DIFFERENTIATION OF INFECTIOUS BURSAL DISEASE VIRUS STRAINS BY RESTRICTION ANALYSIS OF RT-PCR-AMPLIFIED VP2 GENE SEQUENCES

R.S. KATARIA[1*], A.K. TIWARI[1], G. BUTCHAIAH[1], J.M. KATARIA[2]

[1]National Biotechnology Center; [2]Division of Avian Diseases, Indian Veterinary Research Institute, Izatnagar, Bareilly, UP 243 122, India

Received March 22, 1999; accepted April 30, 1999

Summary. – The techniques of reverse transcription-polymerase chain reaction (RT-PCR) and restriction analysis were used to differentiate highly virulent Indian field isolates of infectious bursal disease virus (IBDV) from vaccine strains. Primers were designed to amplify the variable region of VP2 gene coding for major virus neutralizing epitopes. The 552 bp PCR products generated from four vaccine strains and five field isolates were digested with restriction enzymes *Dra*I, *Hha*I, *Mva*I, *Stu*I, *Sty*I, and *Taq*I, which could differentiate field isolates from vaccine strains. Based on restriction enzyme profiles derived from published sequences, Indian field isolates seem to be closely related to highly virulent Japanese, European, and Chinese strains of the virus.

Keywords: infectious bursal disease virus; VP2 gene; RT-PCR; restriction analysis

Introduction

Infectious bursal disease (IBD) is a highly contagious viral disease causing heavy economic losses to the poultry industry worldwide. IBDV, the causative agent of the disease, belongs to the *Avibirnavirus* genus of the family *Birnaviridae*. Association of concurrent bacterial and viral infections is a common feature of the disease because virus multiplies and destroys the lymphoid cells in the bursa of Fabricius, resulting in immunosuppression (Saif, 1991). Out of the two serotypes of IBDV reported, only serotype 1 viruses with many subtypes are pathogenic to chicken, while serotype 2 viruses are apathogenic (Ismail *et al.*, 1988). The virus genome has two segments of double-stranded RNA. Large A segment (approximately 3.3 kb) encodes viral proteins VP5, VP2, VP4, and VP3. VP2-VP4-VP3 encoded as a polyprotein is autocleaved into VP2 and VP3, major structural proteins, and VP4, a viral protease (Hudson *et al.*, 1986). The function of VP5, encoded by upstream overlapping codons, is still unknown (Mundt *et al.*, 1995). Small B segment (approximately 2.8 kb) encodes a single viral polypeptide VP1 considered to be RNA polymerase (Morgan *et al.*, 1988). VP2 is the major immunogenic protein in which most of the conformational neutralizing epitopes are confined to a small variable region between amino acids 206 and 350 (Vakharia *et al.*, 1994). Strains of different antigenic structure and virulence, due to variation in this VP2 region, have been reported from various countries, against which already existing vaccines were found to be ineffective (Snyder *et al.*, 1988; Chettle *et al.*, 1989; Lin *et al.*, 1993).

In India, until late 1992, the disease was well under control by the use of effective attenuated and killed vaccines. Later, appearance of highly virulent virus isolates causing up to 75% mortality in growing layer and broiler flocks led to huge economic losses (Sah *et al.*, 1995). Conventionally, more cumbersome methods like virus neutralization or virulence studies in SPF chicks have been used for the differentiation of IBDV strains of varying antigenicity and virulence. RT-PCR followed by restriction analysis of variable

*E-mail: ivri@x400.nicgw.nic.in; fax: +91-581-440584.

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

Table 1. Description of IBDV strains and isolates

Strain/isolate	Type of strain/isolate	Source
Georgia	Vaccine, intermediate type	Indovax Pvt. Ltd., India
Intermediate plus (Im+)	Vaccine, intermediate type	Ventri Biologicals, Pune, India
Tri-bio	Vaccine, intermediate type	Ventri Biologicals, Pune, India
Lukert	Vaccine, mild type	Ventri Biologicals, Pune, India
UP1/97	Highly virulent, field isolate	Bursa of infected birds from an outbreak in Uttar Pradesh, India.
CH2/97	Highly virulent, field isolate	Bursa of infected birds from an outbreak in Union Territory of Chandigarh, India.
HR1/96	Highly virulent, field isolate	Bursa of infected birds from an outbreak in Haryana, India
MH1/97	Highly virulent, field isolate	Infected bursa from an outbreak in Maharastra, India
JK1/97	Highly virulent, field isolate	Infected bursa from an outbreak in Jammu and Kashmir, India

VP2 region has been found to be an easy and rapid method to differentiate and classify IBDV strains of different virulence and antigenicity (Lin *et al.*, 1993; Jackwood and Jackwood, 1997).

Here, we report differentiation of five highly virulent field isolates of IBDV from different geographical regions of India from four vaccine strains by using RT-PCR and restriction analysis of a 552 bp sequence from variable region of VP2 gene.

Materials and Methods

Virus and cell culture. Four vaccine strains of IBDV already adapted to primary cultures of chicken embryo fibroblasts and five virulent field isolates, obtained as IBDV-infected bursal tissues from the disease outbreaks in different parts of India, were used in this study. Details of the viruses are given in Table 1.

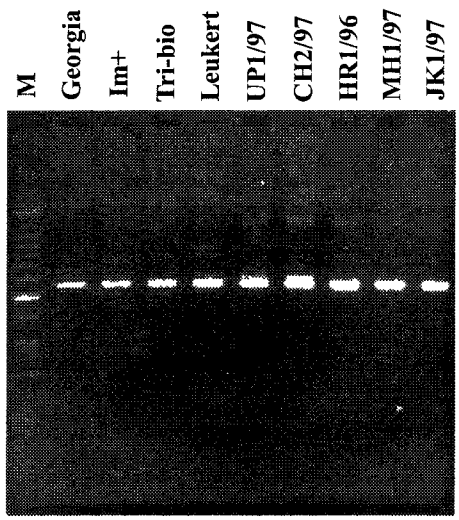

Fig. 1
Agarose gel electrophoresis of purified 552 bp RT-PCR products
DNA size marker, 100 bp ladder (lane M).

Extraction of total RNA from IBDV-infected cell cultures (vaccine strains) showing 60–70% cytopathic effect, was carried out by adding TRIzol reagent (Life Technologies Inc., USA) directly to infected monolayers. RNA from IBDV-infected bursal tissues (field isolates) was extracted by homogenizing the tissues in TRIzol according to the manufacturer's protocol. RNA samples dissolved in nuclease-free water were stored at -20°C until used. For negative controls, RNA from mock-infected chicken embryo fibroblast cultures and healthy bursa was used.

RT-PCR. The reverse transcription was carried out by a method described earlier (Kataria *et al.*, 1998). Briefly, a 20 µl reaction mixture containing approximately 1 µg of heat-denatured total RNA, 50 ng of random hexamer primers, 500 µmoles of dNTPs, 40 U of an RNase inhibitor, and 200 U of Moloney murine leukemia virus reverse transcriptase, was incubated at 20°C for 10 mins and then at 37°C for 1 hr. Reverse transcriptase was inactivated by heating at 95°C for 3 mins.

The amplification was carried out with a 10 µl sample of heat-denatured cDNA in a 50 µl reaction mixture containing 20 pmoles of each primer, 200 µmol/l dNTPs, 1.5 mmol/l $MgCl_2$, and 3 U of Taq DNA polymerase. A primer pair from variable region of VP2 gene of IBDV (forward primer 5'-CGCTATAGGGCTTGACCCA-3' (nt 651-673) and reverse primer 5'-CTCACCCCAGCGACCGTA ACGACG-3' (nt 1179-1202)) was designed to amplify a 552 bp sequence as described earlier (Kataria *et al.*, 1998). The amplification was carried out 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 analysis. The 552 bp RT-PCR products, generated from five field isolates and four vaccine strains, were purified by QIAquick PCR Purification Kit (QIAGEN) and subjected to digestion with restriction endonucleases *Dra*I, *Hha*I, *Mva*I, *Stu*I, *Sty*I, and *Taq*I (Boehringer Mannheim and Life Technologies Inc.). The digested products were analyzed by 1.5% agarose gel electrophoresis.

Results

RT-PCR amplification of RNAs from all the four vaccine strains and five field isolates showed an authentic product of 552 bp while RNAs from mock-infected cells and healthy bursa did not yield any product (Fig. 1). For restric-

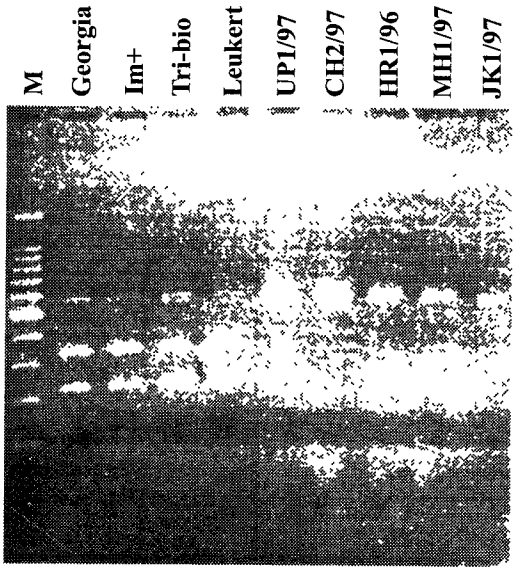

Fig. 2
Agarose gel electrophoresis of *Hha*I-digested RT-PCR products
DNA size marker, 100 bp ladder (lane M).

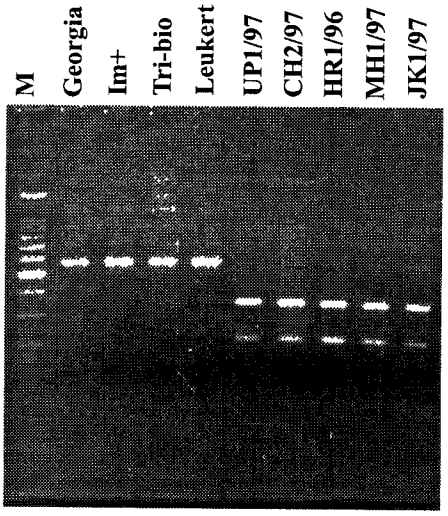

Fig. 4
Agarose gel electrophoresis of *Stu*I-digested RT-PCR products
DNA size marker, 100 bp ladder (lane M).

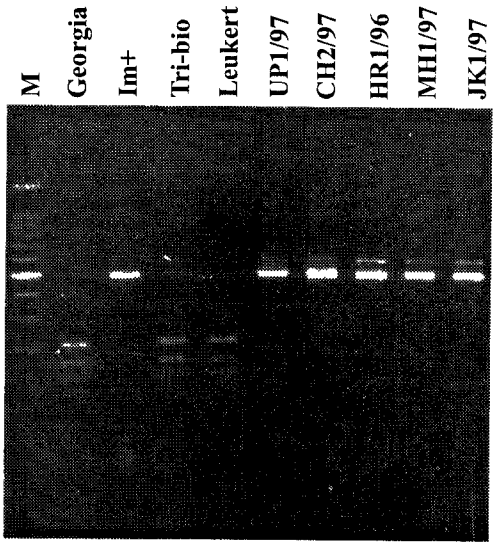

Fig. 3
Agarose gel electrophoresis of *Mva*I-digested RT-PCR products
DNA size marker, 100 bp ladder (lane M).

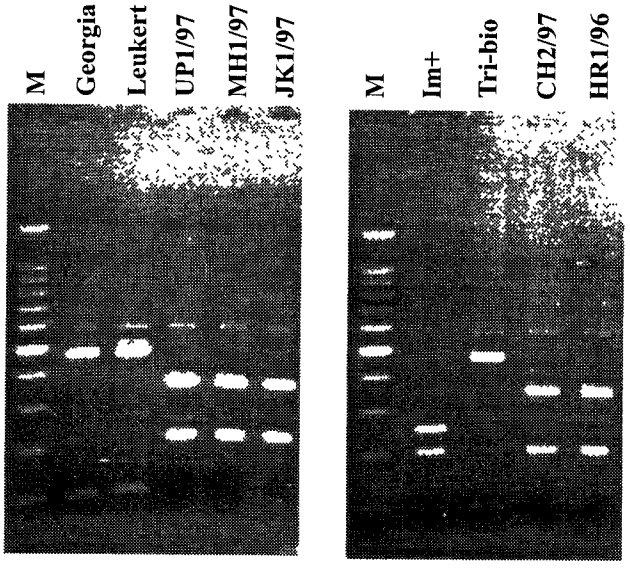

Fig. 5
Agarose gel electrophoresis of *Sty*I-digested RT-PCR products
DNA size marker, 100 bp ladder (lane M).

tion analysis, approximately 1 μg of the PCR products was subjected to digestion with the six restriction enzymes. The digestion with *Hha*I revealed the presence of a single site in all the four vaccine strains and no site in virulent field isolates (Fig. 2). The *Mva*I digestion yielded four fragments with similar pattern for three vaccine strains, Georgia, Tribio, and Lukert, while two fragments with identical pattern were observed for all the field isolates and the Intermediate plus vaccine strain (Fig. 3). The *Stu*I digestion showed no site for all the vaccine strains but a single site with identical pattern for all the field isolates (Fig. 4). The *Sty*I restriction pattern with a single site was identical for all the five field

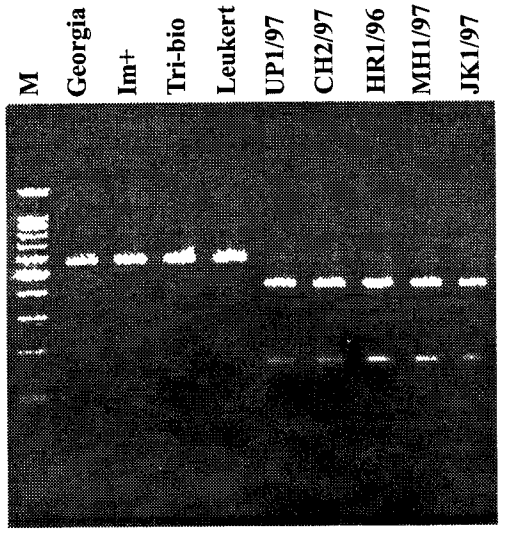

Fig. 6
Agarose gel electrophoresis of *Taq*I-digested RT-PCR products
DNA size marker, 100 bp ladder (lane M).

Table 2. Restriction sites in RT-PCR products from different IBDV vaccine strains and field isolates

Strain/isolate	Number of restriction sites					
	*Dra*I	*Hha*I	*Mva*I	*Stu*I	*Sty*I	*Taq*I
Georgia	0	1	3	0	1	0
Im+	0	1	1	0	2	0
Tri-bio	0	1	3	0	1	0
Lukert	0	1	3	0	1	0
UP1/97	0	0	1	1	1	1
CH2/97	0	0	1	1	1	1
HR1/96	0	0	1	1	1	1
MH1/97	0	0	1	1	1	1
JK1/97	0	0	1	1	1	1

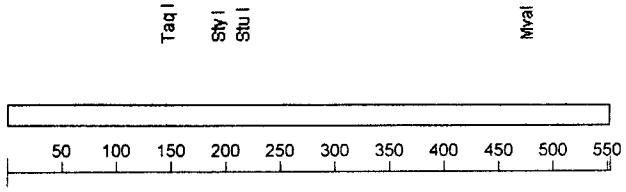

Fig. 7
Restriction map of the 552 bp VP2 gene region of virulent IBDV strains OKYM, UK661, and HK46, based on the published sequences

isolates. Three vaccine strains (Georgia, Tri-bio, and Lukert), had also a single *Sty*I site but at different position, while the Intermediate plus vaccine strain had two sites giving unique pattern of three bands in agarose gel (Fig. 5). The digestion with *Taq*I yielded two fragments with the same pattern for all the field strains, but there was no site for the vaccine strains (Fig. 6). No *Dra*I site was noticed for any of the isolates studied. The number of sites for different restriction enzymes for each virus strain is shown in Table 2.

Discussion

The variable region of VP2 gene has been exploited by many workers for differentiation of IBDV strains by RT-PCR followed by restriction analysis (Lin *et al.*, 1993; Jackwood and Jackwood, 1997). Earlier, we have reported detection of IBDV in clinical samples by RT-PCR and differentiation of two field isolates and one vaccine strain by restriction analysis with *Stu*I (Kataria *et al.*, 1998). In the present study, we could differentiate five virulent field isolates from four vaccine strains by restriction digestion of the purified 552 bp RT-PCR products with five restriction enzymes, *Hha*I, *Mva*I, *Stu*I, *Sty*I, and *Taq*I. The analysis of restriction sites for these enzymes, predicted on the basis of published sequences of highly virulent IBDV strains OKYM, a Japanese strain (Yamaguchi *et al.*, 1997), UK661, a European strain (Brown *et al.*, 1994), and HK46, a Chinese strain (Cao *et al.*, 1998), revealed the same restriction patterns as we found for Indian virulent strains. However, the number of restriction sites found was different from that reported for variant American strains (Jackwood and Jackwood, 1997). Restriction enzymes *Hha*I, *Stu*I, and *Taq*I had different patterns for vaccine strains and Indian field isolates. *Stu*I and *Taq*I restriction enzymes had a single site for field isolates and no site for vaccine strains. Another site, for *Taq*I, located in the forward primer region, which was present in virulent strains OKYM, UK661, and HK46, was absent in our field isolates, because our primers were designed for a classical American strain, STC, having change of one nucleotide in the primer sequence (Kibenge *et al.*, 1990). The presence of *Taq*I restriction sites has been reported for some American vaccine strains as well as for a few field isolates (Jackwood and Jackwood, 1997). Restriction enzyme *Hha*I had a single site with similar pattern in all the four vaccine strains and no site in the field isolates of Indian origin. None of the vaccine strains or field isolates used in this study had a *Dra*I site(s). This is in contrary to American variant strains, which have been reported to have such sites (Jackwood and Jackwood, 1997). A single *Mva*I site of similar pattern was found for the Intermediate plus vaccine strain and all the Indian field isolates tested. Therefore, we could not differentiate the vaccine strains from the field isolates using *Mva*I enzyme. Similarly, Jackwood and Jackwood (1997) were also unable to

differentiate vaccine and USA variant strains of IBDV using *Bst*NI (an isoschizomer of *Mva*I) restriction enzyme. No *Bst*NI sites, located in the variable VP2 region, have been reported for OKYM, UK611, and HK46 strains (Cao *et al.*, 1998). But the analysis of published nucleotide sequences of these strains revealed the presence of a single *Bst*NI site outside of this region of VP2 gene in accordance with our findings. Though *Sty*I restriction enzyme could differentiate all the four vaccine strains from the field isolates, the Intermediate plus vaccine strain was an exception, it had a different pattern. This vaccine strain had one fragment common with other vaccine strains, another common with the field isolates and an additional one neither present in other vaccine strains nor in the field isolates. No differences in restriction patterns were found within the field isolates from different regions of India. The restriction patterns obtained indicated that the Indian field isolates of IBDV are similar to Japanese, European, and Chinese virulent isolates. Sequencing of this region of VP2 gene of the virus containing most of the neutralizing epitopes, will further help in better understanding of the molecular epidemiology of highly virulent Indian IBDV isolates, their origin and antigenic character.

Acknowledgements. The authors thank the Director of the Indian Veterinary Research Institute, Izatnagar, for providing necessary facilities and Mr. N. Prasad for skilful technical assistance.

References

Brown MD, Green P, Skinner MA (1994): VP2 sequences of recent European ‚very virulent' isolates of infectious bursal disease virus are closely related to each other but are distinct from those of ‚classical' strains. *J. Gen. Virol.* **75**, 675–680.

Cao YC, Yeung WS, Law M, Bi YZ, Leung FC, Lim BL (1998): Molecular characterization of seven Chinese isolates of infectious bursal disease virus: classical, very virulent and variant strains. *Avian Dis.* **42**, 340–351.

Chettle N, Stuart JC, Wyeth PJ (1989): Outbreak of virulent infectious bursal disease in East Anglia. *Vet. Rec.* **125**, 271–272.

Hudson PJ, McKern NM, Power BE, Azad AA (1986): Genomic structure of a large RNA segment of infectious bursal disease virus. *Nucleic Acids Res.* **14**, 5001–5012.

Ismail NM, Saif YM, Moorhead PD (1988): Lack of pathogenicity of five serotypes 2 infectious bursal disease viruses in chickens. *Avian Dis.* **32**, 757–759.

Jackwood DJ, Jackwood RJ (1997): Molecular identification of infectious bursal disease virus strains. *Avian Dis.* **41**, 97–104.

Kataria RS, Tiwari AK, Bandyopadhyay SK, Kataria JM, Butchaiah G (1998): Detection of infectious bursal disease virus of poultry in clinical samples by RT-PCR. *Biochem. Mol. Biol. Int.* **45**, 315–322.

Kibenge FSB, Jackwood DJ, Mercado CC (1990): Nucleotide sequence analysis of genome segment A of infectious bursal disease virus. *J. Gen. Virol.* **71**, 569–577.

Lin Z, Kato A, Otaki Y, Nakamura T, Sasmaz E, Ueda S (1993): Sequence comparisons of a highly virulent infectious bursal disease virus prevalent in Japan. *Avian Dis.* **37**, 315–323.

Morgan MM, Macreadie IG, Harley VR, Hudson PJ, Azad AA (1988): Sequence of the small double-stranded RNA genomic segment of infectious bursal disease virus and its deduced 90-kDa product. *Virology* **163**, 240–242.

Mundt E, Beyer J, Muller H (1995): Identification of a novel viral protein in infectious bursal disease virus infected cells. *J. Gen. Virol.* **76**, 437–443.

Sah RL, Kataria JM, Arya SC, Verma KC (1995): Outbreaks of acute infectious bursal disease causing high mortality in chickens. *Indian J. Comp. Microbiol. Immunol. Infect. Dis.* **16**, 7–13.

Saif YM (1991): Immuno-suppression induced by infectious bursal disease virus. *Vet. Immunol. Immunopathol.* **30**, 45–50.

Snyder DB, Lana DP, Cho BR, Morquardt WW (1988): Group and strain- specific neutralization sites of IBD Virus defined with monoclonal antibodies. *Avian Dis.* **32**, 527–534.

Vakharia VN, He J, Ahamed B, Snyder DB (1994): Molecular basis of antigenic variation in infectious bursal disease virus. *Virus Res.* **31**, 265–273.

Yamaguchi T, Ogawa M, Miyoshi M, Inoshima Y, Fukushi H, Hirai K (1997): Sequence and phylogenetic analyses of highly virulent infectious bursal disease virus. *Arch. Virol.* **142**, 1441–1458.