91, other UK or French isolates differed from UK7/91, highlighting the similarity between the Iranian isolates and British and French 793/B [4/91] type isolates. However, the two Iranian isolates were not identical; they differed by 2.5% [10]. In addition, Seyfi *et al* (2002) confirmed the presence of the

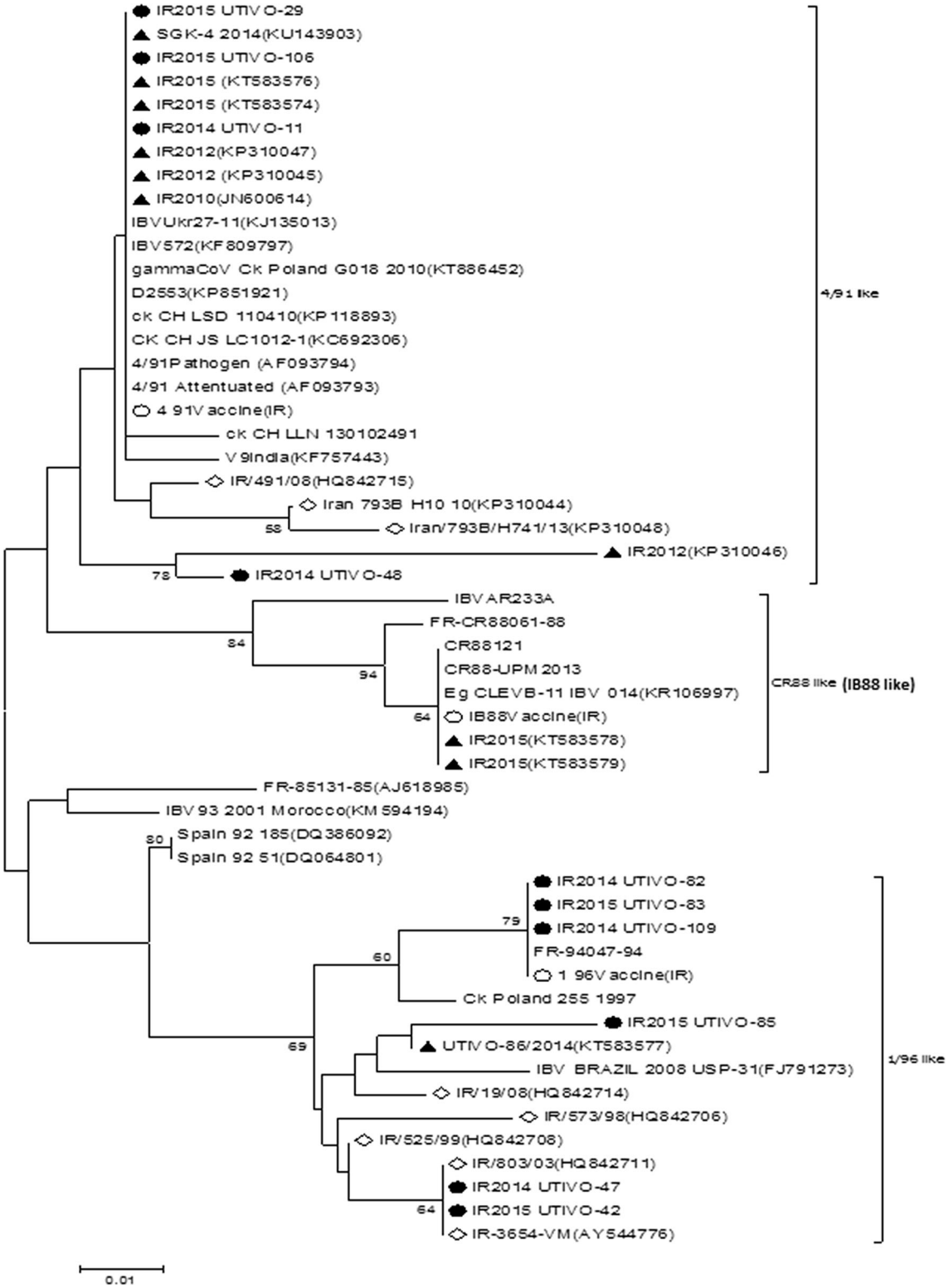

Fig. 1 Amino acid based phylogenetic relationships between the spike proteins of avian infectious bronchitis virus 793/B genotypes isolated in Iran, 2014-2015. The phylogenetic tree was generated using the neighbor joining model with MEGA (version 7.0.14). The numbers below the branches indicate bootstrap values from 1000 replicates. The virus genome characterized in this report is indicated as ●; ▲: indicates previous Iranian IBV isolates that were sequenced in our lab; ○: IBV vaccine strains; ◇: indicates previous Iranian IBV isolates that were sequenced by other Iranian researchers

Table 1 Percentage sequence identities between the different Iranian 793/B IBV isolates, when compared with other IBV strains

COMPARE	4 91Vaccine (IR)	IB88Vaccine (IR)	1 96Vaccine (IR)	4/91 Attenuated (AF093793)	CR88121	CR88-UPM2013	gammaCoVCk Poland G018 2010(KT886452)	FR-94047-94	Ck Poland 255 1997
4 91Vaccine(IR)		94.38202	92.13483	100	94.38202	94.38202	100	95.50562	93.25834
IB88Vaccine(IR)			87.64045	94.38202	100	100	98.8764	94.38202	88.76404
1 96Vaccine(IR)				92.13483	87.64045	87.64045	92.13483	87.64045	97.75281
4/91 Attentuated (AF093793)					94.38202	94.38202	100	92.13483	93.25843
CR88121						100	94.38202	87.64045	88.76404
CR88-UPM2013							94.38202	87.64045	88.76404
gammaCoVCk Poland (KT886452)								92.13483	93.25843
FR-94047-94									97.75281
Ck Poland 255 1997									
Spain 92 51(DQ064801)									
IBV BRAZIL 2008 USP-31(FJ791273)									
IR-3654-VM(AY544776)									
IR2014 UTIVO-11									
IR/491/08(HQ842715)									
IR2015(KT583579)									
IR2015 UTIVO-83									
IR2014 UTIVO-109									
V9India(KF757443)									

COMPARE	Spain 92 51(DQ064801)	IBV BRAZIL 2008 USP-31(FJ791273)	IR-3654-VM(AY544776)	IR2014 UTIVO-11	IR/491/08(HQ842715)	IR2015(KT583579)	IR2015 UTIVO-83	IR2014 UTIVO-109	V9India(KF757443)
4 91Vaccine(IR)	96.62921	92.13483	93.25834	100	98.8764	94.38202	92.13483	92.13483	98.8764
IB88Vaccine(IR)	92.13483	88.76404	88.76404	94.38202	93.25834	100	87.64045	87.64045	93.25834
1 96Vaccine(IR)	95.50562	94.38202	96.62921	92.13483	91.01124	87.64045	100	100	91.01124
4/91 Attentuated (AF093793)	96.62921	92.13483	93.25843	100	98.8764	94.38202	92.13483	92.13483	98.8764
CR88121	92.13483	88.76404	88.76404	94.38202	93.25843	100	87.64045	87.64045	93.25843
CR88-UPM2013	92.13483	88.76404	88.76404	94.38202	93.25843	100	87.64045	87.64045	93.25843
gammaCoVCk Poland (KT886452)	96.62921	92.13483	93.25843	100	98.8764	94.38202	92.13483	92.13483	98.8764
FR-94047-94	95.50562	94.38202	96.62921	96.62921	91.01124	87.64045	100	100	100

Table 1 continued

COMPARE	Spain 92 51(DQ064801)	IBV BRAZIL 2008 USP-31(FJ791273)	IR-3654-VM(AY544776)	IR2014 UTIVO-11	IR/491/ 08(HQ842715)	IR2015(KT583579)	IR2015 UTIVO-83	IR2014 UTIVO-109	V9India(KF757443)
Ck Poland 255 1997	95.50562	94.38202	96.62921	93.25843	92.13483	88.76404	97.75281	97.75281	91.01124
Spain 92 51(DQ064801)		94.38202	96.62921	96.62921	95.50562	92.13483	95.50562	95.50562	95.50562
IBV BRAZIL 2008 USP-31(FJ791273)			95.50562	92.13483	91.01124	88.76404	94.38202	94.38202	91.01124
IR-3654-VM(AY544776)				93.25843	92.13483	93.25843	91.01124	96.62921	92.13483
IR2014 UTIVO-11					98.8764	94.38202	92.13483	92.13483	98.8764
IR/491/08(HQ842715)						93.25843	91.01124	91.01124	97.75281
IR2015(KT583579)							87.64045	87.64045	93.25843
IR2015 UTIVO-83								100	91.01124
IR2014 UTIVO-109									91.01124
V9India(KF757443)									

793-B type in 2001 by type-specific multiplex RT-PCR [27]. Furthermore, Shoushtari *et al* (2008) showed, based on retrospective studies, that 793/B type was the predominant circulating IBV type between 1999 and 2004 in Iran. They concluded that the 793/B type had existed since at least 1999 in Iran, or even earlier [25]. Interestingly, Hashemzadeh *et al* (2013) conducted a survey of IBV genotypes in Iranian flocks between 2009 and 2011 and found two isolates belonged to the 793/B genotype. Razi-HKM891 and Razi-HKM894 shared an amino acid identity of 99.30%, and a similarity of 100% and 99.30% with the pathogenic 4/91 virus, respectively [18]. Hosseini *et al* (2015) reported that the 793/B like virus had an identification frequency of 8% during surveillance of IBV genotypes involved in outbreaks (between 2010 and2014) [19]. In a study performed by Najafi *et al* (2015), 793/B-like viruses had a total prevalence of 21%, ranking second among IBV types in Iranian chicken flocks [22]. In Israel, Massachusetts was the only type detected for many years until the 793/B type of IBV (Israel/793/B/variant 1/96) was identified in 1996 [13]. Similarly, a total of 100 tissue specimens from different commercial broiler flocks in Iraq were collected from 2013 to 2014. The prevalence rate of 793/B-like IBV isolates was 40.62%, second only behind1494-like IBV [26].

Ganapathy *et al* (2015) reported the IBV genotypes circulating in seven Middle East countries between 2009 and 2014. The prominence of 793/B (43.66%) was not surprising given that 793/B vaccine strains are widely used in the Middle East. Sequence analysis demonstrated that the majority of 793/B (67.13%) strains were closely related to vaccine strains, based on high homology (99–100%). After 2012, the 793/B field strain started to show distinct clustering, when compared to strains from earlier years. Indeed 793/B prevalence was 85.2% in UAE, 63.7% in Oman, 43.2% in Saudi Arabia, 16.8% in Egypt and 13% in Lebanon; however, it was not detected in Jordan or Kuwait [17]. In a separate study, two hundred and five samples were collected from broilers and layer chicken farms from all over Egypt in 2012. Sixty-four percent of suspected farms were positive for IBV, by real time RT-PCR. Thirteen IBV-positive samples were selected for further isolation and characterization. Only two isolates had a close relationship with CR/88121 and 4/91 viruses, with identities of 95% and 96%, respectively [28]. The study results showed that about 50% of the 793/B IBV isolates located within the 4/91-like cluster. Capua *et al* analyzed 18 IBV isolates from Italy and Poland from 1997 to 1998. Four types of IBV (793/B, 624/I, B1648 and Massachusetts) were detected in Italy, while the presence of 793/B was confirmed in Poland [7]. Worthington *et al* identified IBV genotypes in Western Europe (France, Holland,

Germany, Belgium, and Spain) between 2002 and 2006. The predominant IBV genotypes detected were again 793/B (33.8%) [33]. Ten IBV isolates collected from commercial chickens in Italy in 1999 were also characterized. Phylogenetic analysis showed five field viruses clustered together with 793/B-type strains, having 91.3–98.5% nucleotide identity within the group [4]. The phylogenetic analysis of IBV isolates from 1997 to 2006 revealed that the 793/B type is present in Poland. One isolate (PL-338-04-, isolated in 2004) grouped in the same cluster as the 4/91 vaccine while others, which were isolated in 1997 and 1999, grouped in another cluster [15]. Twenty-six IBVs isolated in Spain between 1992 and 2005 were also molecularly characterized. Genotype I represented 13 out of the 26 field isolates (isolated between 1992 and 2000) and grouped with 4/91 isolates [14]. Due to the use of a specific vaccine (4/91; Intervet) in the Iranian poultry industry, these strains might be related to the vaccine strain. In addition, 10% of Iranian 793/B IBV isolates were related to the IB88 strain. Interestingly, a significant difference in the detection rate between 4/91 and IB88 groups indicates that the 4/91 strain is more stable than IB88 in chicks. This data may reflect the re-isolation of vaccine strains. It is also possible that these live vaccine strains could have given rise to these genetically-altered field isolates. This is agreement with data from Franzo *et al* that detected 793/B IBV on farms that was derived from two in-use 793/B vaccines. Their evidence showed that the 793/B type became undetectable after the withdrawal of the two 793/B vaccines on several hundred Italian farms; they concluded that 793/B vaccine use is no longer required for 793/B virus control [16]. Finally, 40% of the 793/B IBV isolates were similar to the 1/96-like virus. Since in the period of sampling, iBird (1/96) was not approved for use in Iran, and, the first reported Iranian 793/B IBVs are located within this cluster, our underlying hypothesis and theory is that our 1/96-like IBV viruses are 793/B field isolates. This means that around 40% of 793/B type IBV strains should be considered as field IBV strains. This study examined the molecular epidemiology of the 793/B IBV genotype and studied the molecular dynamics of IBV in Iran and the surrounding region. This will help researchers to differentiate the origin of 793/B like viruses in the future. Of note, full genome sequencing of the different IBV isolates helped us to gather more information about the circulating 793/B IBV type.

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Compliance with ethical standards

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Conflict of interest No conflict of interest.

Ethical approval This article does not contain any studies with animals performed by any of the authors.

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