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Detection of Infectious Bursal Disease Virus in Field Outbreaks in Broiler Chickens by Reverse Transcription-Polymerase Chain Reaction

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Abstract: During the period from July 2002 to June 2003, infectious bursal disease (IBD) was suspected in 101 commercial flocks of broiler chickens on the basis of clinical and post mortem findings in some districts of the Haryana state, India. Bursal samples were collected randomly from 20 flocks for the detection of infectious bursal disease virus (IBDV) by reverse transcription- polymerase chain reaction (RT-PCR) and nested PCR assay. IBDV could be detected in 17 samples as evidenced by amplification of 643 bp fragment of the very variable region of VP2 gene of virus by agarose gel electrophoresis. The authenticity of the amplicons was further confirmed by nested PCR generating amplicons of 552 bp using internal primers. The results of the present study indicated that RT-PCR followed by nested PCR can be used to diagnose field outbreaks of IBD in poultry because of its rapidity, accuracy and sensitivity.

Key words: Infectious bursal disease, diagnosis, RT-PCR, broiler chickens

Introduction

Infectious bursal disease (IBD) also known as Gumboro disease, is an acute, highly contagious viral disease of young chickens. The causative agent is infectious bursal disease virus (IBDV) which belongs to the genus *Avibirnavirus* with in the family Birnaviridae (Dobos *et al.*, 1979). It is a nonenveloped icosahedral, bisegmented, double stranded RNA virus with a diameter of about 55-60 nm (Ismail and Saif, 1990). This virus can be differentiated into two serotypes by virus neutralization test (McFerran *et al.*, 1980). Serotype 1 contains the pathogenic strains where as serotype 2 strains are not pathogenic to chickens (Ismail and Saif, 1990). Pathogenic serotype 1 IBDV field strains can be grouped into classical, antigenic variant and very virulent (vv) strains (Brown *et al.*, 1994).

The genome of IBDV consists of two segments of dsRNA. The larger segment A (3.2 kb) encodes for viral proteins VP2, VP3 and VP4, which are produced by auto proteolysis of a 110 kDa precursor polyprotein from a single large open reading frame (ORF) (Hudson *et al.*, 1986). Segment A also encodes for putative VP5, a 17 kDa protein, encoded from a small ORF, partly overlapping the polyprotein ORF has recently been reported to play role in cell lysis and release of virus (Lombardo *et al.*, 2000). Genome segment B (2.9 kb) encodes for a 90 kDa RNA dependent RNA polymerase protein, VP1 (Azad *et al.*, 1985). VP2 and VP3 are the major structural proteins of the virion. VP2 is major host protective antigen of IBDV and contains the antigenic region responsible for the induction of neutralizing antibodies (Eterradossi *et al.*, 1997), where as, VP3 is considered as a group specific antigen as it is recognized by monoclonal antibodies directed against VP3 from strains of both serotypes 1 and 2 (Becht *et al.*, 1988). VP4 is involved in the processing of 110 kDa precursor polyprotein (Jagadish *et al.*, 1988).

Conventional diagnosis of IBD relies upon clinical findings, pathological changes, and virological and serological methods such as agar gel precipitation test, electron microscopy, fluorescent antibody test, counter-immunoelectrophoresis, indirect ELISA, serum neutralization test etc. The application of molecular techniques like reverse transcription- polymerase chain reaction (RT- PCR) as a tool for the diagnosis of IBDV infection has been reported in the past (Lee *et al.*, 1994; Jackwood and Jackwood, 1997; Banda and Villegas, 2004). The present work aims at detection of IBDV from field outbreaks in broiler chickens in Haryana state, a North Western state of India.

Materials and Methods

Collection of samples: During the period from July 2002 to June 2003, IBD was suspected in 101 commercial broiler flocks on the basis of clinical signs and postmortem findings. Bursal tissues from 20 affected flocks were collected from Hisar, Fatehabad, Sirsa, Bhiwani and Jind districts of Haryana State, in 50% buffered glycerine for diagnosis by RT-PCR. One sample consisted of two to five bursa pooled from the affected birds in a flock. The samples after collection were kept at -20°C till use. Four IBD vaccines (Georgia,

MB, Intermediate and Intermediate Plus) currently used in this region were included as positive control. Bursa samples collected from apparently healthy unvaccinated birds served as negative control.

Total RNA extraction: Total RNA was extracted directly from the bursal tissues and the vaccines using TRIzol reagent (Life Technologies, USA) according to the manufacturer's protocol. The volume of TRIzol used for the vaccine strains corresponded to that of the distilled water required for vaccine reconstitution. Briefly, 500µl of TRIzol reagent was added to approximately 100mg bursal tissue and homogenized using pestle and mortar. The homogenate was centrifuged at 12,000rpm for 15 minutes. The supernatant was collected and mixed with 200µl of chloroform. The aqueous phase was separated by centrifugation at 12,000rpm for 15 min at 4°C. The RNA in the aqueous phase was precipitated with 500µl of isopropanol and collected by centrifugation at 12,000rpm for 10 min. After washing with 70% ethanol, the pellet was dissolved in nuclease free water and stored at -20°C until used.

Reverse Transcription- Polymerase Chain Reaction (RT-PCR): The total RNA extracted was subjected to reverse transcription using 100ng random hexamer primers, 50ng heat denatured viral RNA, 50units RNAase inhibitor, 2µl of 0.1M DTT, 1µl of 10mM dNTPs mix, 4µl of 5X RT buffer and 200 units Superscript II reverse transcriptase (Life Technologies, USA). The 20 µl reaction mixture was incubated at 25°C for 10min and then at 42°C for 50min. Reverse transcriptase was inactivated by heating at 70°C for 15min. The oligonucleotide primers forward- 5'-TCACCGTCCTCAGCTTAC-3' (nucleotide position 587-604) and reverse- 5'-TCAGGATTTGGGATCAGC- 3' (nucleotide position 1212-1229) described by Liu *et al.,* (1994) were used for the amplification of 643 bp amplicons corresponding to very variable region of the VP2 gene of IBDV. For the amplification, 6µl of cDNA was incubated in total volume of 50µl reaction mix containing 5µl 10X PCR buffer, 20pmol each of the forward and reverse primers, 1µl of 10mM dNTPs mix, 3U of *Taq* DNA polymerase (Bangalore Genei, India). The incubation temperature and duration of each cycle of the PCR were 1min at 94°C for denaturation, 1min at 52°C for annealing and 1min at 72°C for extension. The amplification was carried out for 35 cycles with final extension at 72°C for 10 min.

Confirmation of PCR Products: The PCR products (5µl aliquots) were separated on a 1.5% agarose gel stained with ethidium bromide. For determining the DNA segments size, the 100bp DNA marker (Bangalore Genei, India) ranging from 100 to 1000 bp was used. For further confirmation, the nested PCR of the primary amplicons (643 bp) was carried out using forward primer- 5'-CGCTATAGCGCTTGACCCAAAAA-3' (nucleotide position 651- 673) and reverse primer- 5'-CTCACCCCAGCGACCGTAACGACG-3' (nucleotide position 1179-1202) amplifying 552bp internal sequence of the very variable region of VP2 gene of IBDV as described by Kataria *et al.* (1998). Primary amplicons (1µl) of 643 bp diluted 1:10 in nuclease free water were used for nested PCR. Other reagents and cycling conditions were same as for primary PCR except the annealing temperature was 62°C.

Results and Discussion

Clinical findings and post mortem changes: In most of the affected flocks, the birds revealed the signs and symptoms of dullness, depression, anorexia, ruffled feathers, inability to move followed by death. There was yellowish white or greenish yellow diarrhoea in most of the affected birds. Mortality due to the disease increased with the progression of disease and peaked at third and fourth day and then started declining.

In almost all the flocks, the postmortem lesions were observed in bursa of Fabricius. The changes in bursa in acute form of the disease included edematous and swollen bursa, presence of gelatinous exudate around bursa, with hemorrhages (Fig. 1). In addition, severe hemorrhages on thigh and pectoral muscles were also recorded (Fig. 1). In the chronic form of the disease, the bursal changes comprised of atrophy and/or presence of cheesy core inside the bursa. The hemorrhages on muscles were of milder degree in sub-acute form of disease and were mild or absent in chronic form of disease. In some of the flocks, hemorrhages at the junction of proventriculus and gizzard were also recorded. Besides these, swollen kidneys and enlargement of liver were also noticed during postmortem examination of the broiler chicks. More or less similar types of signs and symptoms and/or post mortem findings have been reported earlier (Mohanty *et al.*, 1971; Lukert and Hitchner, 1984; Jindal *et al.,* 2004).

Diagnosis by RT-PCR: Infectious bursal disease virus could be detected in 17 of the 20 field samples and in all the four vaccine strains by RT-PCR. The primary PCR amplicons yielded a single specific band of 643 bp on ethidium bromide stained 1.5% agarose gel without any amplification in apparently healthy unvaccinated bursal sample (Fig. 2a and 2b). Further confirmation by nested PCR amplification resulted in expected 552 bp product in all the primary PCR positive field samples and the vaccine strains. However, there was no amplification in the negative control after nested PCR (Fig. 3a and 3b).

Infectious bursal disease is one of the major problems faced by poultry industry in India and is responsible for considerable economic losses. In Haryana state, the disease has been recorded regularly in broiler chickens

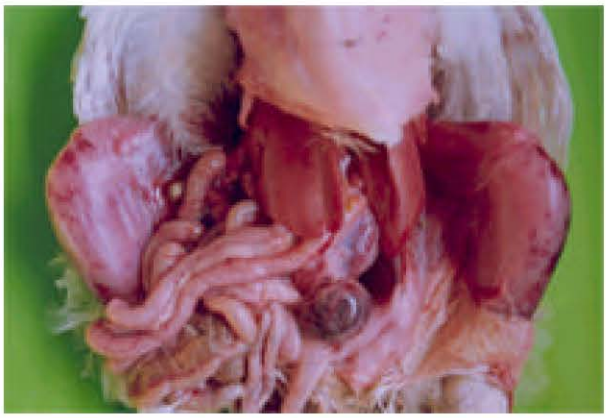
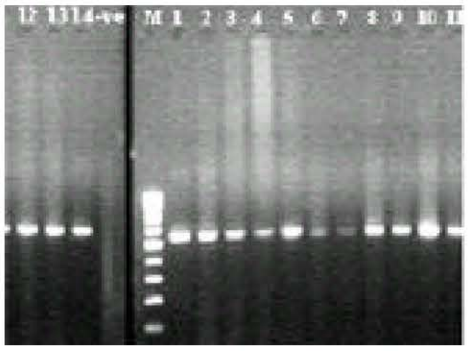


Fig. 1: Hemorrhagic bursa and hemorrhages on thigh muscles in a bird suffering from infectious bursal disease.

2a

643bp



2b

643bp

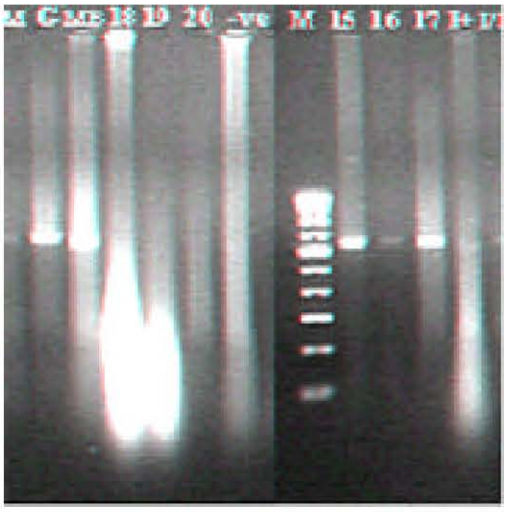


Fig. 2 a, b: Agarose gel electrophoresis of primary PCR products of field samples (1-20) and four vaccine strains (Intermediate Plus, I+; Intermediate, IM; Georgia, G and MB) showing amplicons of 643bp.
Lane M : 100 bp DNA molecular size marker
Lane-ve : negative control

inspite of regular vaccination. During the period from July 1994 to June 2003, the disease affected 8.89% (795) flocks in Hisar and adjoining areas in the state and was recorded both in the vaccinated and unvaccinated flocks. The cumulative mortality and case fatality rates during the nine year period due to this disease ranged from 2.47- 4.40% and 48.60- 70.55%, respectively (Jindal *et al.*, 2004).

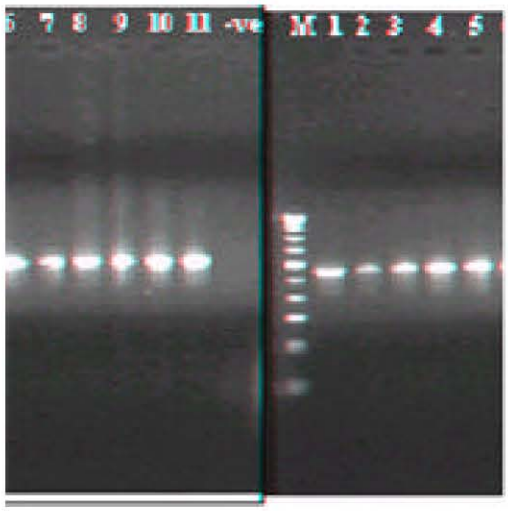
The conventional methods routinely used for diagnosis of IBDV infection are laborious, time consuming and less sensitive. Hindrance for virus isolation is that, most of very virulent field isolates do not replicate in common tissue culture (van den Berg *et al.*, 1991), where as for virus neutralization test, the field strains need to be adapted to grow *in vitro*. Furthermore, there is always a risk of modification of antigenic and pathologic characteristics of the virus during adaptation procedure (Lukert and Saif, 1997).

Knowledge of the molecular make up has led to the development of more sensitive and specific tests. In the present study, efforts were made to assess the diagnostic potential of highly sensitive technique of reverse transcription- polymerase chain reaction (RT-PCR) on clinical field samples. Seventeen of the 20 bursal samples were found positive for IBDV by RT-PCR using very variable region of VP2 gene confirming the presence of IBDV.

Total RNA extracted from infected bursal tissue homogenates by TRIzol reagent yielded sufficiently pure RNA for RT-PCR. Random hexanucleotide primers instead of specific primers used for reverse transcription of viral RNA had an advantage of producing random cDNA fragments which could be amplified by any set of the primers. Use of random primers increases the sensitivity of PCR. Complete denaturation of double stranded RNA to convert it into single stranded prior to c-DNA synthesis is critical during RT-PCR. Many workers have used denaturants like dimethyl sulphoxide (DMSO) or methyl mercuric hydroxide (MMH), instead of heat denaturation alone in order to increase the sensitivity of the test. Vakharia *et al.* (1992) used MMH to denature double stranded RNA prior to cDNA synthesis. Qian and Kibenge (1994) found heat denaturation at 65°C for 90 min along with DMSO to be superior to heat denaturation alone at 65°C for 10 min. In this study, heat denaturation of the double standard RNA (dsRNA) in boiling water bath at 95°C for 5 min followed by snap chilling in ice prior to reverse transcription without using highly toxic denaturants like methyl mercuric hydroxide (MMH) or dimethyl sulphoxide (DMSO) was found to be sufficient for VP2 gene amplification. More or less similar observations were also made by Kataria *et al.* (1998). Also the heat denaturation of template cDNA in the reaction mixture prior to addition of *Taq* DNA polymerase insured improved efficiency and stability of the enzyme over longer cycling period. The problem of non specific

3a

552bp



3b

552b

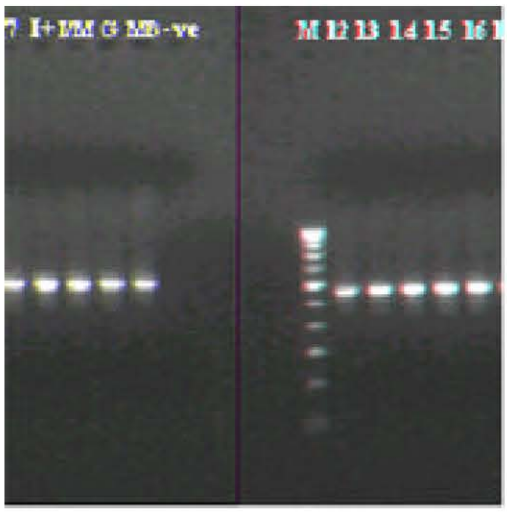


Fig. 3:a, b: Agarose gel electrophoresis of nested PCR products of field samples (1-17) and four vaccines (Intermediate Plus, I+; Intermediate, IM; Georgia, G and MB) showing amplicons of 552 bp
Lane M : 100 bp DNA molecular size marker
Lane-ve : negative control

amplification observed initially was over come by setting the annealing temperature to 52°C for primer pair #1. The magnesium ion (Mg^{++}) concentration of 1.5mM was found most suitable with the primer set. However, Jackwood and Sommer (1998) reported the requirement of 2-4 mM Mg^{++} concentration for IBDV PCR.

The RT-PCR technique thus standardized could easily be used for the amplification of very variable region of VP2 gene of IBDV field isolates. The authenticity of PCR products by the size of the amplicons has been verified by other workers too (Lin *et al.*, 1993; Liu *et al.*, 1994; Kataria *et al.*, 1998). The specificity of RT-PCR was confirmed by absence of amplification in unvaccinated apparently healthy bursal tissue taken as negative control. The specificity of RT-PCR for diagnosis of IBDV was further confirmed by nested PCR. Nested PCR has been considered to be more sensitive for IBDV than conventional RT-PCR (Liu *et al.*, 2002). The RT- PCR followed by nested PCR can be used as a tool for the diagnosis of IBDV infection (Jackwood and Jackwood, 1994; Lee *et al.*, 1994; Tham *et al.*, 1995) because of the existing rapidity, accuracy and sensitivity of these methods. These nucleic acid based techniques are useful tool for detecting IBDVs as the virus can be detected and typed without isolation and propagation in cell cultures or embryonated eggs, even when the virus is present in very minute quantity and has lost its infectivity. The application of these techniques on more numbers of samples followed by further studies using restriction enzyme analysis and sequencing will be helpful in generating epidemiological information in order to formulate a vaccination strategy for effective control of the disease.

References

Azad, A.A., S.A. Barett and K.J. Fahey, 1985. Characterization and molecular cloning of the double stranded genome of an Australian strain of infectious bursal disease virus. Virol., 143: 35-44.

Banda, A. and P. Villegas, 2004. Genetic characterization of very virulent infectious bursal disease viruses from Latin America. Avian Dis., 48: 540-549.

Becht, H., H. Muller and H.K. Muller, 1988. Comparative study on structural and antigenic properties of two serotypes of infectious bursal disease virus. J. Gen. Virol., 69: 631-640.

Brown, M.D., P. Green and M.A. Skinner, 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: 675-80.

Dobos, P., B.J. Hill, R. Hallet, D.T.C. Kells, H. Becht and D. Teninges, 1979. Biophysical and biochemical characterization of five animal viruses with bisegmented double-stranded RNA genomes. J. Virol., 32: 593-605.

Eterradossi, N., D. Toquin, G. Rivallan and M. Guittet, 1997. Modified activity of a VP2-located neutralizing epitope on various vaccine, pathogenic and hypervirulent strains of infectious bursal disease virus. Arch. Virol., 142: 255-270.

Hudson, P.J., N.M. McKeru, B.E. Power and A.A. Azad, 1986. Genomic structure of the large segment of infectious bursal disease virus. Nucleic Acid Res., 14: 5001-5012.

Ismail, N.M. and Y.M. Saif, 1990. Differentiation between antibodies to serotype 1 and 2 infectious bursal disease viruses in chicken sera. Avian Dis., 34: 1002-1004.

Jackwood, D.J. and R.J. Jackwood, 1994. Infectious bursal disease viruses: molecular differentiation of antigenic subtypes among serotype 1 viruses. Avian Dis., 38: 531-537.

Jackwood, D.J. and R.J. Jackwood, 1997. Molecular identification of infectious bursal disease virus strains. Avian Dis., 41: 97-104.

Jackwood, D.J. and S.E. Sommer, 1998. Genetic heterogeneity in the VP2 gene of infectious bursal disease viruses detected in commercially reared chickens. Avian Dis., 42: 321-339.

Jagadish, M.N., V.J. Staton, P.J. Hudson and A.A. Azad, 1988. Birnavirus precuson polyprotein is processed in Escherichia coli by its own virus encoded polypeptide. J. Virol., 62: 1084-1087.

Jindal, N., N.K. Mahajan, D. Mittal, S.L. Gupta and R.S. Khokhar, 2004. Some epidemiological studies on infectious bursal disease in broiler chickens in parts of Haryana, India. Int. J. Poult. Sci., 3: 478-482.

Kataria, R.S., A.K. Tiwari, S.K. Bandyopadhyay, J.M. Kataria and G. Butchaiah, 1998. Detection of infectious bursal disease virus of poultry in clinical samples by RT-PCR. Biochem. Mol. Biol. Int., 45: 315-322.

Lee, L.H., L.J. Tingh, J.H. Shien and H.K. Sheih, 1994. Single tube non-interrupted reverse transcription-PCR for the detection of infectious bursal disease virus. J. Clin. Microbiol., 32: 1268-1272.

Lin, Z., A. Kato, Y. Otaki, T. Nakamura, E. Sasmaz and S. Ueda, 1993. Sequence comparisons of a highly virulent infectious bursal disease virus prevalent in Japan. Avian Dis., 37: 315-23.

Liu, H., J.J. Giambrone and T. Dormitorio, 1994. Detection of genetic variations in serotype 1 isolates of infectious bursal disease virus using polymerase chain reaction and restriction endonuclease analysis. J. Virol. Meth., 48: 281-291.

Liu, J., J. Zhou and J. Kwang, 2002. Antigenic and molecular characterization of recent infectious bursal disease virus isolates in China. Virus Genes, 24: 135-47.

Lombardo, E., A. Maravet, I. Espinosa, A. Fernandez-Arias and J.F. Rodriguez, 2000. VP5, the non-structural polypeptide of infectious bursal disease virus, accumulates within the host plasma membrane and induces cell lysis. Virol., 277: 345-357.

Lukert, P.D. and S.B. Hitchner, 1984. Infectious Bursal Disease. In: Diseases of Poultry, eds. Hofstad M. S., H. J. Barnes, B. W. Calnek, W. M. Reid and H. W.Yoder Jr., 8th edn. Iowa State University Press, Ames, USA, pp: 566-576.

Lukert, P.D. and Y.M. Saif, 1997. Infectious Bursal Disease. In: Diseases of Poultry, eds., Calnek B.W., H.J. Barnes, C.W. Beard, I. R., McDougald and Y.M. Saif , 10th edn. Ames, IA: Iowa State University Press, pp: 721-738.

McFerran, J.B., M.S. McNulty, E.R. McKillop, T.J. Connor, R.M. McCracken, D.S. Collins and G.M. Allan, 1980. Isolation and serological studies with infectious bursal disease viruses from fowl, turkeys and ducks: demonstration of a second serotype. Avian Pathol., 9: 395-404.

Mohanty, G.C., A.P. Pandey and B.S. Rajya, 1971. Infectious bursal disease in chicken. Curr. Sci., 40: 181-184.

Qian, B. and F.S.B. Kibenge, 1994. Observation on polymerase chain reaction amplification of infectious bursal disease virus ds RNA. J. Virol. Meth., 47: 237-242.

Tham, K.M., L.W. Young and C.D. Moon, 1995. Detection of infectious bursal disease virus by reverse transcription-polymerase chain reaction amplification of the virus segment A gene. J. Virol. Meth., 53: 201-212.

Vakharia, V.N., B. Ahmed and J. He, 1992. Infectious bursal disease virus structural proteins expressed in a baculovirus recombinant confer protection in chickens. J. Gen. Virol., 74: 1201-1206.

van den Berg, T.P., M. Gonze and G. Meulemans, 1991. Acute infectious bursal disease in poultry: isolation and characterisation of a highly virulent strain. Avian Pathol., 20: 133-143.