Sequence analysis of the VP1 gene in three very virulent Iranian infectious bursal disease virus strains

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Summary

Infectious bursal disease (IBD) is a highly contagious disease of chickens caused by the infectious bursal disease virus (IBDV). This study was conducted to characterize three IBDV strains from Iran. A reverse transcriptase-polymerase chain reaction (RT-PCR) procedure was used to amplify a 715-bp fragment of the VP1 gene from IBDV strains. Amplified VP1 fragments of the three Iranian IBDV strains were sequenced and compared with published sequences of IBDV strains from around the world, and their phylogenetic relationships were analyzed. Alignment of IBDV strains revealed 23 nucleotide differences between vvIBDV (except for IL and PT) and other non-vvIBDV strains. Two nucleotide positions, 863G and 1023A, were specific as JRMP07IR and JRMP14IR strains. All vvIBDVs differed (except for IL and PT strains) from non-vvIBDVs at aa (amino acids) positions 242E and 287A. In the three Iranian IBDV strains, aa positions 251R in both JRMP07IR and JRMP14IR, and 360L in JRMP14IR differed from those of other vvIBDVs. In phylogenetic analyses, all three Iranian strains clustered together with vvIBDVs. One Iranian strain, JRMP30IR, was more closely related to two European strains (HOL and UK661) and two south-east Asian strains (OKYM and ZJ2000). However, the other two Iranian strains, JRMP07IR and JRMP14IR, were closer to two Turkish strains (OA/G1 and OE/G2) and a Malaysian strain (UPM94). Further comprehensive investigations will provide researchers a better knowledge on the distribution, variability, and phylogenetic relationships of different IBDVs isolated in Iran and other parts of the world.

Key words: Infectious bursal disease virus, Very virulent strains, VP1, Chicken, Iran

Introduction

Infectious bursal disease (IBD) is a highly contagious disease affecting chickens from 3 to 6 weeks of age (Eterradossi and Saif, 2008). The etiologic agent of the IBD is a bi-segmented (A and B), double stranded RNA virus belonging to the *Birnaviridae* family (van den Berg, 2000). The virus genome encodes several proteins including: VP1, VP2, VP3, VP4, and VP5. Two distinct serotypes of the IBD virus (IBDV), serotypes 1 and 2, are known. The first is a pathogenic type, causing IBD in chickens, but the second one produces neither disease nor immunity against the pathogenic strains of serotype 1 (Müller *et al*., 2003; Eterradossi and Saif, 2008). Pathogenic serotype 1 isolates can be classified into standard or classical virulent strains and antigenic variant strains (Synder, 1990; Brown *et al*., 1994). In the late 1980s, very virulent (vv) IBDVs were initially reported in Europe, but later, they were reported from other parts of the world, except Australia. The vvIBDVs are antigenetically very similar to the classical strains but display a marked increase in virulence, causing high mortality in infected flocks (Yamaguchi *et al*., 1997; van den Berg, 2000). The presence of vvIBDVs in Iran has been confirmed in several studies (Hosseini *et al*., 2004; Shamsara *et al*., 2006; Razmyar and Peighambari, 2008a, b; Razmyar and Peighambari, 2009).

Despite being time-consuming and difficult to apply when too many samples are being processed, RT-PCR/RFLP of the VP2 gene has been commonly used by many researchers to identify IBDV and differentiate between classical IBDV and vvIBDVs. Protocols for the amplification of the VP1 gene have also been described in recent years (Tiwari *et al*., 2003). To differentiate classical IBDVs from vvIBDVs, a simple RT-PCR assay was developed based on the VP1 gene, in which no enzymatic restriction was employed (Ashraf *et al*., 2007).

In our earlier studies on bursal samples provided from Iranian poultry flocks, based on the VP2 gene, we differentiated classical IBDVs and vvIBDVs using RT-PCR/RFLP and confirmed our findings by sequence analyses (Razmyar and Peighambari, 2008a, b). Later, we re-confirmed the very virulent pattern of our IBDV field isolates by using VP1 gene amplification without the application of restriction enzymes (Ghaniei *et al*., 2011). To further study the VP1-amplified vvIBDVs, we characterized three vvIBDVs by sequencing and phylogenetic analysis.

Materials and Methods

Viruses

Three IBDV field isolates obtained from Iranian poultry flocks were included in this study during 2005-2006 (Razmyar and Peighambari, 2008a). The preparation of bursal samples has been described previously (Ghaniei *et al*., 2011). Briefly, bursal samples

were placed in tubes containing TNE buffer (10 mM Tris/HCl, 100 mM NaCl, 1 mM EDTA, pH=8.0). The volume was then increased 5 times by the TNE buffer. Penicillin and streptomycin, 10,000 IU and 10,000 mg/ml, respectively, were added to the suspension. The bursal homogenates were vortexed vigorously, frozen and thawed 3 times, and centrifuged at 14000 × g at 4°C for 20 min. The supernatant was removed and used as a substrate in the RNA extraction protocol. The relevant information regarding the three IBDV field isolates used in this study is presented in Table 1.

RT-PCR

A commercial kit (High Pure Viral RNA Kit, Roche, Germany) was used to extract the viral RNA from the bursal samples homogenized in the TNE buffer as recommended by the manufacturer. To make cDNA, a commercial cDNA synthesis kit (RevertAid First Strand cDNA Synthesis Kit, Fermentas Life Science, Germany) was used as recommended by the manufacturer, but with some modifications. Briefly, 5 µl RNA extract, 2.5 µl (10 pM/µl) reverse primer and 2.5 µl diethylpyrocarbonate (DEPC)-treated water were boiled for 2 min and then cooled on ice immediately. Four µl 5 x RT buffer plus 2 µl 10 mM dNTP mix, 1 µl M-MuLV RT (200 u/µl), 0.5 µl RNasin (20 u/µl), and 2.5 µl DEPC-water treated were added to the previous mixture, incubated for 1 h at 42°C and 10 min at 80°C, cooled on ice, and stored at -20°C for further analysis.

To amplify a 715-bp sequence of IBDV VP1 gene, we used AG-F (5'-CCG AGG CCA CAG ATA ACC TTA A <A>-3' and AG-R (5'-CCT CTA AAC GGG TTG AA <C>-3') primers (Ashraf *et al*., 2007), specific to the identification of vvIBDVs. The primers and other materials used in the PCR reactions were provided by Cinnagen, Iran. The amplification was carried out in a 50-µl reaction volume consisting of 5 µl 10 x PCR buffer, 1 µl 10 mM dNTPs, 1.5 µl of each primer (10 pM/µl), 0.5 µl *Taq* DNA polymerase (5 u/µl), 2.5 µl 50 mM $MgCl_2$, 7 µl cDNA, and 31 µl dH_2O and programmed in a thermocycler (Gradient Mastercycler, Eppendorf, Germany) as follows: 35 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 7 min. In all PCR reaction sets, negative controls (dH_2O instead of cDNA) were included. The amplification products were detected by gel electrophoresis (Applex, France) in 1.5% agarose gel in a tris-acetate-ethylenediaminetetraacetic acid buffer.

Sequence and phylogenetic analysis

The 715-bp PCR products of the three very virulent isolates were purified using a Roche purification kit and submitted for an automated sequencing in both directions to the Eurofins MWG Operon (Germany) using PCR primers as the sequencing primers. Nucleotide (nt) and predicted amino acid (aa) sequence data were aligned with clustal alignment algorithms (Table 1). Phylogenetic analysis was conducted based on the nt sequences using a distance method and an unweighted pair group with arithmetic mean and by calculating bootstrap values for 1000 replicates in MEGA version 4.0 (Tamura *et al*., 2007). The sequence data were submitted to GenBank under the accession numbers shown in Table 1 where accession numbers for IBDV sequences used for multiple alignment analysis are also shown.

Results

The RT-PCR amplified a 715-bp fragment of the VP1 gene in all three Iranian vvIBDV strains (JRMP07IR, JRMP14IR, JRMP30IR). Amplified products were sequenced and compared with those of published IBDV strains available in GenBank (Table 2).

Alignment of IBDV strains revealed 23 nt differences between vvIBDV (except for IL and PT) and other non-vvIBDV strains (data not shown). JRMP07IR had a 98% nt identity with HOL (Holland), OKYM and ZJ2000 strains. JRMP14IR was more closely related to HOL, OA/G1, OKYM and ZJ2000 (98%) at the nt level. JRMP30IR also had a 98% nt similarity with HOL, OA/G1, OKYM, ZJ2000. Two nt positions, 863G and

Table 1: Published IBDV sequences of VP1 used for multiple alignment analysis

Virus strain	Pathotype	Origin	Accession No.
Cu-1	Classical cell culture-adapted	Germany	AF362775
D78	Classical attenuated	Netherlands	EU162090
Winterfield 2512	Attenuated vaccine strain	USA	AF083092
Variant E	Virulent	USA	AF133905
GLS	Variant	USA	AY368654
IL	Very virulent	Taiwan	EF576658
PT	Very virulent	Taiwan	DQ679814
UPM94/273	Very virulent	Malaysia	AF527038
HOL	Very virulent	Netherlands	DQ679811
OA/G1	Very virulent	Turkey	DQ679812
OE/G2	Very virulent	Turkey	DQ679813
OKYM	Very virulent	Japan	D49707
UK661	Very virulent	UK	X92761
ZJ2000	Very virulent	China	DQ166818
JRMP07IR	Very virulent	Iran (Tabriz)	HQ735292
JRMP14IR	Very virulent	Iran (Amol)	HQ735293
JRMP30IR	Very virulent	Iran (Tehran)	HQ735294

Table 2: Percentage nucleotide and amino acid identities for the VP1 genes of 17 strains of IBDVs[a]

	Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	JRMP07IR		100	99	99	99	99	99	98	99	99	99	99	99	99	98	98	99
2	JRMP14IR	100		99	99	99	99	99	98	99	99	99	99	99	99	98	98	99
3	JRMP30IR	99	99		100	99	99	99	99	99	99	100	98	99	99	98	99	99
4	HOL	99	99	99		98	99	98	98	98	98	99	99	98	99	98	97	99
5	OA/G1	99	99	99	98		99	97	97	97	97	97	98	97	98	96	96	97
6	OE/G2	98	98	99	99	98		98	98	98	98	98	99	98	99	98	97	99
7	IL	90	90	91	89	89	89		99	99	99	97	97	99	98	99	99	97
8	PT	91	91	91	89	89	89	99		99	99	97	97	99	98	99	99	97
9	D78	91	91	92	89	89	89	97	97		99	97	98	100	98	99	99	98
10	GLS	91	91	92	89	89	89	97	97	97		97	98	99	98	99	99	98
11	OKYM	99	99	99	98	97	98	89	89	89	89		98	97	98	97	96	98
12	UK661	99	99	99	99	97	98	89	89	89	89	98		98	99	97	97	99
13	Winterfield	91	91	92	89	89	89	97	97	99	97	89	89		98	99	99	98
14	ZJ2000	99	99	99	98	97	98	89	89	89	90	98	98	89		98	97	99
15	CU-1	91	91	92	89	88	89	97	97	99	97	89	89	99	89		99	97
16	Variant E	91	91	92	89	88	89	97	96	97	97	88	88	97	89	97		97
17	UPM94/273	98	98	99	98	98	97	89	89	89	89	97	98	89	98	89	89	

[a] Percentage amino acid identity is in upper triangle; percent nucleotide identity is in lower triangle

1023A, were specific to JRMP07IR and JRMP14IR.

All vvIBDVs differed (except for IL and PT strains) from non-vvIBDVs at aa positions 242E and 287A. In the three Iranian IBDV strains, aa positions 251R in both JRMP07IR and JRMP14IR, and 360L in JRMP14IR differed from those of the other vvIBDVs. JRMP07IR had 99% aa identity with Holland and OKYM strains. JRMP14IR was more closely related to HOL, OA/G1, ZJ2000, UPM94/273 (99%) at the aa level. JRMP30IR showed 100% aa similarity with HOL strain (Table 2).

Phylogenetic analyses based on nt sequences formed two main branches, classical-type IBDVs and vvIBDVs. All three Iranian strains of the present study clustered together with vvIBDVs (Fig. 1).

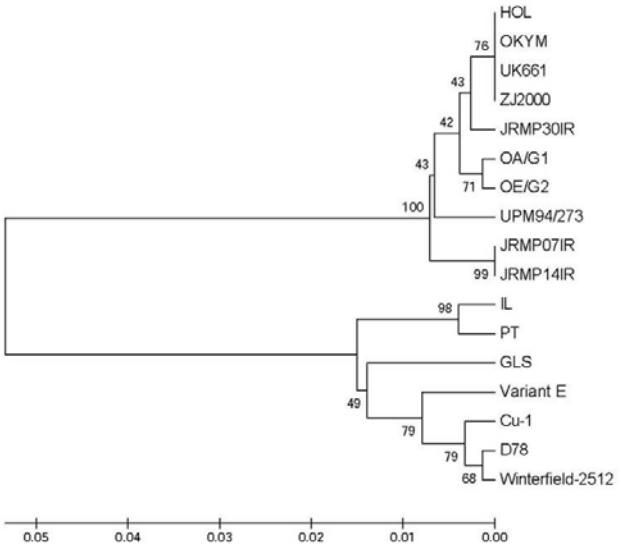

Fig. 1: Phylogenetic tree of selected IBDV strains based on the nucleotide sequence of a part of the VP1 gene. Branched distances correspond to a sequence divergence

Discussion

Infectious bursal disease virus is an important pathogen in the poultry industry causing mortality and immunosuppression. The first isolation of IBDV in Iran dates back to 1981 (Aghakhan *et al*., 1994). Several investigations on IBDV were later carried out in Iran to shed more light on the properties of Iranian IBD viruses (Hosseini *et al*., 2004; Shamsara *et al*., 2006; Bahmaninejad *et al*., 2008; Razmyar and Peighambari, 2008a, b; Haghighi *et al*., 2009).

Previous investigations on IBDV have indicated that VP2 contains sites involved in determining virulence (Fahey *et al*., 1991; Muller *et al*., 2003). However, the important role of VP1 in determining IBDV virulence has also been noted by some researchers (Liu and Vakharia, 2004; Yu *et al*., 2013). VP1, which is the RNA-dependent RNA polymerase of IBDV, is encoded by the segment B of the virus and is a relatively conserved portion of the IBDVs' genome. In recent years, VP1 sequences for many classic and variant viruses and vvIBDVs have been published in GenBank. Several investigations have shown that the VP1 nucleotide sequences of vvIBDVs form a distinct pattern and cluster separately from all the classics, variants, and serotype 2 strains of IBDVs (Yamaguchi *et al*., 1997; Islam *et al*., 2001; Lojkic *et al*., 2008; Yu *et al*., 2010).

Our previous studies on bursal samples provided from Iranian poultry flocks revealed the presence of classical IBDVs and vvIBDVs by using RT-PCR/RFLP and sequence analyses based on the VP2 gene (Razmyar and Peighambari, 2008a, b). Later, we selected several bursal samples from our previous study and re-confirmed the very virulent pattern of the field isolates by amplifying a section of the VP1 gene without applying any restriction enzymes (Ghaniei *et al*., 2011).

In this study, the nt and aa sequence of the RT-PCR-amplified 715-bp VP1 fragment of three Iranian vvIBDV strains (JRMP07IR, JRMP14IR, JRMP30IR) were analyzed and compared with previously published sequences of IBDV strains.

Different amino acids including 242E and 287A of segment B have been specifically attributed to vvIBDVs (Yamaguchi *et al*., 1997; Lojkic *et al*., 2008; Yu *et al*., 2010). In studies on segment B of the Japanese OKYM vvIBDV strain, Yamaguchi *et al*. (1997) stated that

glutamic acid in residue 242 can be a potential virulence marker among vvIBDV strains. However, other researchers speculated that the aa change at position 242 may be more likely associated to origin (Europe) differences rather than an indicator of virulence in vvIBDV strains (Yu *et al*., 2010). These researchers also found 287A (Ala) in all vvIBDVs and suggested this amino acid (Ala) to be a virulence marker in segment B of vvIBDV strains (Yu *et al*., 2010). In Croatia, researchers reported nine characteristic aa residues common to vvIBDVs including 242E (Lojkic *et al*., 2008). In the present study, all three Iranian vvIBDV strains (except for IL and PT strains) were different from non-vvIBDVs at aa positions 242E and 287A. Recently, Yu *et al*. (2013) found that a Valine to Isoleucine substitution at amino acid position 4 of VP1 attenuated viral pathogenicity and reduced viral replication in SPF chickens.

In phylogenetic analysis based on nt sequences, all three Iranian strains clustered together with other vvIBDVs. Interestingly, two Taiwan IBDV strains, IL and PT, which were previously classified among vvIBDVs (Ture *et al*., 1998), were grouped with classical and variant strains in our analysis. The discrepancy regarding these two strains has been also pointed out by Ashraf *et al*. (2007). Among Iranian strains, JRMP30IR was more closely related to two European (HOL and UK661) and two south-east Asian strains (OKYM and ZJ2000). However, the other two Iranian strains, JRMP07IR and JRMP14IR, were closer to two Turkish (OA/G1 and OE/G2) and one Malaysian strain (UPM94/273). It should be noted that these comparisons have been made based on partial sequencing of the VP1 gene of three Iranian strains. For accurate typing of IBDV strains, full-length genomic analysis of more IBDV strains, at both nucleotide and amino acid levels, is required. Such comprehensive investigations will provide researchers with a better knowledge on the distribution, variability, and phylogenetic relationships of different IBDVs isolated in Iran and other parts of the world.

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