DIFFERENTIATION OF CLASSICAL AND VERY VIRULENT STRAINS/ISOLATES OF INFECTIOUS BURSAL DISEASE VIRUS BY REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION

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Summary. – Reverse transcription–polymerase chain reaction (RT-PCR) is a rapid method for identification and differentiation of viruses. It was used to differentiate very virulent from classical (field/vaccine) strains/isolates of Infectious bursal disease virus (IBDV). RT-PCR products of 552 bp were generated by amplification of variable region of VP2 gene in three field classical isolates, two vaccine strains and two very virulent isolates of IBDV. The PCR products were digested with *Sac*I, *Hha*I, *Ssp*I and *Stu*I. Digestion of the PCR products with *Sac*I and *Hha*I revealed the presence of a single restriction site in all the field classical isolates and vaccine strains, but no such a restriction site in very virulent strains. On the other hand digestion of these products with *Ssp*I and *Stu*I showed the presence of a single restriction site in very virulent strains but no such a restriction site in classical field isolates and vaccine strains. Although the restriction profiles of classical field Indian isolates and vaccine strains were identical, all of these enzymes could differentiate very virulent Indian strains from classical field isolates and vaccine strains.

Key words: Infectious bursal disease virus; virulence; reverse trasncription; polymerase chain reaction; restriction analysis

Introduction

IBDV causes a highly contagious immunosuppressive disease in chicken (Lukert and Saif, 1991). Of the two serotypes of IBDV reported, only serotype I with many subtypes are pathogenic for chicken (Ismail *et al.,* 1988). VP2 is the major immunogenic protein in which most of the neutralizing epitopes are confined to its small part known as variable region. RT-PCR-RE is a rapid technique for the identification and differentiation of unknown field isolates of IBDV. This technique is less expensive, less laborious and more reliable than conventional techniques. This technique can also detect genetic variations among IBDV isolates that are closely related serologically but cannot be differentiated using current serological methods. Restriction analysis of PCR products generated from variable region of VP2 has been used to differentiate IBDV strains (Lin *et al*., 1993; Jackwood and Jackwood, 1997). This study was undertaken to differentiate very virulent from classical field isolates and vaccine strains of IBDV in India by using restriction enzyme digestion of a 552 bp region of variable region of VP2 gene.

Materials and Methods

Viruses. Three classical (JM/81,N2/87 and N3/87) and two very virulent isolates of IBDV (B6/99 and HP/2000) were used as infected bursal suspensions. These isolates were collected from IBD outbreaks occurring from 1981 to 2000 in India. Two IBDV vaccine strains were also included. Details of these viruses are given in

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Abbreviations: IBD = infectious bursal disease; IBDV = IBD virus; RT-PCR = reverse transcription–polymerase chain reaction

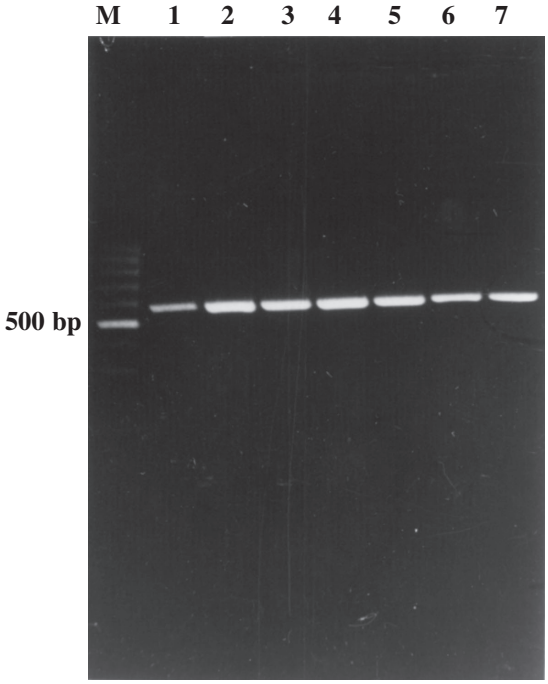

Fig. 1
Agarose gel electrophoresis

Purified 552 bp RT-PCR products of two vaccine strains (lanes 1 and 2), three classical field isolates (lanes 3–5) and two very virulent strains of IBDV (lanes 6 and 7). DNA size marker (lane M).

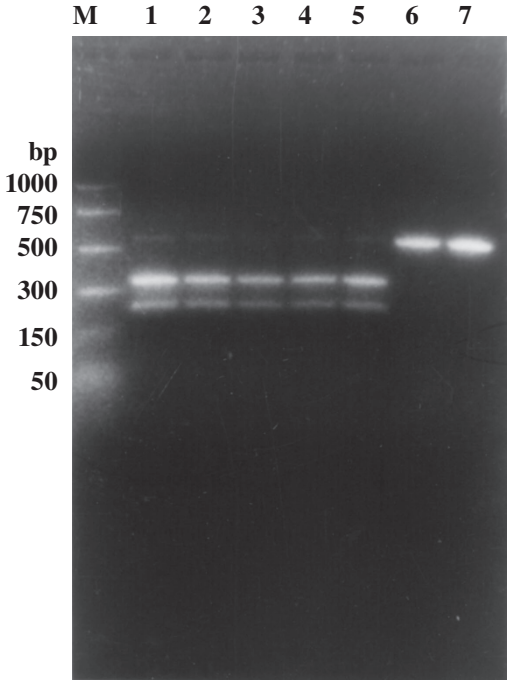

Fig. 2
Agarose gel electrophoresis

Restriction enzyme profiles of purified 552 bp RT-PCR products of two vaccine strains (lanes 1 and 2), three classical field isolates (lanes 3–5) and two very virulent strains of IBDV (lanes 6 and 7). Digestion with *Hha*I (Fig. 2), *Sac*I (Fig. 3), *Ssp*I (Fig 4) and *Stu*I (Fig 5). DNA size marker (lane M).

Table 1. The two very virulent isolates are representatives of 22 sequenced viruses of this type collected from India, Iran and Nepal. These viruses showed similar nucleotide sequences at restriction site of the four enzymes used (Toroghi, 2001; Kataria *et al*., 2001).

Total RNA extraction. Total RNA was extracted from the homogenized bursa or lyophilized vaccine strains using the TRIzol reagent (Life Technologies Inc., USA) according to the manufacturer's instructions. Briefly, 100 mg of bursal tissues was homogenized with 1 ml of TRIzol. After adding 200 ml of chloroform and vigorous shaking the aqueous phase was separated by centrifugation at 12,000 x g for 10 mins. Total RNA in the supernatant was precipitated by adding 0.5 ml of isopropanol and by centrifugation at 12,000 x g for 20 mins. After washing and drying RNA was dissolved in 15 µl of nuclease-free water.

RT-PCR. Total RNA was subjected to reverse transcription using Moloney murine leukemia virus reverse transcriptase and random hexamer primers (Promega) according to the manufacturer's instructions. RT-PCR yielded 552 bp products comprising the complete variable region of VP2 gene as described by Kataria *et al.* (1998). Briefly, the PCR products were generated using the forward primer 5'-CGCTATAGGGCTTGACCCAAAAA-3'and the reverse primer 5'-CTCACCCCAGCGACCGTAACGACG-3'. The amplification was carried out in a 50 µl reaction mixture contai-

Table 1. Details of IBDV strains/isolates used in this study

Strains/isolates	Virulence, type of strain/isolate	Source
Lukert	Vaccine, mild	Ventri, Biological, Pune India
Georgia	Vaccine, intermediate	Indovax, Pvt. Ltd. Hissar, India
JM/81	Classical, field isolate	Outbreak in Andhra Pradesh, India
N2/87	Classical, field isolate	Outbreak in Tamil Nadu, India
N3/87	Classical, field isolate	Outbreak in Tamil Nadu, India
B6/99	Very virulent, field isolate	Outbreak in Uttar Pradesh, India
HP/2000	Very virulent, field isolate	Outbreak in Himachal Pradesh, India

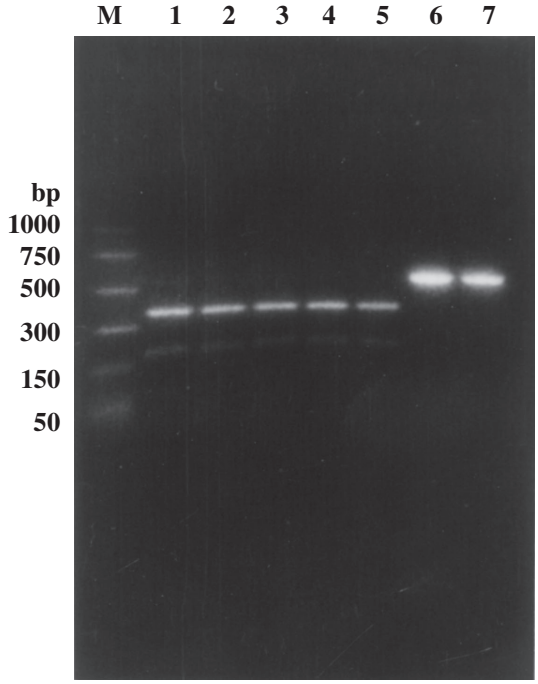

Fig. 3
Agarose gel electrophoresis
For the legend see Fig. 2.

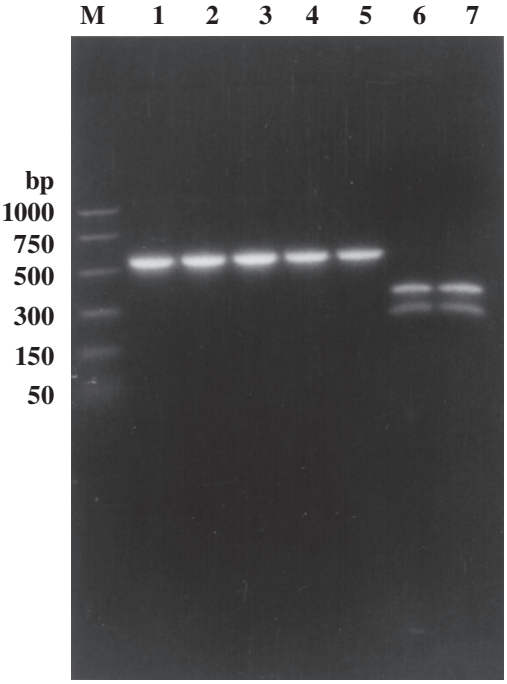

Fig. 4
Agarose gel electrophoresis
For the legend see Fig. 2.

ning 6 µl of cDNA, 15 pmoles of each primer, 200 µmol/l dNTPs, 1.5 mmol/l $MgCl_2$ and 1.5 U of Taq DNA polymerase (Gibco-BRL) in 35 cycles, each cycle consisting of 95°C for 1 min, 52°C for 1 min, and 72°C for 1 min with a final extension at 72°C for 5 mins. The authenticity of the amplicons generated was verified by agarose gel electrophoresis.

Restriction endonuclease digestion. The PCR products were purified using Wizard PCR Prep Purification Columns (Promega) and digested with the restriction enzymes *Hha*I, *Sac*I, *Ssp*I and *Stu*I (Life Technologies Ine and Promega). Approximately 200 ng of the PCR products was digested with 2 U of each enzyme.

Results and Discussion

The generated PCR products of the two vaccine, three classical and two very virulent strains of IBDV showed authentic products of 552 bp. The size of the generated PCR products were confirmed by 1.5% agarose gel electrophoresis (Fig. 1). Restriction enzyme digestion of these products with *Hha*I, *Sac*I, *Ssp*I and *Stu*I could differentiate vaccine and classical field strains from very virulent strains. Digestion of the amplicons with *Hha*I and *Sac*I revealed the presence of a single site in all the classical field isolates and vaccine strains but the absence of a single site in very virulent strains (Figs 2 and 3), while digestion of the PCR products with *Ssp*I and *Stu*I showed no restriction site in the field classical isolates and vaccine strains and a single site in very virulent strains of IBDV (Figs 4 and 5).

The restriction analysis of PCR products generated by amplification of variable region of VP2 gene of IBDV has been employed by many workers to confirm the authenticity of PCR products and to differentiate IBDV strains of different antigenic and virulence characters as well (Lin *et al.*, 1993; Liu *et al.*, 1994; Jackwood and Jackwood, 1997). In the present study, the Indian very virulent strains could be differentiated from vaccine strains and classical isolates on the basis of restriction patterns of the 552 bp PCR products. Only the restriction enzymes *Sac*I and *Hha*I cleaved PCR products of classical field isolates and vaccine strains, while only the restriction enzymes *Ssp*I and *Stu*I cleaved PCR products of very virulent strains strains.

The existence of *Sac*I and *Hha*I double positive and *Stu*I and *Ssp*I double negative in classical field isolates and vaccine strains, and the existence of *Stu*I and *Ssp*I double positive and *Sac*I and *HhaI* double negative in very virulent strains indicated that the latter could be differentiated from classical field isolates and vaccine strains by application of any of these restriction enzymes. However, these enzymes could not differentiate classical Indian field isolates from vaccine strains. It might be due to low pathogenic nature of classical Indian field isolates (Verma *et al.*, 1981). Moreover, comparison of consensus amino acid sequences established

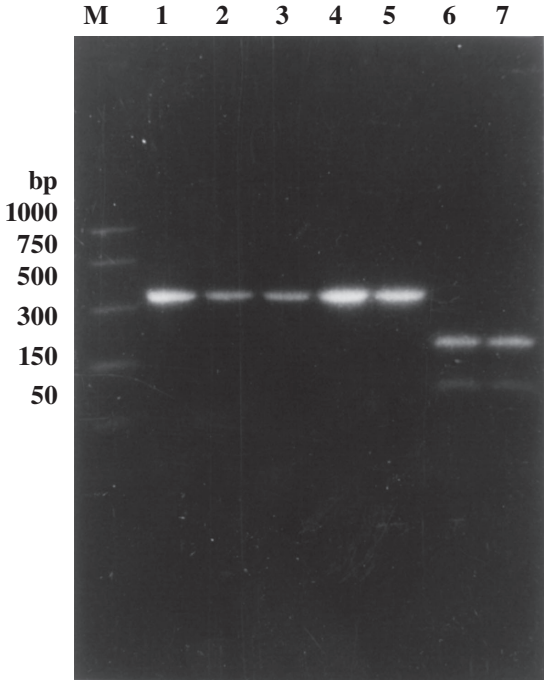

Fig. 2
Agarose gel electrophoresis
For the legend see Fig. 2.

for classical Indian isolates, classical virulent, attenuated and cell culture-adapted strains showed that classical Indian isolates were more similar to attenuated strains than classical virulent strains (Toroghi, 2001). It should be noted the very virulent phenotype of some strains has been predicted on the basis of a *Ssp*I-positive marker (Lin *et al*., 1993; Ture *et al*. 1998; Jackwood and Summer, 1999). The absence of the *Ssp*I site in the sequences of some very virulent strains (Eterradossi *et al*., 1999; Kataria *et al*., 2001; Hoque *et al*., 2001) and the presence of the *Ssp*I site in the sequences of some Australian strains/isolates, in which the very virulent phenotype has not been reported, are exceptional (Sapate and Ignjatovic, 2000). Based on these facts, any *Ssp*I-positive IBDV from India should be considered very virulent. Except some classical virulent and very virulent strains/isolates, *Stu*I can be used for differentiation of very virulent from classical Indian and vaccine strains/isolates.

Recently, Zierenberg *et al*. (2001) have reported differentiation of very virulent from classical virulent strains by using *Sac*I digestion that could be compared with our finding. However, the studies on known sequences of very virulent strains has revealed that 849VB (Eterradossi *et al*., 1999) and Jk1/97 (Kataria *et al*., 2001) with unique *Sac*I sites were the only exceptions.

There is no exception in differentiation of very virulent from classical isolates of Indian origin by *Hha*I. It seems that the presence of *Hha*I site in the sequence of very virulent African IBDV isolate (Eterradossi *et al*., 1999) and the absence of this site in the sequences of two classical strains, vaccine strain Bursin 2 (Eterradossi *et al*., 1998) and 002–73 strain (Sapate and Ignjatovic, 2000) are exceptions for this enzyme marker.

It is evident from the present study that *Hha*I could be employed as a useful marker for differentiation of very virulent from classical (field or vaccine) IBDV strains/isolates originating from India. Moreover, any *Sac*I-negative or *Ssp*I-positive IBDV strain/isolate could be considered very virulent, because so far no *Sac*I-positive or *Ssp*I-negative classical (field or vaccine) IBDV strain/isolate has been reported from India. Although there are a few exceptions concerning the differentiaton of very virulent from classical IBDV strains/isolates using a single restriction enzyme, the RT-PCR method has been found to be rapid in identification and differentiation of unknown field isolates. No exception could be found among these strains/isolates using a panel of restriction enzymes (*Hha*I*, Sac*I*, Ssp*I and *Stu*I). Thus, it is always advisable to digest a PCR product from an unknown isolate with more than one restriction enzyme. Further information on the identity of a isolate can be obtained from sequencing VP2 variable region which is continuously changing in field isolates.

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