

COMPARISON OF AGAR GEL IMMUNODIFFUSION TEST, IMMUNOHISTOCHEMISTRY AND REVERSE TRANSCRIPTION - POLYMERASE CHAIN REACTION FOR DETECTION OF INFECTIOUS BURSAL DISEASE VIRUS

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ABSTRACT

The objective of this study was to compare agar gel immunodiffusion test (AGID) and immunohistochemistry (IHC) with reverse transcriptase-polymerase chain reaction (RT-PCR) in terms of sensitivity and specificity for the detection of infectious bursal disease virus (IBDV). Thirty-five bursal samples collected from field outbreak of IBD were evaluated by all 3 diagnostic tests. Sensitivity and specificity of both AGIDT and IHC with RT-PCR was 94.12% and 100%, respectively. Both AGIDT and IHC showed a 94.29% association with RT-PCR with a kappa value of 0.482, indicating a moderate degree of agreement. The Cochran's Q value was 4.00, which is lower than the critical value, indicating that the methods did not differ significantly ($p > 0.05$) from each other in detection of samples as positive or negative. However, RT-PCR had distinct edge over these two tests employed in the study.

Key words: Sensitivity, specificity, AGID, immunohistochemistry, RT-PCR, IBDV

INTRODUCTION

The confirmative diagnosis of clinical and subclinical cases of IBD is very important for formulating effective strategy for control of the infection. Various assays have been reported for detection of the virus with different levels of sensitivity and specificity (Mahmood and Siddique, 2006), and their comparison in terms of sensitivity has always been found relevant to arrive at proper diagnostic protocols. Of late, the molecular methods like RT-PCR, have also been employed for early and precise detection of the viral nucleic acid. Conventionally, detection of IBDV from field samples is performed serologically by agar gel immunodiffusion (AGID) test, enzyme-linked immunosorbent assay (ELISA) and virus neutralization (VN) test (McFerran, 1993). Several different kinds of ELISA procedures have been described for testing IBDV (Briggs *et al.*, 1986; Silim and Venne, 1989; Keck *et al.*, 1993). The ELISA using a monoclonal antibody enhances the detection and characterization of IBDV (Fahey *et al.*, 1991; Lasher and Davis, 1997). The ELISA allows the quantification of antibodies to IBDV and is therefore used for monitoring the immune status of the chicken flocks (Marquardt *et al.*, 1980), to check response of vaccination, natural field exposure and decay of maternal antibody titer (Lukert, 1986; Lambrecht *et al.*, 2000). The VN is the only serologic test to distinguish the serotypes 1 and 2 of IBDV and also to differentiate the antibodies to different subtypes of IBDV (Macreadie *et al.*, 1990). At least six different antigenic subtypes of IBDV serotype 1 viruses have been identified by the *in vitro* cross-neutralization test. However, VN is laborious and time consuming and therefore its use is limited to research applications. Another serological method used to detect antibodies to IBDV is the AGID test. This test has been adapted to the quantitative format (Cullen and Wyeth, 1975). It is rapid but insensitive. It does not detect serotypic differences and measures primarily group-specific soluble antigens (Lukert, 1986). Molecular techniques like reverse transcription-polymerase chain reaction (RT-PCR) assay and nucleic acid hybridization assay have been used by many workers for detection of IBDV (Wu *et al.*, 1992; Banda *et al.*, 2001; Jackwood and Jackwood, 1994). Moreover, RT-PCR coupled with restriction fragment length polymorphism (RFLP) can be used for genetic characterization of IBDV to determine the serotypes and pathotypes (Jackwood and Sommer, 1999; Zierenberg *et al.*, 2001; Badhy *et al.*, 2004). The purpose of this study was to compare the sensitivity and specificity of agar gel immunodiffusion test (AGID) and immunohistochemistry (IHC) with reverse transcriptase-polymerase chain reaction (RT-PCR) for the detection of infectious bursal disease virus (IBDV).

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MATERIALS AND METHODS

Thirty-five bursal samples collected from field outbreaks of suspected IBD were tested by three tests, agar gel immunodiffusion test, immunohistochemistry and reverse transcriptase-polymerase chain reaction for the detection of infectious bursal disease virus.

Agar gel immunodiffusion test (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 appearance of precipitation lines in between the central and peripheral wells.

Immunohistochemical detection of IBDV

Immunohistochemistry (IHC) was performed with slight modifications following the procedures as described 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 humidified 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) 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.

Extraction of viral RNA

The genomic viral RNA of IBDV was extracted from the reference IBDVs, bursal suspensions using the QIAamp Viral RNA Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions.

Reverse transcription-polymerase chain reaction (RT-PCR)

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 1028 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. 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 10X LA buffer, 2 µl of 10mM dNTP, 2 µl of 25mM 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.

Statistical analysis

Sensitivity and specificity of AGID and IHC with RT-PCR were calculated (Thrusfield, 2005). Cochran's Q value (Q_a) was also determined to find out the significant variation among three tests in detecting a sample as positive or negative. Overall agreement between different methods was estimated using kappa (k) statistic; k is an appropriate measure of the diagnostic agreement between tests beyond the agreement due to chance (Martin, 1977). The results were weighted on the basis of the k value, which ranges from 1 to -1 , where a value of -1 indicates complete disagreement between tests, 0 indicates agreement by chance only, 0.01–0.20 indicates slight agreement, 0.21–0.40 indicates a fair amount of agreement, 0.41–0.60 indicates moderate agreement, 0.61–0.80 indicates substantial agreement, and 0.81–1 indicates almost perfect agreement (Landis and Koch, 1977).

RESULTS AND DISCUSSION

A total of 35 samples were tested by all the three methods i.e. AGIDT, IHC and RT-PCR. Of these 35 samples, 32 (91.43%) were positive and 1 (2.86%) was negative for IBDV by all the three methods (Table 1) showing an agreement of 87.91% among the three methods. Two (5.71%) samples were positive by RT-PCR alone. The Cochran's Q value is 4.00, which is lower than the critical value, indicating that the methods did not differ significantly ($p > 0.05$) from each other in detection of samples as positive or negative. Sensitivity and specificity of both AGIDT and IHC with RT-PCR was 94.12% and 100%, respectively (Table 2). Both AGIDT and IHC showed a 94.29% association with RT-PCR with a k value of 0.482, indicating a moderate degree of agreement.

Table 1. Detection rate of IBDV in 35 bursal samples by three methods

RT-PCR	Methods		No. (%) samples
	AGIDT	IHC	
+	+	+	32 (91.43)
–	–	–	01 (02.86)
+	–	–	02 (05.71)

RT-PCR: Reverse transcription-polymerase chain reaction, AGIDT: Agar gel immunodiffusion test, IHC: Immunohistochemistry, Overall observed agreement was 87.91%, Cochran's Q value (Q_a) was 4.00 for 2 df at $p > 0.05$ ($P = 0.1353$).

Table 2. Agreement between different methods measured by kappa (k) statistic

Methods	RT-PCR		Sensitivity	Specificity	Observed agreement (%)	k-value
	+	–				
AGIDT	+	32	94.12	100	94.29	0.482
	–	02				
IHC	+	32	94.12	100	94.29	0.482
	–	02				

Both AGID and IHC showed same sensitivity with RT-PCR, which is in agreement with the findings of Muhammad *et al.* (1995) and Hamoud *et al.* (2007). Agar gel immunodiffusion test 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). The results of IHC correlated well with the immunoperoxidase detection of IBDV antigen from bursal samples of IBD affected chickens as reported by earlier workers (Jonsson and Engstrom, 1986; Tanimura *et al.*, 1995). However, both AGID and IHC require around 24 hours to complete. Besides, these techniques are dependent on concentration of the virus/antigen in the BF.

Parthiban and Thiagarajan (2000) failed to detect IBDV antigen until 36 hrs PI by AGID. On the contrary, PCR was found to be a sensitive test in detecting presence of the virus at 24 hrs PI and even in frozen bursae for as long as four years (Stram *et al.*, 1994). In the present study, two samples detected negative by AGID and IHC, were found positive by RT-PCR. Thus, RT-PCR was found to be a most sensitive test in detecting IBDV from the bursal samples, which is in accordance with Lin *et al.* (1994) and Elankumaran *et al.* (2002). Therefore, RT-PCR had distinct edge over these two tests employed in the study.

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