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., 1990). Serotype 1 contains the pathogenic strains whereas 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 (Ettraddossi 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 (Jagdish 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’-TCACCCGTCTCAGCTTAC-3’ (nucleotide position 537-604) and reverse- 5’-TCAAGATTGGGATGCAGC-3’ (nucleotide position 1212-1229) described by Liu et al., (1994) were used for the amplification of 643 bp amplicons corresponding to 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’-CGCTATAGCGCTTGACCCAAAA-3’ (nucleotide position 651- 673) and reverse primer- 5’-CTCACCCCGACGGTACCGC-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 843 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 52°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.
inspite of regular vaccination. During the period from July
1994 to June 2003, the disease affected 8.66% (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
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Fig. 3a, 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 overcome by setting the annealing temperature to 52°C for primer pair #1. The magnesium ion (Mg²⁺) concentration of 1.5 mM was found most suitable with the primer set. However, Jackwood and Sommer (1998) reported the requirement of 2-4 mM Mg²⁺ 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 IBD 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
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