

Rapid differentiation between very virulent and classical infectious bursal disease viruses isolated in Iran by RT-PCR/REA

Razmyar, J., Peighambari, S.M.*

Department of Clinical Sciences, Faculty of Veterinary Medicine, University of Tehran, Tehran-Iran.

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Abstract: This study was conducted to characterize infectious bursal disease virus (IBDV) isolates collected from different parts of Iran during 2005-2006. Pooled bursal samples from 49 broiler and layer pullet flocks suspected to IBD infection were collected and processed. A reverse transcriptase-polymerase chain reaction (RT PCR) procedure was used to amplify a VP2 gene fragment (743 bp) from IBDV field isolates. Amplified VP2 fragments were further characterized by two restriction enzymes, *Bsp*MI and *Sac*I. From 49 field samples, 37 (75.5%) samples were IBDV positive by RT PCR. Digestion with two restriction enzymes, *Bsp*MI and *Sac*I, showed patterns compatible with very virulent IBDV and classical IBDV strains in 34 (91.9%) and 3 (8.1%) IBDV-positive samples, respectively. The restriction enzyme analysis of this study was comparable to that of other isolates and reference strains with available nucleotide sequence data in the GenBank. The procedure followed in this study is a useful method to rapidly differentiate the very virulent IBDV and classical IBDV isolates.

Key words: infectious bursal disease, vvIBDV, RT-PCR, broiler, Iran.

Introduction

Infectious bursal disease (IBD) is a highly contagious disease of young chickens (Lukert and Saif, 2003). Chickens are most susceptible to clinical infection from 3 to 6 weeks of age. Lymphoid tissue, in particular bursa of Fabricius, is the primary target of IBD virus (IBDV). Subclinical infection may occur in chickens less than 2 weeks of age leading usually to immunosuppression (Sharma *et al.*, 2000).

Infectious bursal disease virus is a member of the family Birnaviridae. The genome of the virus consists of two double-stranded RNA segments and encodes several proteins including: VP1, VP2, VP3, VP4, and VP5. VP2 is considered the major immunogenic protein of the virus, containing at least two neutralizing sites. It also contains sites that are involved in determining the virulence of the virus (Fahey *et al.*, 1991; Müller *et al.*, 2003). Two distinct

serotypes of IBDV are known, serotypes 1 and 2. The first one is the pathogenic type causing IBD in chickens. Serotype 2 produces neither disease nor immunity against pathogenic strains of the serotype 1 (Lukert and Saif, 2003; Müller *et al.*, 2003). Virus neutralization test is used to differentiate between two serotypes. Pathogenic serotype 1 IBDV field isolates can be grouped into classical virulent, antigenic variant, and very virulent (vv) isolates (Snyder, 1990; Brown *et al.*, 1994; Zierenberg *et al.*, 2000).

Very virulent (vv) IBDVs were initially reported in Europe in the late 1980s (Chettle *et al.*, 1989; van den Berg *et al.*, 1991) then were distributed around the world except the North America and Australia (Sapats and Ignjatovic, 2000; van den Berg, 2000). The vvIBDVs are antigenically very similar to the classical strains but have marked increase in virulence causing high mortality in infected flocks (Yamaguchi *et al.*, 1997; Eterradossi *et al.*, 1998; van

* Corresponding author's email: mpeigham@ut.ac.ir, Tel: 021- 61117150, Fax: 021-66933222



den Berg, 2000).

Many studies using reverse transcription-polymerase chain reaction/ restriction fragment length polymorphism (RT-PCR/RFLP) demonstrated various molecular groups in US isolates and different countries, indicating a wide range genetic diversity among IBDV field isolates (Jackwood and Sommer, 1997, 1998, 1999; Ture *et al.*, 1998; Jackwood *et al.*, 2001; Sapats and Ignjatovic, 2002). Differentiation between classical virulent IBDV and vvIBDV is very important to poultry industry with regard to choosing the appropriate vaccination programs. Serologic tests are laborious, time-consuming and more or less sensitive, although an antigen capture-enzyme linked immunosorbent assay (AC-ELISA) using a panel of monoclonal antibodies has been used to differentiate between serotype 1 IBDV strains (Etteradossi *et al.*, 1999). A variety of restriction enzymes have been used for differentiation of IBDVs by researchers. The application of RT/PCR combined with two restriction enzymes was able to rapidly differentiate between classical and vvIBDVs standard strains (Zierenberg *et al.*, 2001).

In our laboratory, we have focused on characterization of Iranian field IBDVs isolated from infected flocks in recent years. In the work presented here, we were able to rapidly differentiate between classical virulent IBDVs and vvIBDVs isolated in Iran using two restriction enzymes as has been reported previously.

Materials and Methods

Samples: Cases of bursae from chickens suffering acute phase of IBD, atrophied bursae from chickens in flocks prior to administration of IBD commercial vaccines, and unusual hypertrophic bursae found at necropsy were collected from 49 broiler and layer pullet flocks referred to the poultry clinic from different parts of the country. The bursae of each flock (1-5 samples) were pooled after being processed as described below and finally 49 samples were chosen and included in this study. From each bird, the entire bursa was aseptically removed and placed in a sterile 50 ml Falcon tube and carried to

laboratory in cold conditions. In the laboratory, the samples were frozen or placed in tubes containing TNE buffer (1 mM EDTA, 100 mM NaCl, 10 mM Tris HCl, pH 8.0). Each bursa was divided in two halves. One half was cut into fine pieces and the volume increased 5 times by TNE buffer (pH 8.0). Penicillin and Streptomycin, 10000 IU and 10000 mg/ml, respectively, were added. Remaining half was stored at -70°C . The bursal homogenates were prepared as described previously (Rosenberger *et al.*, 1998). Briefly, fine pieces of bursa in TNE buffer were vortexed vigorously, frozen and thawed 3 times, and centrifuged at $14000 \times g$ in 4°C for 20 min. The supernatant was removed, transferred to new 1.5 ml RNase and DNase free microtubes, and stored in -70°C until further use.

RNA extraction: A commercial kit (RNX plus kit, Cinnagen, Iran) was used to extract the total RNA from the bursal samples homogenized in TNE buffer as recommended by manufacturer. Briefly, 200 μl of homogenate suspension was added to 1 ml RNX plus (Guanidine salt base) in a 1.5 ml RNase and DNase free microtube, vortexed vigorously, and kept at room temperature (RT) for 10 min. Then, 200 μl Chloroform:Isoamyl alcohol (24:1) was added to the suspension, mixed gently, and centrifuged at $14000 \times g$ at 4°C for 15 min. The supernatant was removed, transferred to a new microtube, mixed gently with equal volume of the Isopropanol by ten rounds of up and down movements, stored at -20°C for 20 min, and centrifuged as above. The supernatant discarded and the pellet was washed in 1 ml 70% ethanol by centrifugation at $7000 \times g$ at 4°C for 5 min. The supernatant discarded and the pellet was washed once more. The final pellet was resolved in 30 μl DEPC-treated water, incubated at 55°C for 10 min, and stored at -70°C until further use.

Reverse transcription: A commercial cDNA synthesis kit (Fermentas Life Science, Germany) was used to make cDNA. The procedure was as instructed by manufacturer with some modifications. Briefly, 5 μl RNA extract, 2.5 μl (10 pmol/ μl) reverse primer J2 (5'-CCG GAT TAT GTC TTT GAA GCC-3) and DEPC-treated water up to 10 μl were boiled 3 min and then cooled on ice immediately. Four μl 5 x RT buffer



Table 1: Published IBDV strains used in this study for comparison.

Virus strain	Pathotype	Year of isolation	Geographic origin	GenBank accession no.	Reference
F 52/70	Classical virulent	1970	UK	D00869	Bygrave and Faragher (1970)
P2	Classical Cell culture adapted	1973	Germany	X84034	Mundt and Müller (1995)
CU-1	Classical Cell culture adapted	1976	Germany	X16107	Nick <i>et al</i> (1976)
D78	Classical Attenuated	1978	Netherlands	AF499929	-
UK661	Very Virulent	1989	UK	X92760	Brown <i>et al</i> (1994)
GZ96	Very Virulent	Unknown	China	AY598356	Yu <i>et al</i> (2004)
HK46	Very Virulent	1994	Honk Kong	AF092171	Cao <i>et al</i> (1998)
BD 3/99	Very Virulent	1999	Bangladesh	AF362776	Islam <i>et al</i> (2001)

plus 2 µl 10 mM dNTP mix, 1 µl MMLV-RT enzyme (200 u/µl), 0.5 µl RNasin (20 u/µl), 2.5 µl DEPC-treated water were added to the previous mixture, incubated for 1 hour at 42°C and 5 min at 80°C, cooled on ice, and stored at -20°C.

Amplification: To amplify a 743 bp (734-1478) of VP2 hypervariable region (Bayliss *et al.*, 1990; Mundt and Müller, 1995), we used J1 (5'-GGC CCA GAG TCT ACA CCA TAA C-3') and J2 (5'-CCG GAT TAT GTC TTT GAA GCC-3') primers (Sapats and Ignjatovic, 2002). The amplification was carried out in 50 µl reaction volume consisting of 5 µl 10x PCR buffer, 1 µl 10 mM dNTPs, 1.5 µl of each primer (10 pmol/µl), 0.25 µl *Taq* DNA polymerase (5U/µl), 1.5 µl 50 mM MgCl₂, 14.25 µl of dH₂O, and 25 µl cDNA dilution (5 µl cDNA + 20 µl dH₂O) and was programmed in a thermocycler (Gradient Mastercycler, Eppendorf, Germany) as follows: 95°C for 3 min followed by 37 cycles of 94°C for 20 sec, 55°C for 20 sec, 72°C for 40 sec, and a final extension at 72°C for 5 min. In all PCR reaction sets, negative controls (ddH₂O instead of cDNA) and positive control (D78 vaccine strain) were included. The amplification products were detected by gel electrophoresis (Apelex, France) in 1.5% agarose gel in TAE buffer. Gels were run for 1.5 hr at 80 V, stained with ethidium bromide (0.5 µg/ml), exposed to

ultraviolet light and photographed (Visi-Doc-It system, UVP, UK). A commercial 100-bp DNA ladder (Cinnagen) was used as molecular-weight marker in each gel running.

Restriction analysis: The amplification product was purified using a PCR purification kit (Roche Molecular Biochemicals, Germany) and digested with different restriction enzymes (*Bsp*MI and *Sac*I) according to manufacturer's instructions (Fermentas Life Science) as follow: 10 µl pure PCR product, 2 µl RE buffer, 1 µl RE, and 7 µl dH₂O were mixed, incubated at 37°C for 3 hours, and were run in 1.5% agarose gel at 7 v/cm for 100 min.

Results

RT-PCR: Thirty-seven out of 49 bursal-pooled samples were positive in RT-PCR and yielded identical bands (Figure 1). All reactions duplicated.

Restriction enzyme analysis: The products digested with two restriction enzymes: *Bsp*MI, and *Sac*I and the resulted patterns were compared with the restriction sites and patterns of standard strains based on previously published sequence data in the GenBank (Table 1) in which *Sac*I could only cut classical strains and *Bsp*MI could only cut very virulent strains. Using *Bsp*MI and *Sac*I enzymes, 34



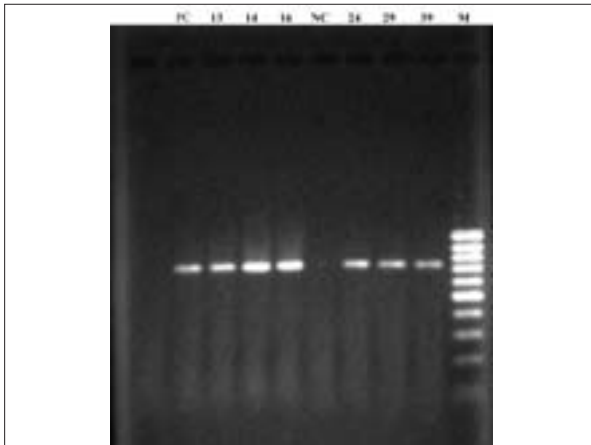


Fig. 1- Electrophoresis of RT-PCR amplified 743 bp products of the VP2 gene on 1.5% agarose gel (7 v/cm, 80 min) and stained with ethidium bromide. Amplified 743 bp product of isolates 13, 14, 16, 24, 29, and 39 are shown. Lanes PC, NC and M indicate positive control (D78 vaccine strain), negative control and 100 bp ladder, respectively.

out of 37 isolates (91.9%) produced the same expected pattern as reference vvIBDV strain, UK661. Three out of 37 (8.1%) yielded expected pattern similar to reference classical IBDV strains, F52/70 and P2. Figure 2 illustrates the electrophoresis of the RT-PCR products after digestion with two restriction enzymes for 3 isolates with classical pattern and 10 isolates with vvIBDV pattern.

Discussion

In Iran, infectious bursal disease virus (IBDV) causing low mortality in a broiler farm was first isolated in 1981 (Aghakhan *et al.*, 1994). Later, they isolated an IBDV that caused 75% and 25% mortality in layer pullet and broiler farms, respectively (Aghakhan *et al.*, 1996). Recent reports have shown the presence of vvIBDV in Iran (Ghoreishi *et al.*, 2003; Hosseini *et al.*, 2004; Shamsara *et al.*, 2006). In the GenBank, there are some reports of vvIBDV sequences belonging to Iran but they have not been published yet.

Hyper variable region VP2 gene of IBDVs is the most interested part of this gene for many researchers. VP2 protein contains serotype-specific neutralization epitopes and also pathotype-specific monoclonal reacting epitopes (Müller *et al.*, 2003). The full sequence of segment A of three IBDV strains

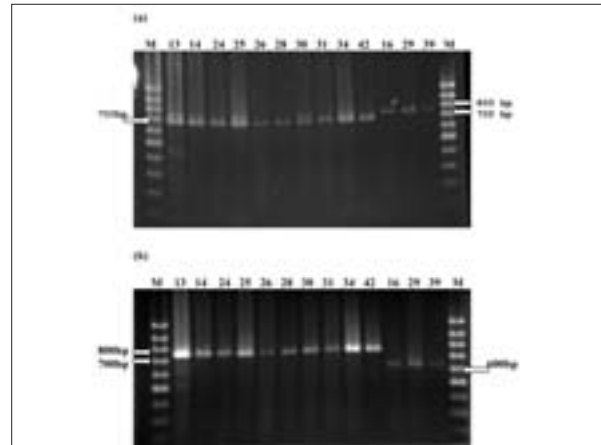


Fig. 2- Electrophoresis of the RT-PCR 743 bp products after digestion with the restriction enzymes *BspMI* (a) and *SacI* (b). Isolates 13, 14, 24, 25, 26, 28, 30, 31, 34, and 42 demonstrate vvIBDV pattern and isolates 16, 29, and 39 show classical pattern. Lane M indicate 100 bp ladder.

published previously (Bayliss *et al.*, 1990) has made it possible to generate a variety primers to amplify fragments on VP2 region for the identification of IBDV viruses by RT-PCR technique. In the present study, we amplified a 743 bp generated from nucleotide position 734 to 1478 of VP2 hypervariable region (Sapats and Ignjatovic, 2002). A majority of bursal samples (37/49=75.5%) were positive in RT-PCR and yielded identical bands that meant neither deletions nor insertions. All samples from atrophic bursae were negative.

There are several reports in which RT-PCR/RE or RT-PCR/RFLP assays have been used to differentiate IBDV isolates and classify them into different molecular groups (Jackwood and Jackwood, 1997; Jackwood and Sommer, 1998; Ture *et al.*, 1998; Ikuta *et al.*, 2001; Banda *et al.*, 2002). Most of the works have focused on VP2 gene but other genes have also been investigated (Tiwari *et al.*, 2003; Gomes *et al.*, 2005). Because of lacking a universal system to choose a unique length of gene sequence and identical REs, it seems difficult to compare results of these reports. Jackwood and co-workers in various studies examined RFLP patterns of a 743-bp RT-PCR amplified fragment of VP2 gene to analyze several vaccine strains and IBDV detected in bursa tissue of commercially reared chickens (Jackwood and Sommer, 1998; 1999; Jackwood *et al.*, 2001). Six molecular groups were found among



the vaccine strains. Field IBDVs demonstrated greater genetic diversity and fell into 22 molecular groups. Further studies revealed that there were additional distinct molecular groups among IBDVs collected from 22 countries, indicating a wide range genetic diversity among IBDV field isolates. They could associate some molecular groups to classical virulent IBDVs, vvIBDVs or variants but many molecular groups did not match with known phenotypic patterns (Jackwood and Sommer, 1998, 1999; Ture *et al.*, 1998; Jackwood *et al.*, 2001).

Australian researchers (Sapats and Ignjatovic, 2002) used also a 743 bp RT-PCR amplified fragment of VP2 gene and found 12 distinct molecular groups among Australian IBDVs in RFLP assay. They used *MboI*, *BstNI* and *SspI* to obtain RFLP patterns of Australian isolates and for comparisons used CS89 strain as a vv IBDV, 52/70 as a classical strain, and variant E strain. Their results showed that only *MboI* was able to produce distinct patterns between these three selected strains. The variant E and CS89 strains with *BstNI*, and 52/70 and variant E strains with *SspI* demonstrated identical digestion patterns. Using this technique, they were able to differentiate all Australian IBDV strains from overseas strains, including vvIBDV strains.

Zierenberg *et al* (2001) used *SacI* and *BspMI* in a restriction enzyme analysis to compare serotype 1 (classical and vvIBDV) and serotype 2 in which *SacI* cut only classical IBDVs and *BspMI* cleaved vvIBDVs except a West African strain, 88180, which lacks the *BspMI* restriction site. Restriction sites for both enzymes, *SacI* and *BspMI*, were not present in RT-PCR products obtained with serotype 2 IBDV strains. Neither of two enzymes, therefore, digested serotype 2. Toroghi *et al* (2003) also used *SacI*, *HhaI*, *SspI*, and *StuI* to differentiate vaccinal IBD strains, classical and vvIBDV field isolates. They found out that only classical virulent and vaccinal strains had restriction site for *SacI*.

In the present work, 34 out of 37 IBDV positive samples (91.9%) showed patterns compatible with vvIBDVs using the restriction enzymes *SacI* and *BspMI* (Figure 2). Three isolates (16, 29, and 39), however, demonstrated patterns compatible with

classical strains which were cleaved with *SacI* but kept intact after several hours reaction with *BspMI*. These findings are clearly compatible with the results reported by Zierenberg *et al.*, (2001). Some of the isolates used in this study were chosen for sequencing and the results (data not shown here) which have been posted in the GenBank completely confirm our restriction enzyme analysis.

The *BspMI* cleavage site found in vvIBDV strains is correlated with the amino acid position 222 (proline to alanine) in the major hydrophilic peak A of the VP2 hypervariable region. This amino acid exchange is conserved in all published typical vvIBDVs (Brown *et al.*, 1994; Cao *et al.*, 1998; Eterradosi *et al.*, 1999; Zierenberg *et al.*, 2000; Kataria *et al.*, 2001). Some isolates, such as the West African strain 88180, may lack the cleavage sites for restriction enzymes due to silent mutations. It is clear that in case of any unexpected results in RE analysis or for the further characterization of possible new IBDV strains, direct sequencing of the RT-PCR product is required.

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تفکیک سریع بین ویروس‌های کلاسیک و فوق حاد بیماری بوریس عفونی جدا شده در ایران به وسیله RT-PCR/REA

جمشید رزم‌پار سیدمصطفی پیغمبری*

گروه علوم در مانگامی دانشکده دامپزشکی دانشگاه تهران، تهران-ایران.

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چکیده

این مطالعه با هدف بررسی خصوصیات جدایه‌های ویروسی بیماری بوریس عفونی (IBDV) که در طی سال‌های ۲۰۰۵-۲۰۰۶ از نقاط مختلف ایران جمع‌آوری شده بودند، صورت گرفت. نمونه‌های بوریس مخلوط شده مربوط به هر کدام یک روز از ۴۹ گله گوشتی و پولت تخمگذار مشکوک به بیماری ویروس عفونی جمع‌آوری و فرآوری شدند. تکنیک RT-PCR برای تکثیر قطعه‌ای به اندازه ۷۴۳ جفت باز از ژن VP2 جدایه‌های IBDV از مزرعه مورد استفاده قرار گرفت و قطعات تکثیر شده توسط دو آنزیم محدود کننده، *BspMI* و *SacI*، هضم گردیدند. از ۴۹ نمونه جدا شده مزرعه، ۳۷ (۷۵/۵ درصد) نمونه در RT-PCR از نظر IBDV مثبت بودند. هضم با دو آنزیم محدود کننده، الگوهای مشابه سویه‌های کلاسیک و فوق حاد IBDV را به ترتیب در ۳ (۸/۱ درصد) و ۳۴ (۹۱/۹ درصد) نمونه‌های مثبت نشان دادند. تجزیه و تحلیل یافته‌های این مطالعه با آنزیم‌های محدود کننده اندونوکلائاز با یافته‌های موجود در مورد جدایه‌ها و سویه‌های رفرانس که اطلاعات توالی نوکلئوتیدی آنها در بانک ژن موجود است، همخوانی داشت. روش مورد استفاده در این مطالعه، روشی مفید برای تفکیک سریع بین جدایه‌های کلاسیک و فوق حاد IBDV می‌باشد.

واژه‌های کلیدی: بیماری بوریس عفونی، ایران، جوجه گوشتی، IBDV، RT-PCR.

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