<Original Article>

Isolation and identification of infectious bursal disease virus from broiler and layer chickens during the outbreak year 2007 in Bangladesh

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Abstract

The objective of the present study was to isolate and identify infectious bursal disease viruses (IBDV) from broiler and layer chickens of outbreaks of infectious bursal disease (IBD) in three districts of Bangladesh. A total of 70 bursal samples were collected from dead broiler (n=40) and layer (n=30) chickens showing specific lesions of IBD from seven commercial poultry farms of three different districts (Mymensingh, Chittagong and Tangail) of Bangladesh during the year 2007. Five representative bursal samples from each farm were used for the isolation of IBDVs using 9-day-old embryonated eggs of seronegative flock of layer birds and for identification the samples were subjected to agar gel immunodiffusion test (AGIDT), immunohistochemistry (IHC) and reverse transcription-polymerase chain reaction (RT-PCR). Out of 35 bursal samples, IBDVs were successfully isolated from 28 (80%) samples. By AGIDT, 32 (91.4%) samples were found positive for IBDV antigen. Results of AGIDT clearly indicated that IBDVs detected in 29 bursal samples of six affected farms were identical to each other but not to IBDVs present in the remaining three samples of another farm. Indirect immunoperoxidase staining of the bursal sections revealed the presence of IBDV antigen in 32 (91.4%) samples and the IBDV antigen was detected mainly in the cortex of the lymphoid follicles of the bursal tissues. In histopathology, cell depletion, atrophy and necrosis were observed in many bursal follicles with severe edema of interfollicular septa. Of the 35 bursal samples, 34 (97.1%) samples generated 254 bp product by RT-PCR. In conclusion, the results of virus isolation and identification by AGIDT, IHC and the analysis of viral genome by RT-PCR confirmed the outbreaks of acute IBD in commercial poultry of Bangladesh. Moreover, histopathological findings and results of AGIDT gave a clear indication that the isolates from six outbreaks were different from classical strain and it seems to be of very virulent strain. On the other hand, the isolates from the other outbreak were similar to the classical strain.

Key words : Infectious bursal disease virus, Isolation, Immunohistochemistry, RT-PCR

INTRODUCTION

Infectious bursal disease virus (IBDV) is a causative agent of a highly contagious disease of young chickens (Infectious bursal disease, commonly known as Gumboro

disease) characterized mainly by severe changes in the bursa of Fabricius followed by immunosuppression (Faragher et al, 1974; Islam et al, 2005). IBDV is a non-enveloped icosahedral double-stranded RNA (dsRNA) virus with a diameter of about 55~60 nm belongs to the genus *Avibirnavirus* in the family *Birnaviridae* (Leong et al, 2000). The dsRNA genome consists of

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two segments, namely, A and B (Müller et al, 1979). The smaller segment (segment B), with a length of about 2,900 bp, encodes the multifunctional protein VP1 of 90-kDa with RNA-dependent RNA polymerase (Spies et al, 1987). The larger segment (segment A) at about 3,200 bp in length, encodes a polyprotein that is cleaved by autoproteolysis to form mature viral proteins VP2, VP3 and VP4 (Hudson et al, 1986). The VP2 and VP3 are the major structural proteins of the virion which are responsible for the regulation of pathogenicity and immunogenicity.

There are two distinct serotypes of IBDV, designated as serotype 1 and serotype 2 (Jackwood and Saif, 1987). All subtypes of serotype 1 strains are pathogenic to chickens and vary markedly in their virulence, whereas, all known subtypes of serotype 2 are non-pathogenic for both turkeys and chickens (Jackwood et al, 1985). According to antigenic variation and virulence property, IBDVs of serotype 1 can be divided into classical virulent (mild, intermediate and intermediate-plus/hot strains), very virulent (vv) and antigenic variant strains. Recently emerged vvIBDV can cause up to 70% or higher mortality in the field (Chettle et al, 1989; Van den Berg et al, 1991).

IBD has been considered as an existing threat to the poultry industry in Bangladesh since the first isolation of IBDV strain by Rahman et al (1996) from bursa of Fabricius of layer chickens during the period of 1992-1993. Since then, the disease was satisfactorily controlled by routine vaccination with imported live-attenuated and inactivated IBDV vaccines in Bangladesh. However, outbreaks of acute IBD noticed in 1999 and continued thereafter sporadically (Islam et al, 2001; Islam and Samad, 2003). The emergence of vvIBDV strains among the poultry population of Bangladesh was confirmed by Islam et al (2001). In 2007, many outbreaks of acute IBD were observed both in vaccinated and non-vaccinated broiler and layer flocks in different parts of the country. It is not quite unexpected that high mutation rate of IBDV, segmented nature of the IBDV genome and high immune selection pressure due to intensive vaccination can lead to the emergence of IBDV with new properties (Müller et al, 2003; Jackwood and Sommer-Wagner, 2005). Therefore, it is essential to iso-

late, identify and characterize IBDVs when there is any outbreak.

The present study describes the isolation and identification of IBDV from broiler and layer chickens of recent outbreaks of IBD in three districts of Bangladesh.

MATERIALS AND METHODS

Reference IBDV

The vaccine strains Nobilis[®] Gumboro 228E (Intervet International, Netherlands) and BAU 404 of classical strain (DLS, Dhaka, Bangladeshi) were used as reference IBDVs in agar gel immunodiffusion (AGIDP) test and reverse transcription-polymerase chain reaction (RT-PCR) assay.

Field bursal samples

Bursal samples of dead birds showing specific lesions of IBD were collected from seven field outbreaks (four in broiler farms and three in layer farms) during the year 2007 from Mymensingh, Chittagong and Tangail districts. From each outbreak of IBD, 10 bursal samples were collected. A portion of each bursal sample was preserved at -86°C while another portion was kept in 10% buffered normal saline for histopathology and immunohistochemistry. Bursal suspension preserved at the Department of Microbiology and Hygiene, BAU, Mymensingh which were previously confirmed negative for IBDV by AGIDT and RT-PCR were used as negative control.

Inoculation of bursal samples into embryo-nated chicken eggs

Five representative bursal samples from each outbreak were homogenized with sea sand and 20% bursal homogenates were prepared in phosphate buffered saline (PBS). After centrifugation of the suspension at 3,000 rpm for 15 min, the supernatants were treated with penicillin (10,000 IU/ml) and streptomycin (10,000 $\mu\text{g/ml}$), which were used as inoculum. 0.2 ml of each sample

was inoculated into 9-day-old embryonated chicken eggs through chorioallantoic membrane (CAM). The eggs were collected from seronegative layer birds of 68 to 70 weeks old. Five embryonated eggs were used for each sample. Allantoic fluids, CAM and the embryo proper were harvested aseptically from the embryonated eggs dying after 24 hr and within 5 days after inoculation and were stored at -86°C until used. Embryos were processed to make 50% suspensions for the extraction of viral RNA.

Hyperimmune sera preparation

Hyperimmune sera for using in agar gel immunodiffusion test and immunohistochemistry were raised in 3-week-old cockerels against the intermediate plus IBDV vaccine strain (Nobilis[®] Gumboro 228E, Intervet International, Netherlands). Six chickens were immunized with the vaccine intraocularly at weekly interval for four occasions. After 10 days of last vaccination, the chickens were bled and sera were separated following the method as described earlier (Jackwood and Saif, 1987). Similarly, chicken anti-NDV serum was also prepared with F-strain of Newcastle disease virus (Baby Chick Ranikhet Disease Vaccine, DLS, Dhaka, Bangladesh). The prepared hyperimmune sera were aliquoted into 1 mL volume and stored at -20°C until used.

AGIDT

The test was performed following the procedures described by Wood et al (1979). Briefly, the central well of a glass slide coated with melted agarose gel was loaded with known hyperimmune sera against IBDV and peripheral wells with reference antigen of IBDVs and bursal suspensions. Slides were kept in moist chamber for 48-72 hours at 4°C and observed for antigen antibody reaction in the form of precipitation lines between the central and peripheral wells.

Immunohistochemical detection of IBDV

Immunohistochemistry (IHC) was performed with slight modifications following the procedures as de-

scribed by Tanimura et al (1995). Formalin fixed bursal tissues were embedded in paraffin and 5-micron-thick sections were cut using a sliding microtome (MIC 509, Euromex, Japan) and mounted on glass slides. The sections were deparaffinized twice for 5-minute treatments in 100% xylene and hydrated through a series of descending graded ethanols (twice for 3-minute treatments in 100% ethanol and once for 3-minute treatment each in 95%, 90%, 80% and 70% ethanols) at room temperature. Hydrated samples were rinsed in distilled water and reacted with 5% formaldehyde in PBS for 30 min instead of 0.3% hydrogen peroxide in 99.6% methanol for fixation. Then the samples were rinsed with PBS and treated with 1% NP40 in PBS for 1 hr at room temperature instead of 0.1% actinase E. The samples were washed with PBS and blocked with 2% normal rabbit serum (Biosource, Camarillo, California, USA) for 2 hr at room temperature. Serum was blotted and slides were incubated overnight with undiluted chicken anti-IBDV hyperimmune serum in a humid chamber at 4°C . The slides were then washed in PBS and reacted for 1 hr with HRP-conjugated rabbit anti-goat IgG (1 : 500) (Bethyl Lab., Inc., USA) diluted in PBS. After washing in PBS, the sections were incubated for 5 min in 0.02% diaminobenzidine tetrahydrochloride dihydrate (AppliChem, Darmstadt, Germany) in Tris-HCl buffer (pH 7.6) supplemented with 0.03% hydrogen peroxide. The slides were then washed with tap water for 5 min, counter stained with hematoxylin, dehydrated through ascending graded ethanols (70%, 80%, 90%, 95% and 100%) and cleared with xylene, and mounted with DPX for microscopic examination.

Histopathology

One or two tissue sections of each bursal sample were stained with hematoxylin and eosin according to the procedures as described by Luna (1968).

Extraction of viral RNA

The genomic viral RNA of IBDV was extracted from the reference IBDVs, bursal as well as embryo suspensions using the QIAamp Viral RNA Mini Kit (QIAGEN

GmbH, Hilden, Germany) according to the manufacturer's instructions.

Oligonucleotide primers

Primers Vv-fp775 (forward primer, 5'-AATTCTCATCACAGTACCAAG-3') and Vv-rp1028 (reverse primer, 5'-GCTGGTTGGAATCACAAT-3') specific for a 254 bp sequence located at nucleotide position 775 to 1,028 of VP2 hypervariable region of segment A of serotype 1 IBDV genome were used in RT-PCR. These primers were originally designed and used by Kusk et al (2005) for detection of IBDV.

RT-PCR

RNAs from bursal samples, embryo suspension and reference IBDVs were reverse transcribed to cDNA and amplified by a one-step RT-PCR (Ikuta et al, 2001) in 50 µl of reaction mixture containing 5 µl of 10× LA buffer, 2 µl of 10 mM dNTP, 2 µl of 25 mM MgCl₂, 1 µl prime RNase inhibitor, 0.3 µl AMV-RT, 1 µl of each primer, 13 µl RNA, 0.2 µl LA-Taq DNA polymerase and 24.5 µl DEPC water. Initially, 13 µl of RNA and required amount of DEPC water were taken in a PCR tube, mixed and placed on thermocycler (MJ Mini Thermocycler, Bio-Rad, USA), heated at 68°C for 5 min for initial denaturation of double stranded RNA followed by snap cooling on ice. Then reaction mixture was added to PCR tube, mixed gently and returned to thermocycler. Reverse transcription was performed at 42°C for 1 hr followed by activation of *Taq* polymerase at 94°C for 2 min, then 39 cycles of denaturation at 94°C for 30 sec, annealing at 45°C for 1 min and elongation at 60°C for 1.5 min. A final extension step was performed at 60°C for 10 min. After amplification, RT-PCR product was subjected to electrophoresis on 2% agarose gel containing ethidium bromide (5 µg/ml). The resulting band was examined under UV-transilluminator.

RESULTS

Isolation of IBDV in chicken embryos

Out of 35 bursal samples, 28 (80%) were found positive for virus isolation. Mortality of the embryos started 48 hr postinoculation (PI) and 20~60% embryos died within 5 days of PI. The dead embryos were edematous with cutaneous congestion and had some petechial hemorrhages. Thickening of the CAMs was observed in most cases. The isolates from three outbreaks of Mymensingh district were designated as BML-2/07, BML-8/07 and BMB-11/07, and those of two outbreaks of Chittagong district were designated as BCB-13/07 and BCL-14/07 and the isolates of two outbreaks of Tangail district were named as BTB-4/07 and BTB-5/07 respectively.

AGIDT

By AGIDT, 32 (91.4%) bursal samples were found positive for IBDV. Prominent line of precipitation was noticed between known positive anti-IBDV hyperimmune serum of the central well and bursal homogenates of the peripheral wells due to antigen and antibody reaction within 24~48 hr. The pattern of precipitation lines indicated that IBDVs detected in 29 bursal samples of six affected farms were identical to each

Fig. 1. Agar gel immunodiffusion test demonstrating the presence of IBDV in bursal samples of affected chickens. Ab: Known hyperimmune serum against IBDV, A: BML-2/07 isolate, B: BML-8/07 isolate, C: BCB-13/07 isolate, D: BCL-14/07 isolate, E: BTB-4/07 isolate, F: BTB-5/07 isolate, G: BMB-11/07 isolate, H: Positive control (Nobilis® Gumboro 228E, Intervet, Netherlands), I: BAU 404 vaccine strain (classical strain), N: Negative control (NDV). Arrow indicates spur formation.

other but were different from BAU 404 vaccine strain (classical strain) and IBDVs present in the remaining three samples of another farm (Fig. 1). However, IBDVs present in these three samples showed a line of complete identity with the reference IBDV antigens Nobilis® Gumboro 228E strain. No line of precipitation was observed in 3 (8.6%) samples making them negative for IBDV antigen.

Histopathology

The lesions typical of IBD were observed in all the 35 bursal samples. Moderate to marked cell depletion,

atrophy and necrosis in many follicles were observed. Severe edema of interfollicular septa was also evident (Fig. 2, Fig. 3).

Immunohistochemistry

Indirect immunoperoxidase staining of the bursal sections revealed the presence of IBDV antigen in 32 (91.4%) samples. The IBDV antigen was detected by brown colour deposit with 3, 3 diaminobenzidine (DAB) precipitate in tissue sections. The staining reaction was found mainly in the cortex of the lymphoid follicles (Fig. 4). Positive reactions were absent in bursal sections stained with nonimmune chicken serum and chicken anti-NDV serum in place of chicken anti-IBDV se-

Fig. 2. Section of bursa of Fabricius of 23-day-old broiler chickens naturally infected with IBDV showing follicular atrophy (arrow) and edema of the interfollicular septa (arrow head) (H&E stain, $\times 82.5$).

Fig. 3. Section of bursa of Fabricius of 33-day-old layer chickens naturally infected with IBDV showing necrotic follicle with lymphoid cell depletion (arrow head) and edematous interfollicular septa (arrow) (H&E stain, $\times 330$).

Fig. 4. Section of bursa of Fabricius of 33-day-old layer chickens naturally infected with IBDV showing brown colour materials (IBDV antigens, arrow) following indirect immunoperoxidase staining (H&E stain, $\times 330$).

Fig. 5. Section of bursa of Fabricius of 33-day-old layer chickens naturally infected with IBDV showing no brown colour materials (IBDV antigens) following indirect immunoperoxidase staining with chicken anti-NDV serum in place of chicken anti-IBDV serum (H&E stain, $\times 330$).

rum (Fig. 5).

RT-PCR

Of the 35 bursal samples, 34 (97.1%) samples generated 254 bp amplicon by RT-PCR as ascertained by agarose gel electrophoresis (Fig. 6). One (2.9%) sample was negative for IBDV showing no amplified product in RT-PCR. Same amplicon size of DNA was also observed by RT-PCR amplification from RNA samples of two vaccine strains of IBDV i.e. BAU 404 (DLS, Dhaka, Bangladeshi) and 228E (Intervet International, Netherlands) as well as of the corresponding embryo suspensions of 28 bursal samples which were positive

for virus isolation in chicken embryos (Fig. 7). No amplified products of RT-PCR were detected from the bursal sample of the uninfected control birds.

DISCUSSION

After the emergence of vvIBDV strains among the poultry population of Bangladesh (Islam et al, 2001), there were some sporadic outbreaks of IBD in commercial flocks in Bangladesh (Islam and Samad, 2003). However, acute IBD has not been reported for some years since 2003. In 2007, many outbreaks of acute IBD throughout the country have caused concern in the

Fig. 6. RT-PCR amplified products from bursa of Fabricius following 2% agarose gel electrophoresis and ethidium bromide staining. M: DNA marker (100 bp), lane 1: Negative control (bursal suspension negative for IBDV), lane 2: Positive control (BAU 404, DLS, Dhaka), lane 3: Negative control (NDV), lane 4: Positive control (Nobilis[®] Gumboro 228E, Intervet), lane 5: BML-2/07 isolate, lane 6: BML-8/07 isolate, lane 7: BMB-11/07 isolate, lane 8: BCB-13/07 isolate, lane 9: BCL-14/07 isolate, lane 10: BTB-4/07 isolate, lane 11: BTB-5/07 isolate.

Fig. 7. RT-PCR amplified products from isolates from embryo inoculation following 2% agarose gel electrophoresis and ethidium bromide staining. M: DNA marker (100 bp), lane 1: Negative control (bursal suspension negative for IBDV), lane 2: Positive control (Nobilis[®] Gumboro 228E, Intervet), lane 3: BML-2/07 isolate, lane 4: BML-8/07 isolate, lane 5: BMB-11/07 isolate, lane 6: BCB-13/07 isolate, lane 7: BCL-14/07 isolate, lane 8: BTB-4/07 isolate, lane 9: BTB-5/07 isolate.

poultry industry. The present study reports the isolation and identification of IBDVs circulating in the field that caused acute outbreaks of IBD in four broiler and three layer farms during the year 2007.

In the present study, 9-day-old embryonated chicken eggs were used for isolation of viruses and 28 (80%) bursal samples were found positive for IBDV using eggs from seronegative birds. The rate of isolation of viruses could be higher if SPF eggs were used. The viruses induced 20~60% mortality within 2~5 days of PI, with lesions similar to those described earlier (Rosales et al, 1989; Abdel-Alim and Saif, 2001). Abdel-Alim and Saif (2001) reported 100% mortality of SPF embryos within 3~4 days of PI with very virulent strains of IBDV.

The presence of IBDV antigen was confirmed in 32 (91.4%) bursal samples by AGIDT. The higher rate (>80%) of detection of IBDV by AGIDT was reported by Nachimuthu et al(1995). AGIDT clearly indicated that IBDVs of six outbreaks were identical to each other but not to IBDVs of another one outbreak and BAU 404 vaccine strain (classical strain) as they formed spurs at the junction between the lines of precipitation. However, IBDVs of that outbreak were identical to Nobilis® Gumboro 228E vaccine strain which led to assume that there might have occurred vaccine failure. AGIDT has been reported by a number of workers to be useful in easy screening of the field samples, prior to either isolation of the virus or to applying other techniques for virus detection and characterization (Rosales et al, 1989; Kadam and Jhala, 2003).

Histological changes observed in all the bursal sections were similar to the observations of Rosales et al (1989) and Hassan et al(1996). Moderate to severe bursal atrophy because of lymphoid cell depletion observed in this study may be indicative of classical or highly virulent nature of IBDVs as reported earlier (Sharma et al, 1989). Diagnosis of IBDV infections using histopathology is not always reliable, because other infectious and non-infectious agents can cause bursal atrophy (Riddell, 1987) as well. Therefore, immunohistochemical demonstration of viral antigen may be useful for differential diagnosis (Cruz-Coy et al, 1993). In the present study, 32 (91.4%) samples were found positive for IBDV by immunohistochemistry. IBDV antigens were

detected mainly in the cortical portion of the bursal follicle. The results correlated well with the immunoperoxidase detection of IBDV antigen from bursal samples of IBD affected chickens as reported previously by earlier workers (Jonsson and Engstrom, 1986; Tanimura et al, 1995).

Thirty four (97.1%) bursal samples and the positive control samples produced amplicon of approximately 254 bp as expected with Vv-fp775 and Vv-rp1028 primer pair by RT-PCR. Kusk et al (2005) has also successfully targeted the same gene sequences using the same primer pair used in the present study. The application of RT-PCR as a tool for the diagnosis of IBDV infection has been widely reported (Wu et al, 1992; Kabell et al, 2005).

In conclusion, results of isolation and identification by AGIDT, IHC and the analysis of viral genome by RT-PCR confirmed the recent outbreaks of IBD in commercial poultry of Bangladesh. Moreover, histopathological findings and results of AGIDT gave a clear indication that the isolates of six outbreaks were different from classical strain and seems to be of very virulent strain. On the other hand, the isolates from the other outbreak seems to of classical strain. Further research on characterization of the isolates of IBDV of Bangladesh at molecular level by nucleotide sequencing of hypervariable region of the VP2 gene in order to determine the possible emergence of IBDV strains with modified antigenic and/or virulence properties is in progress.

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