CLONING AND NUCLEOTIDE ANALYSIS OF THE VP2 GENE OF A VERY VIRULENT INFECTIOUS BURSAL DISEASE VIRUS ISOLATE FROM IRAN

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Summary. – An Iranian field isolate (IR01) of Infectious bursal disease virus (IBDV) was characterized by sequence analysis of its VP2 gene and protein. Comparison of the obtained sequences with those of IBDV isolates from other countries revealed that IR01 was similar to very virulent IBDV (vvIBDV) strains with the identities at nucleotide and amino acid levels reaching 98.1–98.9% and 99.1–99.3%, respectively. On the other hand, it was less similar to non-vvIBDV strains; with nucleotide and amino acid identities of 95.2–95.7% and 96.0–97.3%, respectively. Out of nine unique nucleotide differences found between IR01 and some other serotype 1 strains only two resulted in amino acid substitutions (Ile296Val and Thr359Lys). In phylogenetic analysis, IR01 was closely related to Asian and European vvIBDV strains. Based on these results, IR01 obviously belongs to vvIBDV strains.

Key words: Infectious bursal disease virus; VP2 gene; sequence analysis; phylogenetic analysis

Introduction

IBDV is the causative agent of an avian disease originally known as Gumboro (Cosgrove, 1962). Based on cross-neutralization, two serotypes of IBDV have been identified. Serotype 1 strains are pathogenic to chickens (Winterfield and Thacker, 1978) while serotype 2 strains are commonly non-pathogenic to chickens (Ismail *et al*., 1988). The virulence of serotype 1 strains is variable (van den Berg *et al*., 1991). The target organ for IBDV is the bursa of Fabricius in young birds. The virus replication causes a depletion of B cells due to apoptosis, resulting in severe immunodepression in chickens (Vasconcelos and Lam, 1995). The acute form of infectious bursal disease (IBD) caused by vvIBDV was first reported from Europe (Chettle *et al*., 1989). Then it rapidly spread all over Asia and other parts of the world (van den Berg, 2000), characterized by severe clinical signs and high mortality. Emergence of the acute forms has changed the epidemiology of IBD and become the cause of heavy losses to the poultry industry.

IBDV belongs to the genus *Birnavirus*, the family *Birnaviridae*. Its genome consists of two segments (A and B) of double-stranded RNA (Dobos *et al*., 1979). The larger A segment (3.3 kbp) contains two partly overlapping ORFs. The small ORF encodes non-structural protein VP5 (Mundt *et al*., 1995) while the large one encodes a polyprotein, which is proteolytically cleaved into pVP2/VP2, VP4, and VP3 proteins (Hudson *et al*., 1986). VP2 contains the antigenic regions responsible for neutralizing antibodies and serotype specificity (Azad *et al*., 1987; Becht *et al*., 1988; Fahey *et al*., 1989). The epitopes recognized by neutralizing antibodies are conformational and have been mapped to amino acids 206–353 (Azad *et al*., 1987). The smaller B segment (2.9 kbp) contains one large ORF encoding VP1 protein, an RNA-dependent RNA polymerase (Bruenn,

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Abbreviations: IBD = infectious bursal disease; IBDV = IBD virus; non-vvIBDV = non-very virulent IBDV; vvIBDV = very virulent IBDV

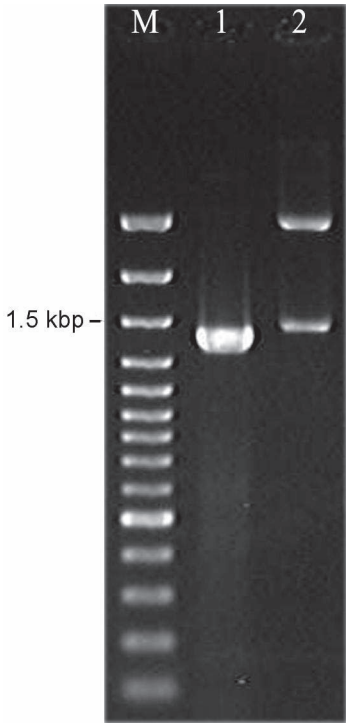

Fig. 1

Agarose gel electrophoresis of RT-PCR product and digested recombinant vector

The RT-PCR product (lane 1); the recombinant vector digested with *Eco*RI and *Xho*I (lane 2); DNA size marker (100 bp ladder, lane M).

1991). Little is known about genetic and antigenic properties of vvIBDV strains occurring in Iran. The aim of this study was to characterize an Iranian field isolate of IBDV by sequence analysis of its VP2 gene.

Materials and Methods

Virus isolation. Bursal tissue samples were collected from affected flocks in eastern Iran. In the acute phase, the disease signs included severe depression with ruffled feathers, watery of white diarrhea, pallor of the comb and wattles, and sudden death. Bursal samples were used for RNA extraction.

Total RNA extraction. Tissue samples (50–100 mg) were homogenized in a mortar in DEPC-treated distilled water (1 ml) and extracted with phenol-chloroform (Chomczynski and Sacchi (1987). The obtained RNA was precipitated with isopropanol, washed with 70% ethanol, dried, and dissolved in 20 µl of DEPC-treated distilled water. The RNA was immediately subjected to RT-PCR or stored at -70°C.

RT-PCR. To amplify the IBDV VP2 gene (nt 122–1486, 1365 bp), the following specific primers, designed from the sequence of the VP2 gene of IBDV CEF94 strain (Acc. No. AF194428), were employed: 5'-GAATTCATCGC AGCGATGACAAAC-3' (forward, nt 122–139) and 5'-CTCGAGCCTTATGGCCCGGATTATGT-3' (reverse, nt 1467–1486). The primers contained *Eco*RI and *Xho*I sites (underlined). In the RT step, the reaction mixture (20 µl) contained 0.5–2 µg of total RNA, 30 pmoles of the reverse primer, 200 µmol/l dNTPs, 40 U of an RNase inhibitor, and 40 U of Moloney murine leukemia virus reverse transcriptase in the RT buffer (all from Roche Molecular Biochemicals, Germany). The reaction conditions were already described earlier (Beladi *et al.*, 2005). In the PCR step, the reaction mixture (50 µl) consisted of 5 µl of the RT product, 200 µmol/l dNTPs, 20 pmoles of the forward and reverse primers, 2 U of Taq DNA polymerase, 2.0 mmol/l MgCl2, and the PCR buffer. The PCR consisted of initial denaturation, 35 cycles of amplification, and final elongation as described earlier (Beladi *et al.*, 2005). The PCR products were analyzed by 1% agarose gel electrophoresis.

Cloning and sequencing. The desired PCR fragment was isolated from the agarose gel using the High Pure PCR Purification Kit (Roche Molecular Biochemicals, Germany) and was cloned in the pTZ57R/T vector (Fermentas, Lithuania) using a T/A cloning system according to the manufacturer's instructions. The presence of the insert (1365 bp) in the clones was confirmed by isolation of plasmid DNA, its digestion with *Eco*RI and *Xho*I (Fermentas, Lithuania), and agarose gel electrophoresis. Four independent clones of VP2 gene were sequenced by direct method using an ABI PRISM 310 genetic analyzer (PE Biosystems).

Sequence and phylogenetic analyses. The sequence data were assembled using a Seqman (DNASTAR, USA). Nucleotide and deduced amino acid sequences were aligned using the MegAlign program (DNASTAR, USA). Phylogenetic trees were generated by the neighbor-joining method and 100 bootstrap replications using the CLUSTAL-X program version 1.8.

Results

Cloning of the VP2 gene

The product of RT-PCR, when analyzed by agarose gel electrophoresis, appeared to be of the expected size (1365 bp) (Fig. 1). It was cloned into the pTZ57R/T vector and the obtained recombinant was checked for the presence of the correct insert by digestion with *Eco*RI and *Xho*I and agarose gel electrophoresis. The results demonstrated that recombinant vector contained the VP2 gene, as the digestion

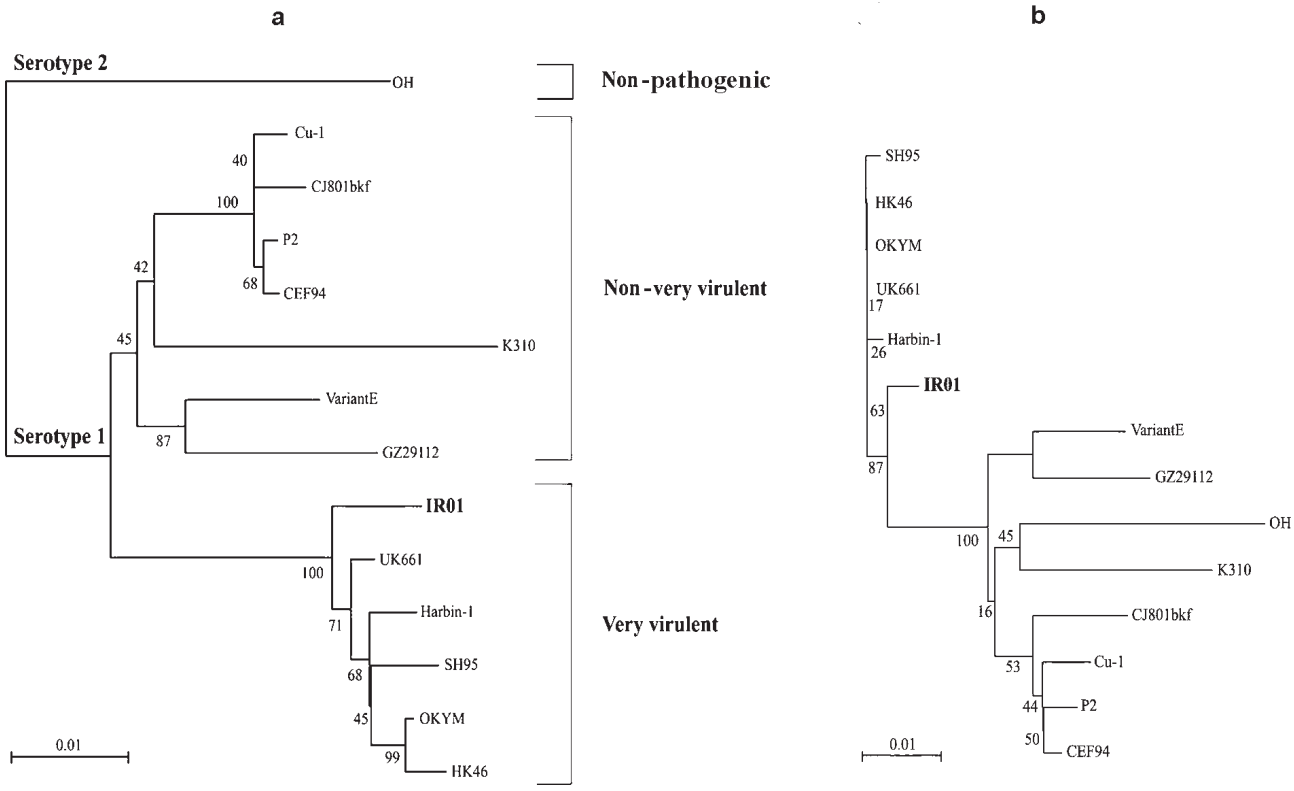

Fig. 2

Phylogenetic trees of IBDV strains based on nucleotide (a) and deduced amino acid (b) sequences of VP2 gene

The trees were generated by neighbor-joining method and 100 bootstrap replications. Bootstrap values are shown besides the nodes. The following IBDV strains (Acc. Nos.) were compared: SH95 (AY134874), OKYM (D49706), UK661 (X92760), HK46 (AF092943), Harbin-1 (AF454945), Variant E (AF133904), Cu-1 (AF362771), CEF94 (AF194428), K310 (AF165149), GZ29112 (AF051837), P2 (X84034), CJ801bkf (AF006694), and OH (U30818).

products had the size of the original vector (2.9 kbp) and the VP2 gene (1365 bp) (Fig. 1).

Sequence and phylogenetic analyses

Four independent clones containing the VP2 gene were completely sequenced. The derived consensus sequence of 1365 nts was considered authentic and was deposited in GenBank under Acc. No. AY704912. IR01 showed 15–81 nucleotide differences in the VP2 gene when compared with some other serotype 1 IBDV strains; however, only 9 differences were unique for IR01 (data not shown).

The results of phylogenetic analysis based on the VP2 gene sequence showed a 98.1–98.9% identity and hence a very close relationship of IR01 to vvIBDV strains such as UK661, HK46, OKYM, SH95, and Harbin-1 (Fig. 2a). On the other hand, IR01 was found to be less similar to non-vvIBDV strains (95.2–95.7% identity) (Table 1).

Table 1. Similarity of IR01 isolate to other IBDV strains based on the VP2 nucleotide and amino acid sequence identities

Serotype	Pathogenicity/virulence	Strain	Identity (%)	
			Nucleotide	Amino acid
2	Non-pathogenic	OH	79.4	86.7
1	Non-very virulent	K310	93.7	94.5
		GZ29112	95.2	96.0
		CJ801bkf	95.4	96.5
		Cu-1	95.4	96.9
		Variant E	95.7	96.7
		P2	95.7	97.1
		CEF94	95.7	97.3
	Very virulent	SH95	98.1	99.1
		HK46	98.3	99.3
		Harbin-1	98.5	99.1
		OKYM	98.6	99.3
		UK661	98.9	99.3

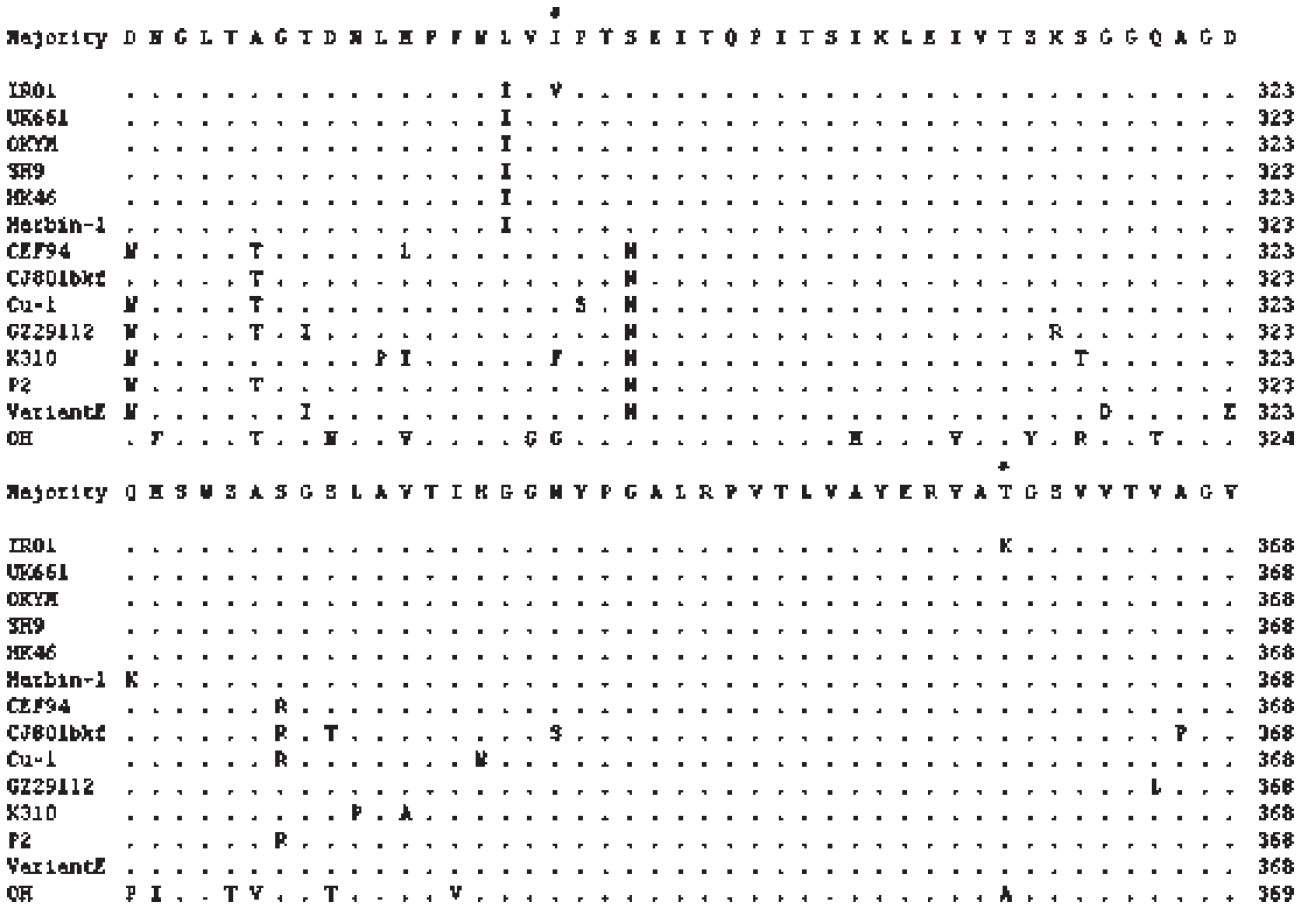

Fig. 3

Comparison of VP2 amino acid sequences of different IBDV strains

Amino acids at the positions 279–368 were compared. Two unique amino acid substitutions are marked by asterisk.

The VP2 gene of IR01 corresponded to a protein of 453 deduced amino acids. The VP2 protein exhibited 3–25 amino acid differences as compared to those of some other serotype 1 strains, and two unique substitutions Ile296Val and Thr359Lys (Fig. 3). In phylogenetic analysis based on the VP2 amino acid sequence, IR01 revealed a 99.1–99.3% identity and hence a very close relationship to vvIBDV strains such as UK661, OKYM, HK46, and Harbin-1 (Fig. 2b). On the other hand, when compared to non-vvIBDV strains, it showed only a 96–97.3% identity (Table 1).

Discussion

The acute form of IBD first appeared in Europe at the end of the 1980s (Chettle *et al*., 1989) and then rapidly spread all over Asia and other major parts of the world (van den Berg, 2000). The VP2 is one of the structural proteins of virion and is the major antigen containing the epitope, which induces neutralizing antibodies (Azad *et al*., 1987; Becht *et al*., 1988; Fahey *et al*., 1989). Due to high mutation rate in the VP2 variable domain, its comparison in various IBDV strains provides a powerful tool to monitor and trace its evolution in the nature. Molecular, epidemiological and mortality studies have suggested that vvIBDV strains belong to the same genetic lineage (Yamaguchi *et al*., 1997).

In this study, the comparison of Iranian IR01 isolate with other IBDV strains world-wide on the basis of VP2 sequence led to the its assignment to the very virulent lineage including Asian and European vvIBDV strains like UK661, HK46, OKYM, SH95, and Harbin-1. Based on the VP2 gene sequence, the compared IBDV strains grouped in two major phylogenetic lineages: one included a serotype 2 strain (OH)

while the other included serotype 1 strains; IR01 appeared in the latter lineage. In the serotype 1 lineage, two groups could be observed: vvIBDV and non-vvIBDV strains.

Two major phylogenetic lineages were also observed in the tree based on the VP2 amino acid sequence, but this time the serotype 2 OH strain clustered together with vvIBDV and non-vvIBDV strains. This could be due to the fact that the nucleotide differences were silent (did not result in amino acid differences).

UK661 strain, whose sequence was the first published among IBDV strains, is considered the reference for European very virulent strains (Brown and Skinner, 1996). Asian very virulent strains probably originated from European ones and then spread to Asia (Yamaguchi *et al*., 1997; Cao *et al*., 1998; Chen *et al*., 1998). The results of this study indicate that IR01 is a vvIBDV isolate, which could originate from European vvIBDV strains.

The VP2 gene of IBDV is highly conserved except for a hypervariable domain in the middle of the molecule, which causes the most genetic variability of IBDV strains (Pitcovski, 1998). According to previous studies, all IBDV strains with four specific amino acids (222Ala, 256Ile, 294Ile, and 299Ser) within the hypervariable domain had a very virulent phenotype (Brown *et al*., 1994; van den Berg *et al*., 1996; Yamaguchi *et al*., 1997; Cao *et al*., 1998; Eterradossi, 1998; Zierenberg *et al*., 2000, 2001). Meanwhile, the studies on the amino acid sequence of the hypervariable region indicated that Gln253, ASP279, and Ala284 were involved in the virulence, cell tropism and pathogenic phenotype of vvIBDV strains (Brandt *et al*., 2001). The mutations Gln253His and Ala284Thr in vvIBDV strains resulted in tissue culture adaptation and attenuation for chickens (van Loon *et al*., 2002). Through the mutations Asp279Asn and Ala284Thr in VP2 achieved by site-directed mutagenesis the very virulent HK46 strain could be adapted to CEF cultures (Lim *et al*., 1999); mutations of this kind are commonly found in attenuated strains of IBDV (Yamaguchi *et al*., 1996; Cao *et al*., 1998). We found two unique substitutions, namely Ile296Val and Thr359Lys in the amino acid sequence of VP2 protein of IR01. As the former is located in the hypervariable region it may cause an antigenic variation. The comparison of the amino acid sequences of VP2 protein of IR01 with those of vvIBDV and non-vvIBDV strains demonstrated that the VP2 amino acids in IR01 isolate and vvIBDV strains were mostly conserved (Table 2).

Table 2. VP2 amino acid differences between vvIBDV and non-vvIBDV strains

Position	Amino acid	
	Very virulent strains	Non-very virulent strains
222	Ala	Pro
253	Gln	His
256	Ile	Val
279	Asp	Asn
284	Ala	Thr
294	Ile	Leu
299	Ser	Asn

In conclusion, the VP2 gene/protein sequence and phylogenetic analyses suggested that IR01 was a vvIBDV isolate. Molecular characteristics of IR01 obtained in this study along with further pathogenetic studies can provide valuable information, which will be useful for improving control measures against IBD in Iranian poultry farms.

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