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

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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, BspMI and SacI. From 49 field samples, 37 (75.5%) samples were IBDV positive by RT PCR. Digestion with two restriction enzymes, BspMI and SacI, 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 vvIBDVVs are antigenitically 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
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 (Eterradossi 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 x g in 4°C for 20 min. The supernatant was removed, transferred to 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 (Guadrine 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 x 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 x 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
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 42ºC and 5 min at 80ºC, cooled on ice, and stored at -20ºC.

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 (5 U/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, Eppendorff, Germany) as follows: 95ºC for 3 min followed by 37 cycles of 94ºC for 20 sec, 55ºC for 20 sec, 72ºC for 40 sec, and a final extension at 72ºC 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 (BspMI and SacI) 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 37ºC 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: BspMI, and SacI 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 SacI could only cut classical strains and BspMI could only cut very virulent strains. Using BspMI and SacI enzymes, 34
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 vvIBDVs 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 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 IBD 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 vvIBDV 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; Eterradossi 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.

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

چکیده

این مطالعه با هدف بررسی خصوصیات جدایی‌های ویروس بیماری بورس عفونی (IBDV) که در طی سال‌های 2009-2010 از نقاط مختلف ایران جمع‌آوری شدند، صورت گرفت. نمونه‌های بورس مخلوط شده مربوط به هر کدام یک بک روز از 49 گله گوشته و پایه تخم‌گذار مشکوک به بیماری IBDV ویروس عفونی جمع‌آوری و قرایر آن‌ها شدند. تکنیک RT-PCR برای تکثیر قطعه‌های مربوط به آن‌ها جدایی‌های 2VP بلافاصله از آن‌ها برای بررسی و نتایج مورد استفاده قرار گرفت. نتایج نشان داد که گروه تکثیر IBDV مثبت بودند. همچنین با تغییر جفت‌شدن، این نتایج با بلندی‌های سوابق هم‌پیوسته کلاسیک و فوق حاد را به‌طور تشویق‌آمیز در مورد جدایی‌های IBDV انجام دادند. یک نتیجه کلیدی این مطالعه روش‌های مقیاس بین جدایی‌های کلاسیک و فوق حاد IBDV را تکثیر می‌نماید.

RT-PCR/REA

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