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308 Lasani Town, Sargodha Road, Faisalabad - Pakistan
Mob: +92 300 3008585, Fax: +92 41 8815544
E-mail: editorijps@gmail.com

Molecular Characterization of Infectious Bursal Disease Virus Isolated from Broiler Chickens in Bangladesh

M.A. Islam¹, M.M. Rahman¹, M.R. Islam² and M.M. Khatun¹

¹Department of Microbiology and Hygiene, Bangladesh Agricultural University,
Mymensingh-2202, Bangladesh

²Department of pathology, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh
E-mail: arifmicro2003@yahoo.com

Abstract: An attempt was undertaken for molecular characterization of infectious bursal disease virus (IBDV) field isolates. In order to isolate the virus, bursae of thirty five dead chicken with clinical infectious bursal disease (IBD) were collected from Bangladesh Agricultural University (BAU), Mymensingh. Isolation of field strain of IBDV was carried out in chickens of 5-week-old. Five IBDV isolates were obtained from chicken inoculation. Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed to detect IBDV isolates in the bursal tissues. RT-PCR couple with restriction enzyme (RE) analysis was carried out for molecular characterization of IBDV isolates to determine the pathotype. 677 bp fragments from IBDV genome segment A corresponding to the hyper variable domain of outer capsid protein VP₂ was amplified by RT-PCR. Two restriction endonuclease (REs), *SspI* and *SacI* were used for digestion of RT-PCR products. RT-PCR product was digested by *SspI* but not *SacI*. The presence of *SspI* restriction site in the 677 bp RT-PCR fragment indicated that IBDV isolates belonging to very virulent (vv) pathotype.

Key words: Molecular characterization, Infectious bursal disease virus, broiler chickens

Introduction

Infectious bursal disease popularly known as Gumboro disease is a highly contagious, immunosuppressive infection of immature chickens (McFerran, 1993) caused by a double stranded RNA virus belonging to the genus Birnavirus and subgenus Avibirnavirus under the family Birnaviridae (Leong *et al.*, 2000). The IBDV has bi-segmented genome; segment A encodes for the outer capsid protein VP₂, inner capsid protein VP₃, a protease VP₄ and a non structural protein VP₅. The segment B encodes for the polymerase protein VP₁. The virus has two serotypes, of which only serotype 1 is pathogenic for chicken and comprises several pathotypes such as classical virulent, antigenic variant and very virulent (vv) strains (Muller *et al.*, 2003). The primary target organ for IBDV is the bursa of Fabricius (Lukert and Saif, 1997). Molecular technique like reverse transcription-polymerase chain reaction (RT-PCR) has recently been used for the detection of IBDV. Moreover RT-PCR coupled with restriction enzyme analysis can be used for genetic characterization of IBDV to determine serotypes and pathotypes (Zierenberg *et al.*, 2001). Three Bangladeshi isolates of IBDV (BD 1/99, BD 2/99, and BD 3/99) were characterized at molecular and antigenic level and they were antigenically and genetically related to vvIBDV reported from Europe, Asia and Africa (Islam *et al.*, 2001a). Present communication was aimed to characterize IBDV field isolate at molecular level.

Materials and Methods

The experiment was conducted in the Department of

Microbiology and Hygiene and Department of Pathology, Faculty of Veterinary Science, BAU, Mymensingh during the period from May 2003 to April 2004.

Collection of field samples: Bursa of Fabricius of thirty five dead birds suspected to be infected with IBD were collected from BAU poultry farm, Mymensingh and stored at -20°C.

Preparation of samples: 10% bursal homogenate was prepared from five bursae in phosphate buffered saline (PBS). The suspension was centrifuged at 3000 rpm for 30 minutes. The supernatant was treated with broad spectrum antibiotic (Gentamycin) @ 50µg/ml and kept at room temperature for 30 minutes.

Isolation of virus in chickens: In order to isolate the IBDV field isolates five chickens of 35 days old were inoculated with 10% bursal homogenate @ 100µl through intranasal, intraocular and intra cloacal routes. Five chickens of same age were kept as uninfected control. At day 3 post infection (P.I.) all chickens were sacrificed and bursae were collected aseptically.

Reference virus: BD-3wt representing a vvIBDV isolate and a vaccine virus Nobilis D-78 representing an attenuated classical strain were used as reference virus.

Isolation of RNA: Total RNA was isolated from bursal tissues. The procedure was based on guanidine lysis, phenol-chloroform-isoamyl alcohol extraction, and

Table 1: Amplification of cDNA from VP₂ gene of segment-A of IBDV following RT-PCR

Sample No.	Sample ID	RT-PCR result
1	Water control	-
2	Uninfected control	-
3	BD-3wt (vvIBDV)	+
4	Nobilis D78 vaccine virus	+
5	M1 (Unknown field strain)	+

+ Indicates cDNA amplification, - indicates absence of cDNA amplification

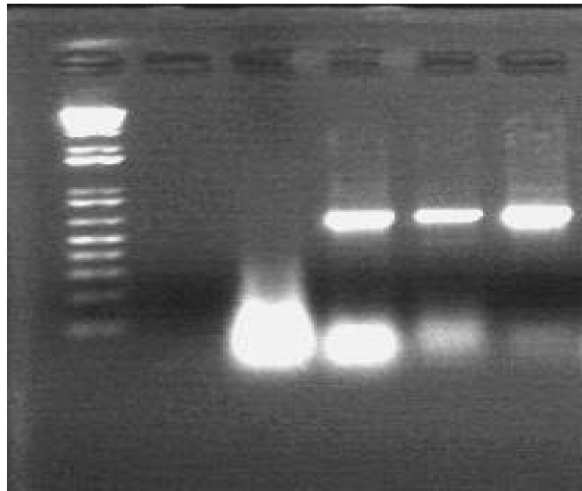


Fig. 1: cDNA of 677 bp long corresponding to the hyper variable domain of the VP₂ gene synthesized by RT-PCR and subjected to electrophoresis on 1.2% agarose gel and stained with ethidium bromide. Lane 1: Marker, Lane 2: Water control, Lane 3: Uninfected bursa of day 3 p.i., Lane 4: BD-3wt infected bursa of day 3 p.i., Lane 5: Nobilis D-78 vaccine, Lane 6: Local isolate of IBDV (M₁) infected bursa of day 3 p.i.

isopropanol precipitation described by Zierenberg *et al.* (2000) and Malik *et al.* (2001). For isolation of RNA from the vaccine virus Nobilis D-78, the lyophilized vaccine (1000 dose) was resuspended in 1ml distilled water and processed as the tissue homogenate.

RT-PCR: One- tube RT-PCR in 50 µl volume was performed following the procedure described by Zierenberg *et al.* (2000) and Islam *et al.* (2001b) after some modifications. RT-PCR products were analyzed by electrophoresis in 1.2% agarose gel.

Restriction enzyme analysis: PCR products were subjected to restriction enzyme (RE) analysis without further purification. PCR products were digested with the use of restriction enzymes *SspI* and *SacI* as described by Lin *et al.* (1993) and Meir *et al.* (2001).

Analysis of RE digestion: The cleavage of DNA by RE digestion was analyzed in 2% agarose gel by electrophoresis described by Jackwood and Sommer (1998). The results were examined and recorded by using the image documentation system.

Results and Discussion

A molecular virological technique, reverse transcription-polymerase chain reaction (RT-PCR), was performed to identify IBD viral RNA in the bursal tissue of the experimentally infected chicken at day 3 post infection. Two reference viruses namely BD-3wt belonging to vvIBDV and Nobilis D-78 vaccine virus belonging to cvIBDV were also used in this study to compare the pathotype with the field strain of IBDV. The RT-PCR was used to amplify a 677-bp product, encompassing the hypervariable region of VP₂ gene of the segment A. The RT-PCR products of 677-bp were digested with two restriction enzymes, *SacI* and *SspI* to determine the pathotype of the local isolate. The cDNA of expected 677-bp was amplified from the local field isolate of IBDV by RT-PCR. A similar cDNA of 677-bp was also amplified from the Nobilis D-78 vaccine virus and BD-3wt virus. On the other hand, no cDNA was amplified from the bursal tissue of uninfected control chickens by RT-PCR. Water control was also negative indicating the absence of any contamination. The results of electrophoretic analysis of RT-PCR products are shown in Table 1 and Figure 1.

RT-PCR technique used in this study was based on the protocol described by Zierenberg *et al.* (2000) and Islam *et al.* (2001a) with some modifications. Instead of more conventional SDS-proteinase K digestion method guanidine lysis based approach was used for RNA isolation in the present study. It has been suggested that guanidine based approach could provide more clear RNA preparation (Malik *et al.*, 2001). In addition to guanidine, β-mercaptoethanol and N-lauryl sarcosine were also present in the denaturing solution used for RNA isolation in the present study. Guanidine thiocyanate and N-lauryl sarcosine act for disruption of nucleoprotein complexes, allowing RNA to be released into protein free solution. Guanidine thiocyanate and β-mercaptoethanol have also two potent RNase inhibitors (Chirgwin, 1979). One tube RT-PCR was used in the present study, in which reverse transcription was immediately followed by PCR in the same tube. RT-PCR employs two enzymes: a reverse transcriptase such as avian myeloblastosis virus-reverse transcriptase (AMV-RT) and a heat stable DNA polymerase. RT-PCR for IBDV requires heat-denaturation of double stranded RNA before commencement of reverse transcription. However, denatured RNA must be cooled rapidly before adding enzyme mix, as the reverse transcriptase is relatively heat labile. This rapid cooling is best done by sharp freezing in liquid nitrogen (Zierenberg *et al.*, 2000; Islam *et al.*, 2001b). However, in the present study, the

Table 2: Restriction enzyme analysis of RT-PCR products

Sample No.	Sample ID	Digestion profile		Interpretation
		<i>SacI</i>	<i>SspI</i>	
1	BD-3wt(wIBDV)	-	+	Very virulent
2	Nobilis D-78 vaccine	+	-	Classical virulent
3	M1 (unknown field strain)	-	+	Very virulent

+Indicates digestion of RT-PCR products, - indicates no digestion of the RT-PCR products

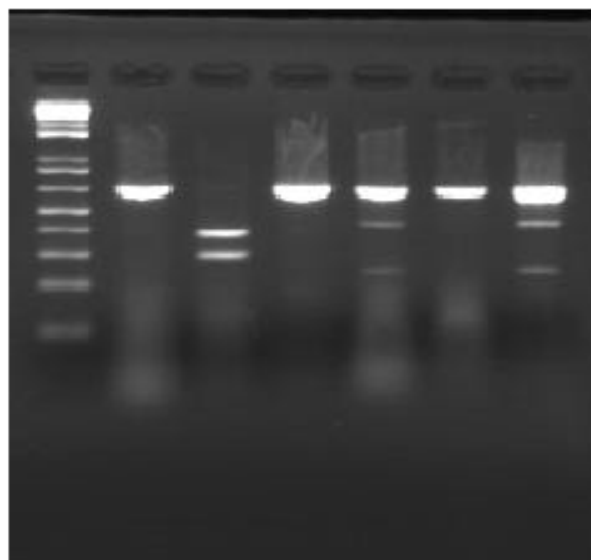


Fig. 2: Analysis with the restriction enzymes (*SspI* and *SacI*) of the cDNA synthesized by RT-PCR, corresponding to the hypervariable domain of the VP2 gene. Electrophoresed on 2% agarose gel and stained with ethidium bromide. Lane 1: Marker, Lane 2: BD-3wt infected bursa of day 3 p.i., Lane 3: Nobilis D78 vaccine, Lane 4: Local isolate (M1) infected bursa of day 3 p.i., Lane 5: BD-3wt infected bursa of day 3 p.i., Lane 6: Nobilis D78 vaccine, Lane 7: Local isolate (M1) infected bursa of day 3 p.i., Lanes 2-4: digested with *SacI*; Lanes 5-7: digested with *SspI*.

cooling of denatured RNA was done by transferring PCR tubes rapidly in ice (Sellers *et al.*, 1999; Yehuda *et al.*, 1999) and this procedure worked perfectly.

Restriction enzyme analysis involves restriction endonuclease enzymes, which cut the amplified RNA into fragments that can be separated and visualized by the process of electrophoresis (Jackwood and Sommer, 1999). Restriction enzyme recognizes a specific nucleotide sequences in the genome and cut the amplified genome at site or adjacent to it. RT-PCR followed by restriction enzyme analysis is now widely used for IBDV strain differentiation and characterization. The amplified genome usually corresponds to the VP2

variable fragment domain and restriction enzyme digestion is performed with various sets of enzymes. Various restriction enzymes have been used to discriminate wIBDV and classical IBDV strains. In the present study, *SspI* and *SacI* were used to differentiate between wIBDV and classical IBDV strains. These two enzymes have successfully discriminated D-78 (a classical IBDV) from BD-3wt(wIBDV) and field strain of IBDV. The PCR products of 677-bp amplified from BD-3wt(wIBDV) and field strain of IBDV (M1) when digested with *SspI* enzyme generated two fragments 432-bp and 245-bp in length. *SacI* enzyme could not digest the same PCR product amplified from BD-3wt(wIBDV) and field strain of IBDV. The PCR products synthesized from BD-3wt(wIBDV) and field strain of IBDV (M1) exhibited RE digestion profile of wild phenotype i.e., cleavage by *SspI* but not by *SacI*. On the other hand PCR product of 677-bp amplified from vaccine virus Nobilis D-78 when digested with *SacI* enzyme generated two fragments with size of 364 bp and 293-bp. The result of restriction enzyme (RE) analysis of RT-PCR products are shown in Table 2 and Fig. 2.

The RFLP Pattern resulting from digestion of PCR product synthesized from vaccine virus Nobilis D-78 with restriction enzymes indicating RE digestion profile of classical phenotype i.e., cleavage by *SacI* but not by *SspI*. This result is similar with finding observed by Zierenberg *et al.* (2001) and Jackwood (1994). The RFLP pattern resulting from digestion of the PCR product of BD-3wt(wIBDV), field strain of IBDV indicating RE digestion profile of w phenotype i.e., cleavage by *SspI* but not by *SacI*. Lin *et al.* (1993) and Meir *et al.* (2001) stated that *SspI* RE site is found exclusively in wIBDV.

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