# CHARACTERIZATION OF THREE INFECTIOUS BURSAL DISEASE VIRUS ISOLATES OBTAINED FROM LAYER CHICKENS IN IRAN

M.A. BAHMANINEJAD[1], M. HAIR-BEJO[2*], A.R. OMAR[2], I. AINI[2], R. TOROGHI[1]

[1]Department of Poultry Disease Research and Diagnosis, Razi Vaccine and Serum Research Institute, P.O. Box 11365-1558 Tehran, Iran; [2]Faculty of Veterinary Medicine, University Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

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**Summary. –** Three infectious bursal disease viruses (IBDVs) were isolated from field outbreaks in IBDV-vaccinated and non-vaccinated layer chicken flocks. Agar gel precipitation test (AGPT), immunoperoxidase staining, transmission electron microscopy (TEM), inoculation into embryonated eggs, and chicken embryo fibroblasts (CEFs) confirmed that the isolates were IBDVs. RT-PCR, restriction fragment length polymorphism (RFLP), and phylogenetic analysis demonstrated that the isolates were very virulent IBDV (vvIBDV) and showed a nucleotide sequence similarity of 96.3 to 99.8% in comparison with other vvIBDV strains. It was concluded that the Iranian isolates represented vvIBDV of serotype 1 originating from Europe, Japan, and China.

**Key words:** infectious bursal disease virus; restriction fragment length polymorphism; sequence and phylogenetic analysis

## Introduction

Infectious bursal disease (IBD) is a highly contagious disease of young chickens characterized by severe lesions in the bursa of Fabricius followed by an immunosuppression (Saif, 1991; Hirai *et al*., 1974; Allan *et al*., 1972). The causative agent, Infectious bursal disease virus (IBDV) is a member of the family *Birnaviridae*, the genus *Avibirnavirus* (Fauquet *et al*., 2005). Since the first outbreak of IBD in 1957, (Cosgrove, 1962) the disease was satisfactorily controlled by a vaccination (Lasher and Shane, 1994). In 1980's, the variant (vaIBDV) and very virulent (vvIBDV) strains of IBDV emerged in USA (Snyder *et al*., 1988) and Europe (van den Berg *et al*., 1991; Chettle *et al*., 1989). These field IBDV strains exhibited different degrees of pathogenicity in chickens (Tsukamuto *et al*., 1995b). The classical strains of IBDV (caIBDV) induced mortality up to 5%, while vvIBDV strains caused high mortality up to 90–100% (Chettle *et al*., 1989; Hirai *et al*., 1973). Subclinical IBD or immunosuppression usually occurred in chickens that were infected with caIBDV at the age less than 2 weeks (Allan *et al*., 1972), but vvIBDV caused a severe immunosuppression even if the infection occurred in the chickens older than 2 weeks (Otaki, 1993; Nakamura *et al*., 1992). The vaIBDV strains do not induce clinical IBD, but are able to break through the maternal derived immunity against caIBDV and induce immunosuppression (Snyder, 1990).

IBDV has bi-segmented genome designated as the segment A and B placed within a non-enveloped single-shelled icosahedral capsid about 60 nm in diameter (Bottcher *et al*., 1997; Dobos *et al*., 1979). The segment A of IBDV is composed of 3.0–3.3 kbp containing two ORFs. The small ORF encodes the non-structural polypeptide VP5 (Mundt *et al*., 1995; Kibenge *et al*., 1990). The large ORF encodes 109 K (Kibenge

*Corresponding author. Email: mdhair@vet.upm.edu.my; fax: +603-89486317.

**Abbreviations:** AGPT = agar gel precipitation test; CAM = chorioallantoic membrane; CEF(s) = chicken embryo fibroblast(s); caIBDV = classical strains of IBDV; HE = hematoxylin and eosin; HPVR = hypervariable region; IBD = infectious bursal disease; IBDV(s) = infectious bursal disease virus(es); RFLP = restriction fragment length polymorphism; SPF = specific pathogen free; TEM = transmission electron microscopy; vaIBDV = variant IBDV; vvIBDV = very virulent IBDV

*et al*., 1990) polyprotein (NH2-VPX-VP4-VP3-COOH) (Azad *et al*., 1987; Hudson *et al*., 1986) that is cleaved and processed into two structural proteins VP2, VP3, and viral protease VP4 (Fahey *et al*., 1989; Becht *et al*., 1988). Among the five genes of IBDV, the VP2 gene has been widely studied, since it encodes the major structural protein that contains the main antigenic site responsible for eliciting of neutralizing antibodies (Oppling *et al*., 1991; Becht *et al*., 1988; Azad *et al*., 1987). The identified nucleotide sequence of VP2 within AccI and SpeI restriction sites like a hypervariable region located between the amino acid residues 206 to 350 is responsible for several neutralizing epitopes (Oppling *et al*., 1991; Bayliss *et al*., 1990). Furthermore, the serine rich heptapeptide domain (SWSASGS) is conserved within the hypervariable region of most IBDV strains except for the vaccine strains. It is believed that the heptapeptide region plays an important role in the pathogenicity of the virus (Dormitorio *et al*., 1997; Yamaguchi *et al*., 1996; Brown *et al*., 1994; Vakharia *et al*., 1994; Lana *et al*., 1992; Heine *et al*., 1991; Bayliss *et al*., 1990). The segment B encodes a VP1 protein of 90 K acting as a RNA dependent RNA polymerase (Muller and Nitschke, 1987; Spies *et al*., 1987).

Conventional serological methods such as AGPT (Hirai *et al*., 1972), ELISA (Briggs *et al*., 1986), and virus neutralization test (Thayer *et al*., 1987) have been used to diagnose IBD. However, these techniques are not able to differentiate among IBDV strains. Recently, molecular biology techniques including RT-PCR (Davis and Boyle, 1990), nucleotide sequencing, and RFLP are widely used to identify and differentiate IBDV isolates (Ture *et al*., 1998; Jackwood and Jackwood, 1997; Jackwood and Sommer, 1997). The RT-PCR-RFLP analysis of hypervariable region of VP2 is a rapid technique for identification and differentiation of unknown field IBDV isolates. The nucleotide sequencing can provide more information on the identity of the virus strain especially a newly emerging strain that cannot be determined by conventional methods. Phylogenetic analysis is a common method that is used to differentiate and determine the serotypes, pathotype strains, and evolutionary relationship as well as the origin of the different field IBDV isolates (Cao *et al*., 1998; Yamaguchi *et al*., 1997; Vakharia *et al*., 1994; Kibenge *et al*., 1991; Bayliss *et al*., 1990).

This study was aimed at the biological and molecular characterization of the three IBDV isolates obtained from IBDV-vaccinated and non-vaccinated commercial layer chickens during outbreaks of the disease in Iran.

## Materials and Methods

*Virus isolates*. Three IBDV isolates were obtained from IBD outbreaks in commercial layer chickens in Iran in 1997 to 1999 and designated as IR199, IR299, and IR398. The IR199 and IR229 were isolated from non-IBDV vaccinated flocks, whereas the IR398 was isolated from a flock vaccinated against the disease at 12, 22, and 32 days of age.

*Necropsy.* The gross lesion was recorded and samples of the bursa of Fabricius were collected for isolation, detection, identification, and molecular characterization of IBDV. The organs were fixed in 10% buffered formalin for histological examination.

*Histopathology*. The bursa of Fabricius was fixed in 10% buffered formalin for 24 hrs, processed, and stained with hematoxylin and eosin (HE) for histological examination using a standard procedure (Lussier, 1993; Lillie, 1965).

*Immunoperoxidase staining*. The bursa of Fabricius was stained according to the technique of Bratthauer (1994) and Bellonick (1993). The primary antibody was prepared in SPF chicken inoculated with purified Malaysian vvIBDV isolate P97/302 with accession No. AF 464901 (Phong *et al.*, 2002).

*Transmission electron microscopy*. Bursa of Fabricius was collected and processed according Hair-Bejo (1993).

*Bursa of Fabricius inoculums*. The bursal tissues were ground and homogenized according Hoque *et al.* (2001).

*Agar gel precipitation test*. The test was carried out according Hirai *et al*. (1972).

*Virus inoculation in embryonated eggs*. The IBDV inoculation in SPF embryonated eggs followed the standard procedure of Senne, 1998. The chorioallantoic membrane (CAM) from dead embryos was harvested and processed similarly as the bursa of Fabricius (Hoque *et al*, 2001).

*Chicken embryo fibroblast (CEF) cell culture* was prepared (Schat and Purchase, 1998) and the inoculum prepared from the CAM was inoculated. The infected cells were observed daily for CPE.

*Viral RNA extraction, primers, and RT-PCR*. The primers designated as P1 (forward) (5´-TCACCGTCCTCAGCTTAC-3´) and P2 (reverse) (5´-TCAGGATTTGGGATCAGC-3´) described by Liu *et al*. (1994) were used for amplification of 643 bp amplicon corresponding to the nt positions 587 to 1229 of the IBDV VP2 gene according to the numbering system of Kibenge *et al*. (1990). The total RNA was extracted using TriPure Isolation Reagent (Roche). The reverse transcriptase reaction and amplification of hypervariable region by PCR were also conducted (Liu *et al*., 1994; Kataria *et al*., 1998; Toroghi *et al*., 2001). DNA size marker 100 bp ladder used in agar gel electrophoresis was obtained from MBI Fermentas.

*Nucleotide sequencing, sequence, and phylogenetic analysis*. The PCR products were purified using a High Pure PCR Production purification kit (Roche) and sequenced on an ABI PRISM® 377-96 automated DNA sequencer (Applied Biosystem). The sequence comparison was performed by the Clustal method and phylogenetic analysis was carried out by the neighbor-joining method with the Megalign software package (DNAstar version 5.1). The hypervariable region sequences of IBDV isolates were analyzed and compared with other IBDV strains (Table 1).

*RFLP analysis.* The analysis of the Iranian isolates sequences were performed using restriction enzyme digestion with computer-generated software. The sequence of hypervariable region of VP2 gene of Iranian isolates was submitted to restriction enzymes mapping software of NEBcutter V2 (http://tool.neb.com/NEBcutter2/index.php) and the restriction site for SspI, BstNI, BspMI, MboI, StyI, SacI, NaeI, and TaqI.

**Table1. Characteristic of IBDV isolates used for sequence and phylogenetic analysis**

| Isolate | Virulence | Country of origin | Acc. No. | Reference |
|---|---|---|---|---|
| IR199 | Very virulent | Iran | EU082024 | This paper |
| IR299 | Very virulent | Iran | EU082025 | This paper |
| IR398 | Very virulent | Iran | AY772164 | This paper |
| K406/89 | Very virulent | Egypt | AF159218 | Zierenberg *et al*. (2000) |
| K357/88 | Very virulent | Germany | AF159216 | Zierenberg *et al*. (2000) |
| UK661 | Very virulent | Britain | X92760 | Brown *et al*. (1994) |
| OKYM | Very virulent | Japan | D49706 | Yamaguchi *et al*. (1996) |
| UPM97/61 | Very virulent | Malaysia | AF247006 | Chong *et al*. (2001) |
| HK46 | Very virulent | Hong Kong | AF092943 | Lim *et al*. (1999) |
| D6948 | Very virulent | Holland | AF240686 | Boot *et al*. (2000) |
| 849VB | Very virulent | Belgium | X95883 | van den Berg *et al*. (1996) |
| STC | Classical | USA | D00499 | Kibenge *et al*. (1990) |
| 52/70 | Classical | Britain | D00869 | Bayliss *et al*. (1990) |
| Kal2001 | Classical | Egypt | AY311479 | Unpublished |
| T2/China | Classical | China | AF312371 | Liu *et al*. (2001) |
| 002-73 | Classical | Australia | X03993 | Hudson *et al*. (1986) |
| Variant E | Variant | USA | D10065 | Heine *et al*. (1991) |
| GLS | Variant | USA | M97346 | Vakharia *et al*. (1994) |
| PBG98 | Attenuated | Britain | D00868 | Bayliss *et al*. (1990) |
| P2 | Attenuated | Germany | X84034 | Mundt *et al*. (1995) |
| D78 | Attenuated | Holland | Y14962 | Unpublished |
| CEF94 | Attenuated | Holland | AF194428 | Boot *et al*. (1999) |
| 23/82 | Avirulent | Britain | Z21971 | Schnitzler *et al*. (1993) |
| OH | Avirulent | USA | M66722 | Kibenge *et al*. (1991) |

## Results

*Clinical signs and gross lesions*

The isolates IR199 and IR229 caused about 60% mortality in 33- and 55-day-old non-vaccinated commercial layer chickens. The infected chickens showed severe depression, weakness, whitish diarrhea, anorexia, ruffled feather, and prostrations. The bursa of Fabricius was hemorrhagic and enlarged, while the kidneys were swollen and the urates were present in the ureters. Some chickens displayed the symptoms of moderate to severe hemorrhage in the thigh and pectoral muscles and at the junction of proventriculus and gizzard. The isolate IR398 caused 25% mortality after infection in 45-day-old layer chickens vaccinated against IBDV at 12, 22, and 32 days of age. The infected chicken showed the same symptoms as chicken infected with isolates IR199 and IR229. The bursa of Fabricius was edematous with gelatinous transudate covering the surface and areas of petechial hemorrhage present in the mucosa. Hemorrhage was also observed at the thigh and pectoral muscles.

*Histopathology and transmission electron microscopy*

IR199, IR299 and IR389 isolates: mild to moderate hemorrhages with follicular cells degeneration, necrosis and cysts formation, and infiltration of inflammatory cells especially heterophils and macrophages in the interstitial connective tissues were observed in the bursa of Fabricius (Fig. 1).

The transmission electron microscopy detected IBDV particles about 60 nm in diameter as a closely packed array in the cytoplasm of lymphoid cells coming from bursa of Fabricius (Fig. 2).

*Immunoperoxidase staining and agar gel precipitation test*

Clusters of the IBDV antigens were visualized as brown to golden stain in the cytoplasm of lymphoid and necrotic cells in the bursal follicles infected with the IBDV isolates by immunoperoxidase staining (Fig. 3).

The precipitation lines in AGPT were observed between the positive antibody against known IBDV and the tested IBDV isolates (data not shown).

*Infection of embryonated chicken eggs and CEF cells*

IBDV isolates IR199 and IR299 caused facial edema and hemorrhage in the embryos with mortality of 100% at day 5 p.i. The CAM was slightly thickened, edematous, and hemorrhagic. The IR398 IBDV isolate caused thickened, hemorrhagic CAM at the puncture mark and the infected embryos showed severe hemorrhage with facial edema and 100% mortality at day 3 p.i.

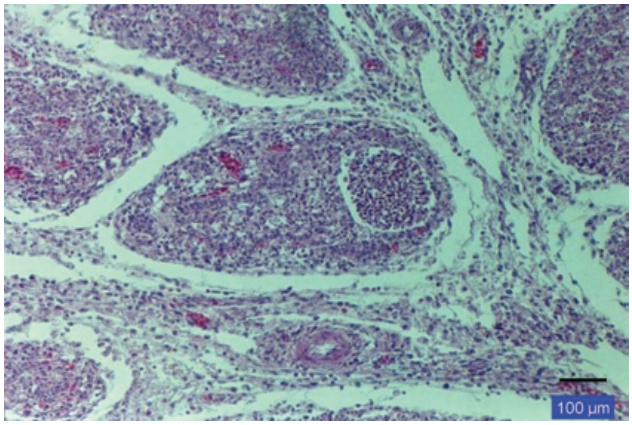


**Fig. 1**

**HE staining of the IR398-infected bursa of Fabricius**

Follicular necrosis, infiltration of inflammatory cells and enlargement of the interstitial space. Magnification 100x.

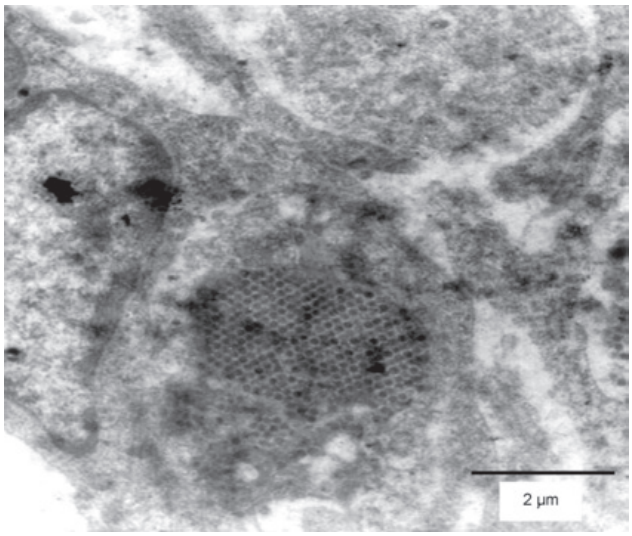


**Fig. 2**

**Transmission electron microscopy of the IR398-infected bursa of Fabricius**

IBDV particles seen in the cytoplasm of the lymphocyte. Magnification 30,000x.

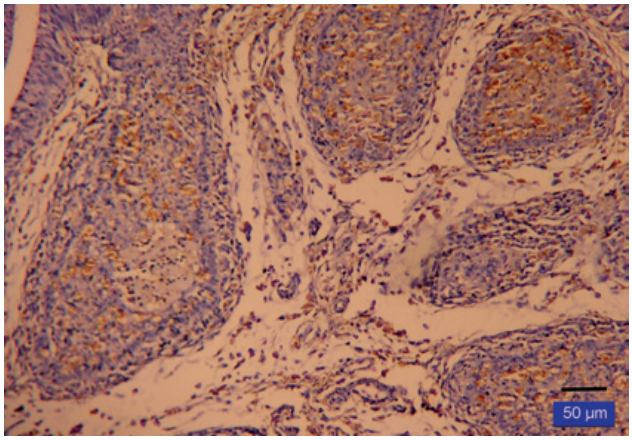


**Fig. 3**

**Immunoperoxidase staining of the IR398-infected bursa of Fabricius**

IBDV antigens seen as brown to golden stains in the cytoplasm of the lymphoid and necrotic cells. Magnification 200x.

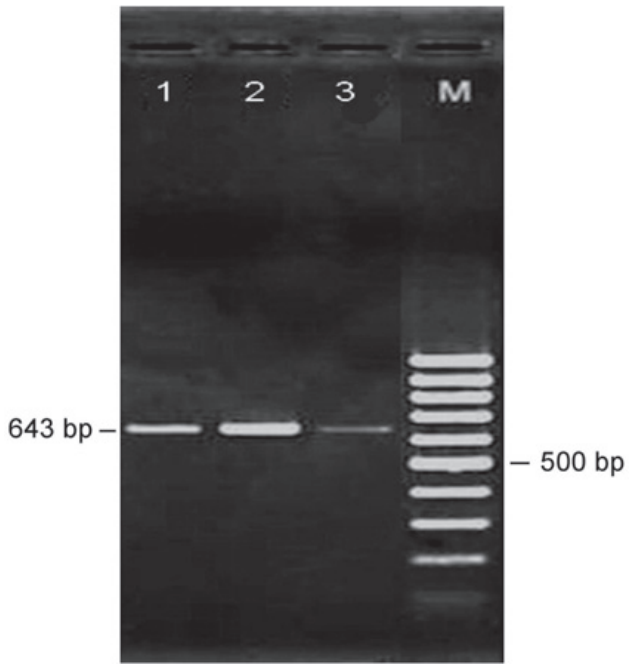


**Fig. 4**

**Agarose gel electrophoresis of RT-PCR products of IBDV isolates**

IR199 (lane 1), IR398 (lane 2), IR299 (lane 3), DNA size marker 100 bp ladder (lane M).

None of the three isolates caused CPE after 4 passages in the CEF cells.

*Amplification of VP2 hypervariable region of the IBDV isolates by RT-PCR*

The hypervariable region of VP2 gene of the three IBDV isolates; IR398, IR199, and IR299 were successfully amplified and showed identical mobility after electrophoresis on a 1% agarose gel. The PCR amplification using primer pairs P1-P2 generated a specific DNA band of 643 bp (Fig. 4).

*Sequence and phylogenetic analysis of the IBDV isolates*

The IBDV isolates IR199, IR299, and IR398 showed 99.3 to 99.8% similarities due to the nucleotide changes at positions (C951A) and (G992A). These isolates showed a similarity of 96.3 to 99.8% with very virulent strains (K406/89, K357/88, UK661, OKYM, UPM97/61, HK46, D6948, and 849VB), 86.9 to 95.6% with the classical strains (STC, 52/70, Kal2001, T2/China, and 002-73), 93.2 to 93.7% with the variant strains (variant E and GLS), 92.8 to 93.9% with the vaccine strains (PBG98, P2, D78, and CEF94), and 71.9 to 72.3% with the avirulent strains (23/82 and OH).

The deduced amino acid sequences were also aligned and compared with other available IBDV strains (Table 1). The three Iranian IBDV isolates showed 100% similarity with each other, 99.0 to 100% with very virulent strains, 90.4 to 97.1% with classical strains, 91.3 to 92.3% with variant strains, 90.4 to 92.3% with vaccine strains, and 5.8% with avirulent strains.

The nucleotide sequences of the three Iranian IBDV isolates reported in this paper have been deposited in the GenBank nucleotide database under Acc. Nos. EU082024, EU082025, AY772164 for isolates IR199, IR299, IR398, respectively.

Phylogenetic analysis showed that the isolates IR199, IR299, and IR398 were included in a subgroup of vvIBDV strains (data not shown).

*RT-PCR-RFLP*

All tested IBDV isolates contained BspMI, BstNI, MboI, SspI, StyI, TaqI sites, but lacked SacI site. In addition, the IR199 IBDV isolate contained NaeI site, while isolates IR299 and IR398 lacked it.

## Discussion

This study demonstrated that according to the clinical signs, histological lesions, and identification of the virus using conventional techniques, the three Iranian IBDV isolates (IR199, IR299, and IR398) were very similar to those of vvIBDV strains belonging to the serotype 1 (Hair-Bejo *et al*., 2004; Tanimura *et al*., 1995; Inoue *et al*., 1994; Hair-Bejo, 1993; van den Berg *et al*., 1991). This assumption was further confirmed using the molecular characterization of the isolates.

The common lesions observed in the infected chicken was hemorrhage in the bursa of Fabricius, in the thigh and pectoral muscles, and quite frequently in the mucosa at the junction of the proventriculus and gizzard. The cause of the hemorrhage was not fully understood, although it has been suggested that it could be caused by several factors including depletion of a hemolytic component (Skeeles *et al*., 1979), depletion in some of clotting factors (Skeeles *et al*., 1980), and destruction of thrombocytes (Payne, 1994). The cause of death in the acute vvIBDV infection can be caused by a severe circulatory failure resulting from excessive and severe hemorrhage. A severe dehydration due to the diarrhea and reduction in a water intake could also lead to a circulatory failure and death (Hair-Bejo, 1993).

Generally, a similar signs and gross lesions were recorded in the embryos infected with the virus obtained either from the IBDV-vaccinated or non-vaccinated flocks (Lukert and Saif, 1997; Takase *et al*., 1996). None of the tested isolates were able to multiply in CEF cells up to 4$^{th}$ passage. It was reported that vvIBDV could not grow in cell culture without adaptation, what indicated that the virus had to undergo many passages in CAM prior to the inoculation into the cell culture (Yamaguchi *et al*., 1996).

Occurrence of IBD in IBDV-vaccinated flocks could result from several factors. Live attenuated vaccines are generally derived from serial passages of caIBDV or vaIBDV strains in embryonated eggs or in primary cell cultures such as CEF cells (Lim *et al*., 1999). The attenuated strains derived from the classical and variant strains usually protect birds from infections by their parental strains (Lim *et al*., 1999). However, many of these vaccines could not induce 100% protections against the vvIBDV strains. The vvIBDV strains could break through higher level of maternal antibody induced by the current caIBDV or vaIBDV vaccine strains (Tsukamoto *et al*., 1995a). Improper administration of vaccine may lead to a failure of antibody production against the virus. Other factors like individual difference of the host, biological variation of the seed vaccine, inadequate vaccine, delayed immunization, immunosuppressive bird, and presence of maternal antibodies may play an important role in the vaccination failure (Tizard, 2000).

The nucleotide and amino acid sequences of the hypervariable region revealed that all the Iranian IBDV isolates were very similar to vvIBDV strains (K406/89, K357/88, UK661, OKYM, HK46, D6948, 849VB and UPM97/61). The three Iranian IBDV isolates shared the same amino acids marker for vvIBDV strain at positions A222,

I242, Q253, I256, A284, and I294. These amino acid residues are unique for the vvIBDV strains (Rudd *et al*., 2002; Cao *et al*., 1998; Brown *et al*., 1994). The serine rich heptapeptide (326SWSASGS332) region located immediately downstream of the second hydrophilic region was believed to be involved in the virulence of IBDV strains (Vakharia *et al*., 1994; Heine *et al*., 1991). However, this region was conserved in the Iranian IBDV isolates.

The RFLP pattern of all IBDV Iranian isolates were obtained with SspI and TaqI enzymes similar to the vvIBDV isolates coming from Japan (Lin *et al*., 1993). Moreover, RFLP of several vvIBDV strains isolated in Japan, China, Turkey, Malaysia, and Europe had conserved SspI, TaqI, and StyI sites at the hypervariable region (Chong *et al*., 2001; Hoque *et al*., 2001; Cao *et al*., 1998; Ture *et al*., 1998; Lin *et al*., 1993). It was reported that vvIBDV strains had restriction site for BspMI related to the amino acid substitution (P222A) in the major hydrophilic peak A of the hypervariable region (Zierenberg *et al*., 2001). Similarly, the three IBDV Iranian isolates had this restriction enzyme site. In addition, some of the vvIBDV strains contained BstNI negative site in their sequences. For instance, Nigerian vvIBDV (N4, N6, N7, N8, N9, N10, N11, N13, and N14), Egyptian vvIBDV (K406/89), and German vvIBDV (K357/88) were negative for BstNI site (Zierenberg *et al*., 2000). However, the Turkish, Dutch, and Taiwanese vvIBDV strains comprised this site (Ture *et al*., 1998). Similarly, the three Iranian isolates showed this restriction site in their sequence.

In conclusion, the three Iranian IBDV isolates were categorized as vvIBDV strains belonging to the serotype 1. We confirmed that three isolates have a close relationship with vvIBDV isolates originated from Europe, Japan, and China.

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