Restriction fragment length polymorphism analysis of isolates of infectious bursal disease viruses from Turkey

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ABSTRACT: Infectious bursal disease Virus (IBDV) specific reverse transcriptase/polymerase chain recation (RT/PCR) positive 40 broiler bursa fabricius samples obtained from a commercially reared flock were investigated for genetic diversity by PCR-RFLP assay. The assay amplifies a 743 bp fragment of the IBDV VP2 gene. The RFLP profiles of 40 of these positive samples were determined using the enzyme *MboI*. Most of the viruses had the same RFLP with the *MboI* enzyme. RFLP analysis of the isolates produced two different band profiles. The results of this study showed that little genetic heterogeneity exists among IBDV strains in a infected flock.

Keywords: IBDV; RT/PCR; commercially reared chickens

For many years, infectious bursal disease (IBD) (Gumboro disease) has constituted a serious problem for the poultry industry (van den Berg, 2000). Different antigenic and pathogenic types of this virus have been identified (Jackwood and Nielsen, 1997). Because antigenic typing of IBDV strains using cross virus neutralization requires adaptation of the virus to grow in cell culture and production of antiserum to the new strain, new methods for characterization of these viruses were sought (Jackwood *et al.*, 2001). Monoclonal antibodies have also been used to characterize the antigenic differences among IBDV strains (Snyder *et al.*, 1988a). The antigen-capture enzyme linked immunosorbent assay (AC-ELISA) was used to differentiate IBDV strains (Snyder *et al.*, 1988b). These procedures are laborious, time consuming and more or less sensitive (Zierenberg *et al.*, 2001). It can be difficult to adapt some IBDV isolates to propagate in cell culture. These assays can detect the presence of IBDV in a sample but do not identify the serotype or antigenic subtype (Jackwood and Jackwood, 1994).

In recent years, the use of molecular techniques to detect genetic changes in IBDV has increased greatly (Wu *et al.*, 1992; Stram *et al.*, 1994; Jackwood and Jackwood, 1997; Akin *et al.*, 1998). The current technologies for typing IBDV include molecular methods that amplify specific segments of the IBDV genome by the reverse transcriptase-polymerase chain reaction (RT/PCR) followed by restriction fragment length polymorphism (RFLP) (Dybing and Jackwood, 1996; Jackwood and Nielsen, 1997; Jackwood and Sommer, 1997; Ture *et al.*, 1998). These assays examine nucleotide sequences of the VP2 gene responsible for encoding the major antigenic determinants of the virus (Schnitzler, 1993). The RT/PCR-RFLP assays appear to be more sensitive than other diagnostic assays including AC-ELISA. Using RT/PCR and RFLP, researches were able to characterize antigenic subtypes as well as differentiate pathogenic isolates from vaccine virus (Lin *et al.*, 1993; Liu *et al.*, 1994; Jackwood and Jackwood, 1994, 1997; Jackwood and Nielsen, 1997).

Different restriction enzymes have been used for RFLP of IBDV. The enzymes *BstNI* or *EcoRII*, *Sty1*, *DraI*, *SspI*, *SacI*, *Sau3AI* or *MboI*, *StuI*, *HaeIII*, *TaqI* and *BspMI* are all currently used in various combinations or alone (Jackwood and Jackwood, 1997; Jackwood and Nielsen, 1997; Dormitorio *et al.*, 1997; Jackwood and Sommer, 1998, 1999; Banda *et al.*, 2001).

The aim of this study was to investigate the genetic variation among IBDV isolates from commercially reared chickens in Elazig province located in the East of Turkey with the use of restriction enzyme *MboI* in RT/PCR-RFLP.

MATERIAL AND METHODS

Bursa samples

RT-PCR positive bursa samples were obtained from a commercially reared chicken flocks suspected of IBD. The samples were collected during slaughtering from an abattoir in Elazig province located in the East of Turkey. Each sample was from same chicken flock. A total of 40 IBDV positive bursa samples used to determine genetic diversity of IBDV's.

Tri Reagent method

Before use, bursae thawed, and a 50–100 mg tissue sample of each bursa was homogenized in 1 ml Tri Reagent™ solution (Sigma, Product No. T9424, Technical Bulletin MB-205) using a mini hand-homogenizer. This homogenate was used for RNA extraction.

Viral RNA from vaccine strain (live attenuated IBDV vaccine Hipragumboro-CH/80 used as positive control) and bursa tissue was extracted using Tri Reagent solution, according to manufacturer's instructions. The homogenate was stored for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes. Next, 0.2 ml chloroform was added to per 1 ml homogenate. The resulting mixture was kept at room temperature for 15 min and centrifuged at 13.000 × g for 15 minutes. After centrifugation, the aqueous phase was transferred to a new tube. RNA was precipitated from the aqueous phase by mixing with 0.5 ml isopropanol. The mixture was stored at room temperature for 10 min and centrifuged at 13.000 × g for 8 minutes. The supernatant was removed and the RNA pellet washed with 70% ethanol and centrifuged at 10.500 × g for 5 minutes. Finally, the pellet was dried for 3–5 min, resuspended in 50 μl of diethylpyrocarbonate (DEPC) treated sterile distilled water (DEPC-sdH$_2$O) and stored at –20°C until further use.

Primers

A primer pair designed by Jackwood and Sommer (1997) was used in this study. These primers designated 700-5' and 700-3' were designed to amplify a 743-bp fragment of the VP2 gene from 701 bp to 1 444 bp (Kibenge et al., 1990). The concentration of primers was 1.0 μM.

Reverse transcription

The RevertAid™ First Strand cDNA Synthesis kit (MBI, Fermentas, Lithuania) was used according to the manufacturer's instructions to produce cDNA from the RNA in these samples.

20 μl reaction mixture contained 2 μl extracted RNA sample (boiled for 5 minutes); 4 μl of 5× reaction buffer, 2 μl each of 10 mM dNTPs; 1 μl of Ribonuclease inhibitor (20 u/μl); 1 μl of random hexamer primer; 1 μl of RevertAid M-MuLV reverse transcriptase (200 u/μl). The reaction was allowed to proceed for 10 min at 25°C, followed by incubation at 42°C for 1 hour. The temperature was then raised to 70°C for 10 min, after which the sample was rapidly cooled to 4°C.

PCR

Five μl of the cDNA was added to 50 μl of PCR reaction mixture containing 2.5 μl of 25 mM MgCl$_2$; 5 μl of 10 × PCR buffer; 4 μl each of 10 mM dNTPs; 4 μl of primer (50 pM); 0.25 μl of Taq Polymerase and 23 μl distilled water. Amplification was carried out at 95°C for 2 min, 35 cycles at 95°C for 1 min, and 53°C for 1 min 30 s, 72°C for 1 min and final extension at 72°C for 7 minutes. A 10 μl sample of each reaction was analyzed on a 2.5% agarose gel containing 0.5 μg/ml ethidium bromide. The DNA fragments were visualized by UV illumination and photographed.

RT/PCR products were digested with the restriction enzyme *MboI*. The enzyme was used to generate RFLP patterns that distinguished viruses into molecular groups.

Ten microliter aliquots of RT/PCR reaction products were digested with 1 μl of the restriction enzyme (*MboI*; 10 U/μl) (Fermentas, Lithuania) and incubated for 2 h at 37°C. The *MboI* digested products were separated on 2.5% agarose gels and visualized with ethidium bromide staining. Sizes of the RFLP bands were determined by comparison with a 100 bp DNA ladder (Fermentas, Lithuania).

RESULTS

IBDV was detected in 40 out of a total of 56 bursa tissue samples from commercially reared chicken flocks. In the agarose gel electrophoresis of the RT/PCR-RFLP products digested with *MboI*, two
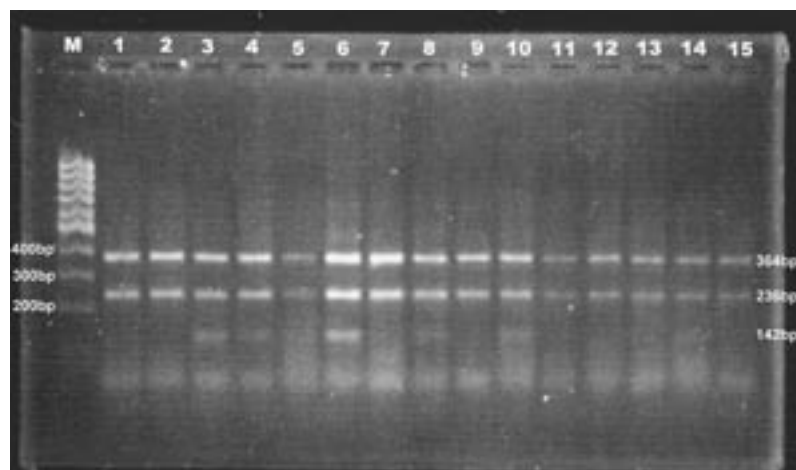
Figure 1. Restriction fragment length polymorphisms results of IBDV isolates; 1–15 = IBDV isolates, M = 100 bp DNA ladder

different band profiles were obtained (Figure 1). The one profile generated two distinct bands and another profile generated three bands. The bands in length 364 bp and 236 bp were obtained with each 40 samples. But the band in length 142 bp was obtained with only ten samples. Results of the RT/PCR-RFLP assay indicated that little genetic heterogeneity exists among strains of IBDV.

DISCUSSION

RT/PCR-RFLP has become a popular diagnostic assay for IBDV because it can be used to quickly detect these viruses (Jackwood *et al.*, 2001). To detect the genetic variations among IBDV isolates, the hypervariable region located in VP2 gene of IBDV was amplified by PCR (Liu *et al.*, 1994). In the present study, 40 viruses were detected by RT/PCR, and the RFLPs of the 743 bp VP2 fagment.

RFLP has successfully been applied in the hypervariable region of VP2 gene of IBDV. The RT/PCR-RFLP assay examines the 743 bp product for the presence and location of multiple *BstNI* or *MboI* restriction sites by comparing the sizes of the resulting restriction fragments (Jackwood and Sommer, 1997). Different enzymes have ben used for RFLP analysis of IBDV. Among them, *BstNI* or *MboI* appear to the provide best discrimination for IBDV isolates (Jackwood and Sommer, 1998). Jackwood and Sommer (1998) reported that fewer RFLP profiles were observed using the *BstNI* enzyme compared with the *MboI* enzyme. The *BstNI* enzyme recognizes a six-base sequence in the RT/PCR products compared with *MboI*, which recognizes a four base-sequence. Thus the probability of observing a new *BstNI* RFLP is lower compared with observing a new *MboI* RFLP because there is a lower probability of observing the six-base recognition sequence for *BstNI* in the 743 bp RT/PCR product compared with the four-base *MboI* recognition sequence (Jackwood and Sommer, 1998). For this reason, we used *MboI* for restriction analysis to investigate genetic diversity among IBDV isolates.

Some studies indicated that little genetic heterogeneity exists among domestic and international IBDV samples and vaccine strains tested using RT/PCR-RFLP (Jackwood and Sommer, 1997, 1998). In contrast to the conserved genetic heterogeneity among vaccine strains of IBDV in a study showed that a relatively large amount of genetic heterogeneity existed in field viruses from commercially reared chicken flocks (Jackwood and Sommer, 1998). All the isolates were successfully typed using RT/PCR-RFLP in this study. Two different RFLP's were detected among 40 IBDV isolates. Results of this study were in agreement with the reports that little genetic heterogeneity exists in domestic and international IBDV samples with RT/PCR-RFLP assay.

The results of this study indicated that little genetic heterogeneity exists among IBDV samples from commercially reared chickens. Our samples were taken from same chicken flock and this may due to the differences in the results of another studies. If strains were isolated from different chicken flocks, a larger heterogeneity could have been obtained in this study.

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