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COMPARATIVE EFFICACY OF RT-PCR, AGPT AND REVERSE PASSIVE HAEMAGGLUTINATION ASSAY FOR THE DETECTION OF INFECTIOUS BURSAL DISEASE VIRUS IN BROILERS

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ABSTRACT

The reverse transcription polymerase chain reaction (RT-PCR), reverse passive haemagglutination assay (RPHA) and agar gel precipitation test (AGPT) were compared for the detection of infectious bursal disease virus (IBDV) in various organs of experimentally infected broilers. RT-PCR and RPHA required a time of 8 and 1 hour, respectively, whereas AGPT showed precipitation reaction at least after 24-48 hours of incubation. RT-PCR and RPHA were found sensitive to detect IBDV in tissues, whereas AGPT was less sensitive in the detection of IBDV. The RT-PCR was significantly more sensitive to detect IBDV as compared to RPHA, which was significantly more sensitive than AGPT in bursa of Fabricius, liver, kidneys, spleen and thymus samples. RPHA has given economical results for the detection of IBDV in samples from various organs and can be used in field conditions. However, RT-PCR was found to be the most sensitive test among the three tests compared in this study.

Key words: Broilers, Infectious bursal disease virus, RT-PCR, RPHA, AGPT.

INTRODUCTION

Infectious bursal disease (IBD) is an acute, infectious disease of young chicken characterized by high mortality, morbidity and immunosuppression (Cullen and Wyeth, 1975). Immunosuppression by the virus may also result in increased mortality and morbidity from other associated diseases (Fussell, 1998) and infection causes destruction of lymphoid organs especially the bursa of Fabricius. Infectious bursal disease virus (IBDV) induces a clinical disease in the susceptible chicken at 15-22 days of age (Hussain *et al*., 2003, Muhammad *et al*., 1995). The IBDV replicates in the growing cells of target organs (Mcllroy *et al.,* 1992).

Early detection of IBDV is the best method by which avian health professionals may attempt for vaccination or other symptomatic treatment programmmes (Fussell, 1998). The disease may be diagnosed by gross lesions (Snyder *et al*., 1988) and detection of antigens by nucleic acid probes in tissues (Jackwood, 1988). In a study using several procedures for detection of the virus in bursa of experimentally infected chickens, the reverse transcription polymerase chain reaction (PT-PCR) has been reported to be the most sensitive test (Abdel-Alim and Saif, 2001). The other method used for the detection of IBDV antigen is agar gel precipitation test (AGPT; Kwang *et al*., 1987). This test measures primarily group specific soluble antigens but does not detect serotypic differences (Cullen and Wyeth, 1975).

IBDV itself is not a directly haemagglutinating virus, however, the agglutination may be induced after coating the erythrocytes with any coupling reagent and sensitizing these coupled erythrocytes with specific hyperimmune serum against IBDV. Various tests like AGPT (Faraghar *et al*., 1974), fluorescent antibody technique, RPHA (Nachimuthu *et al*., 1995) and RT-PCR (Zahoor *et al*., 2005) have been applied for the detection of IBDV in different organs of the birds. The present study describes the evaluation of RPHA for the detection of the IBDV as compared to RT-PCR and AGPT.

MATERIALS AND METHODS

Virus and experimental design

Fifty one-day-old broiler chicks were procured from the local market and reared in the experimental animal house, Department of Veterinary Microbiology, University of Agriculture, Faisalabad, Pakistan. The feed, drinking water and housing conditions were maintained homogenous for all the groups. The chicks were not vaccinated against IBD and confirmed to be negative for antibodies against IBDV. At 18 days of age, 42 birds were orally inoculated with 10^5 EID_{50} of very virulent infectious disease virus (vvIBDV) isolated and characterized from the field outbreaks in Pakistan (Zahoor *et al*., 2005) and 8 birds were kept as uninoculated control. Six birds were sacrificed each on 1, 3, 5, 7, 10, 14 and 21 days post inoculation and the

bursa of Fabricious, spleen, kidneys, liver and thymus were collected. Each of these individual organs was homogenized in equal volume (w/v) of phosphate buffer saline (PBS) and clarified by centrifugation at 400xg for 15 minutes. The supernatant was collected and extracted with chloroform and stored at -20°C (Hussain *et al*., 2003) till subjected to RT-PCR, RPHA and AGPT.

Hyperimmune serum

Hyper-immune serum against vvIBDV was raised following Hussain *et al.* (2004). Ten one-day-old chicks were reared. The birds were primed at 10 days of age with IBDV live vaccine (Merial) and were given first booster on day 20 and second on day 30 with the oil based killed vaccine (Merial). On 14th day of the last booster, blood samples were collected, serum was separated and stored at –20°C for further use.

Sensitization of sheep erythrocytes

Using 0.5 ml of sodium citrate solution, 5 ml of sheep blood was collected and centrifuged to sediment the cells, and the supernatant was discarded. This procedure was repeated thrice and finally 2% sRBCs suspension in normal saline was prepared. An equal volume of 1:20,000 freshly prepared tannic acid solution was mixed with 2% washed sRBCs. The mixture was incubated at 37°C for 10 minutes, washed with normal saline and a suspension of 2% sensitized sRBCs was made to be incubated with the homogenates of samples suspected for IBDV.

Reverse Passive Haemagglutination (RPHA) test

The RPHA test was performed following the method of Nachimuthu *et al.* (1995) with some modifications. The test was carried out in 96 well (U-shaped bottom) microtitration plates using two fold dilutions of the antigen in 50 µl of PBS, followed by addition of 50 µl of 2% sensitized sRBCs. Then the microtitration plates were incubated at 37°C for 30 minutes and examined. A positive reaction consisted of marked haemagglutination, while a negative reaction was evidenced by button formation at the bottom of the well.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

A set of following primers (forward and reverse primers) with a sequence specific for VP2 gene amplification (Wang *et al*., 2004) was procured from Invitrogen Life Technologies, USA:

Forward (P1) =5'GGTCTAGAACGATCGCAGCGATGAC3'
Reverse (P2) =5'GGTCTAGACTACACCTTCCCCAATTG3'

RNA was extracted by TRIZOL® LS Reagent (Invitrogen Life Technologies, USA), following the manufacturer's instructions. The viral RNA was subjected to one-step RT-PCR for the amplification of VP2 using specific primers. RT-PCR cocktail composition and profile was followed as recommended (Invitrogen Life Technologies, USA, the manufacturer of "SuperScript® One-Step RT-PCR kit" cat #10928-042). The reaction mix contained 25 pmol of each primer, 0.2mM of each dNTPs, 2mM $MgCl_2$, 1 µl of Rt/Platinum® Taq Mix and 1× RT-PCR buffer (Invitrogen Life Technologies, USA). The RT-PCR profile was an initial step of predenaturation at 94°C for 2 minutes, followed by 40 cycles of denaturation at 94°C for 15 seconds, annealing at 60°C for 30 and extension at 72°C for 1.5 minutes. The final cycle was followed by a long extension at 72°C for 10 minutes. RT-PCR products after incubations were analyzed on 1% agarose gel electrophoresis.

Agar Gel Precipitation Test

The test was performed following the procedure described by Cullen and Wyeth (1975) with some modifications. The test was standardized for 0.9% Noble Agar composition in distilled water at 37°C, keeping pH of the gel at 7.0 and time for incubation of 24-48 hours.

Statistical analysis

The data obtained from all the three tests in both the groups were analyzed by one way analysis of variance. Duncan's Multiple Range Test was applied for multiple means comparisons. Statistical analysis system computer programme (SAS, 1987) was used for these analyses.

RESULTS AND DISCUSSION

Amplification of VP2 gene by RT-PCR is obvious by the presence of 1.44 kbp band (Figure 1). Preliminary AGPT method indicated the precipitation line, which was formed between IBDV-infected tissue samples and homologous antiserum. No line was formed between the samples prepared from uninoculated control chicks and antiserum to IBDV. In case of RPHA, a titer of 1:4 was considered negative because sometimes a negative tissue may give nonspecific agglutination. Hence a titre of 1:8 was considered as positive (Nachimuthu *et al*., 1995).

All the three tests showed higher number of positive samples for IBDV from all the different tissues on the third and fifth day post inoculation. The results

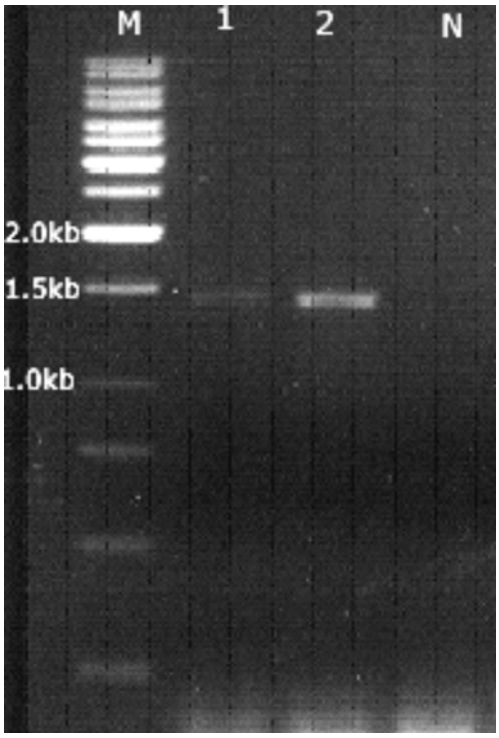

Figure 1: Gel electrophoresis of RT-PCR showing 1.44 kbp band in the samples collected from inoculated birds and no band (no VP2 gene was amplified) was observed in negative control birds. Lane M: 1kbp marker; lane 1 and 2: Samples from inoculated birds; Lane N: Negative control.

obtained with RT-PCR, AGPT and RPHA in detecting IBDV in the samples tested are summarized in Table 1. RT-PCR detected IBDV in more specimens than RPHA, followed by AGPT, which was found to be significantly less sensitive method for the detection of IBDV as compared with both of the other tests.

In RPHA test, all the samples showed reaction within one hour whereas in AGPT, the results of precipitation reaction could be finally declared after 24-48 hours of incubation. However, the most sensitive test (RT-PCR) took 5-7 hours duration to complete the analysis and this was expensive too.

All the 3 tests employed in the detection of IBDV are sensitive, though the RT-PCR and RPHA were significantly more sensitive than AGPT, whereas the former two methods were comparable to each other. RT-PCR was found to be the most sensitive but RPHA can be recommended for the routine use in the diagnostic laboratories because a large number of samples can be screened by this method and the results can be obtained within 1 to 3 hours. RT-PCR was found to be the most sensitive but at the same time it was the most costly test and required a well-equipped laboratory. AGPT can provide results in one to two days and it stands significantly less sensitive for routine screening studies. The results of the present study agreed with the findings of Samekata *et al.* (1979), who detected Rotavirus from faecal samples by using RPHA. The sensitivity of AGPT agreed with the results of Muhammad *et al.* (1995) and that of RPHA with Rajeswar and Dorairajan (1998).

In conclusion, RPHA test is an economical test for detection of IBDV and the shelf life of sensitized sheep RBCs stored at refrigeration makes it an ideal pen side diagnostic test for the screening of IBDV even though this test is less sensitive as compared to RT-PCR. The RPHA test enables better detection of IBDV, because it eliminates false positive reactions causing non-specific agglutination, as the titer of 1:8 is considered as positive one.

Table 1: Comparative sensitivity of RPHA, RT-PCR and AGPT for the detection of IBDV from different tissues of the inoculated broiler birds on various days post inoculation

PID[a]	Bursa			Spleen			Kidneys			Liver			Thymus		
	RP[b]	RT[c]	AG[d]	RP[b]	RT[c]	AG[d]	RP[b]	RT[c]	AG[d]	RP[b]	RT[c]	AG[d]	RP[b]	RT[c]	AG[d]
1	3	5	1	2	5	0	4	6	0	3	6	0	1	2	0
3	4	6	2	5	6	1	3	5	1	3	6	0	1	5	0
5	4	6	2	3	6	2	3	5	0	1	4	0	2	1	0
7	3	5	1	4	5	0	3	6	0	0	5	0	0	0	0
10	3	6	1	3	5	0	0	4	0	0	2	0	0	0	0
14	2	5	0	0	2	0	0	1	0	0	3	0	0	0	0
21	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0
	**	*	***	**	*	***	**	*	***	**	*	***	**	*	***

[a]Post-inoculation days; RP[b]: Reverse passive haemagglutination test; RT[c]: Reverse transcription-polymerase chain reaction; AG[d]: Agar gel precipitation test.

Columns with different number of stars for each organ are significantly different ($P<0.05$).

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