

TRYPSIN-INDUCED HEMAGGLUTINATION ASSAY FOR THE DETECTION OF INFECTIOUS BRONCHITIS VIRUS

M. S. Mahmood, M. Siddique, I. Hussain and A. Khan¹

Department of Microbiology, ¹Department of Pathology, Faculty of Veterinary Science, University of Agriculture, Faisalabad-38040, Pakistan

ABSTRACT

A trypsin-induced hemagglutination (THA) assay was standardized to detect infectious bronchitis virus (IBV) in allantoic fluid (AF) of embryonated eggs. The test was used in 20 samples, each collected from 5 different layer farms suspected for IBV. Allantoic fluid from inoculated embryos was harvested and treated with reagent grade trypsin at the percentages of 0.25, 0.50, 1.0 and 2.0 for 30 minutes to 3 hours at pH 7.2. The IBV in trypsinized AF was identified by clear and consistent agglutination of chicken red blood cells within 5 minutes of incubation at 37°C. The results indicated that AF treated with equal volume of 1.0% reagent grade trypsin elicited the hemagglutinating (HA) activity in 3.0 hours whereas 2.0% reagent grade trypsin elicited the HA activity only after 30 minutes incubation at 37°C. Sensitivity of THA was 92% as compared with 76% for agar gel precipitation test. Gross pathological lesions (curling and dwarfing) in chick embryo, intracerebral inoculation of un-weaned mice and pathogenicity test in one-day-old broiler chicks showed 79, 84 and 77% sensitivity, respectively.

Key words: - Infectious bronchitis virus, trypsin-induced hemagglutination, allantoic fluid.

INTRODUCTION

Avian infectious bronchitis (IB), caused by a *Coronavirus*, is an acute and highly contagious disease of the respiratory and urogenital tracts of chickens (Cavanagh and Naqi, 1997). The infection is confirmed by the isolation of IBV in either embryonated chicken eggs or tracheal organ cultures (Gelb and Jackwood, 1998). The IBV can also be demonstrated in sections of the tracheal mucosa and other tissues by immunofluorescence or immunoperoxidase assays (Naqi *et al.*, 1993). Serologic tests such as hemagglutination inhibition (HI), agar gel precipitation test (AGPT) and the enzyme-linked immunosorbent assay (ELISA) are used as well (deWit *et al.*, 1997). Recently, IBV has been detected from either tracheal swabs of infected birds or allantoic fluid (AF) from embryonated chicken eggs inoculated with the virus (Kwon *et al.*, 1993; Keeler *et al.*, 1998). Another recent advancement in IBV diagnostics is the detection and serotyping of AF containing IBV with the use of monoclonal antibodies. This is accomplished either by direct immunofluorescence or by antigen-capture ELISA (Yagyu and Ohta, 1994).

Great variation exists among members of the family Coronaviridae in their ability to agglutinate red blood cells. Although members of group II are efficient hemagglutinating agents, the others in groups I or III (where IBV is classified) are not so (Schultze *et al.*, 1991a). Several attempts have been made to make IBV

hemagglutinate chicken red blood cells (CRBCs) (Bingham *et al.*, 1975). Hemagglutination (HA) activity by IBV was first revealed after trypsin treatment, which was observed with IBV strains and was specifically inhibited by immune serum (Corbo and Cunningham, 1959). Moreover, IBV was reported to acquire hemagglutinating activity after treatment with phospholipase C (King, 1984). This activity could be inhibited by specific antiserum (Bingham *et al.*, 1975). However, the phospholipase C did not consistently induce HA activity on IBV (Schultze and Herrler, 1992), which has been postulated to be caused by the removal of a contaminating crude enzyme in preparations of phospholipase C (Schultze *et al.*, 1991b). Neuraminidase was successfully introduced to remove sialic acid residues from the spike glycoprotein of the virus envelope and to induce HA activity in IBV (Schultze *et al.*, 1991b).

Hemagglutination of trypsinized AF was reported to be efficient, specific, sensitive and economical to perform in our routine diagnostic practices. Trypsinized AF could be stored at -65°C for more than 3 weeks without any loss in hemagglutinating activity of virus (Corbo and Cunningham, 1959).

The present study was conducted to standardize the THA that can detect IBV by the treatment of inoculated AF with reagent grade trypsin enzyme and to compare its sensitivity and specificity with AGPT and other routinely practiced diagnostic procedures.

MATERIALS AND METHODS

A total of 100 samples (trachea and kidneys mixed) were collected from five different layer farms suspected of IB infection, 20 samples from each farm, in test tubes containing sterilized phosphate-buffered saline (PBS) supplemented with penicillin (10,000 IU per ml), streptomycin (10,000 ug per ml) and nystatin sulphate (1000 IU per ml). The pH of this transportation media was adjusted at 7.2. After collection, the samples were stored in the refrigerator at -4°C . They were reconstituted in PBS, centrifuged at 5000 rpm for 20 minutes and the supernatant was stored at -4°C for further use.

Embryonated chicken eggs inoculation

Nine-day-old embryonated chicken eggs (ECEs) were obtained from Experimental Poultry Farm, Department of Poultry Husbandry, University of Agriculture Faisalabad. Each virus sample, purified from the specimens following the method of Doherty (1967), was inoculated into a set of four nine-day-old ECEs via the chorioallantoic membrane.

Trypsinization

Working solutions of reagent grade trypsin (Sigma Chemical Company St. Louis, MO, USA) were prepared containing 0.25, 0.5, 1.0 and 2.0 percent trypsin in PBS with pH adjusted at 7.2. Allantoic fluid from inoculated dead and live embryos was collected 72 hours post-inoculation (PI) and treated directly by mixing 0.25 ml of AF from each set of inoculated embryos with 50 ul from each of the working solutions. After the addition of trypsin, all samples were held at 37°C for 30 minutes and 3 hours to determine optimal trypsin and AF incubation time. After incubation at 37°C for both time durations, all samples were placed at 4°C for 5 minutes.

Trypsin-induced Hemagglutination assay

Trypsin-induced Hemagglutination assay was performed for all the samples separately by gently mixing 50 ul of trypsin-treated AF with 50 ul of a 2% solution of CRBCs in a microtitration plate. Direct agglutination of the CRBCs was read within 5 minutes (Corbo and Cunningham, 1959). Clear and consistent HA was considered as positive.

Agar gel precipitation test

Samples positive for THA assay were subjected to AGPT using known hyperimmune serum against IBV. The procedure for AGPT was followed as described by deWit *et al.* (1997).

Specificity and sensitivity

Newcastle disease virus (NDV) and infectious bursal disease virus (IBDV) were inoculated individually into a set of three 9-day-old embryos via the chorioallantoic sac. Allantoic fluid from each set of embryos was treated with trypsin and subjected to THA assay.

A dual embryonic infection containing IBVH-120 was considered as positive control and each of the aforementioned samples was subjected to the same procedures. This assay was established to determine trypsin specificity for IBV in mixed dual infections.

The sensitivity of the THA to determine virus titer was compared with conventional embryo lesions. Each of the virus, vaccine and field samples was titrated in 9-day-old embryos in dilutions ranging from 10^{-2} to 10^{-6} and the results were calculated on the basis of embryo lesions observed 7 days PI. Typical lesions included membrane-bound embryos, clubbed down, stunting and urates of the mesonephros. The THA was performed 2 days PI on each individual embryo and the titer was calculated by the method of Reed and Muench (1938).

Un-weaned mice and embryonated chicken egg inoculation

Samples from all the groups were inoculated intracerebrally into un-weaned mice along with negative controls kept parallel for comparison, following the methods described by Ahmad *et al.* (1985). The same samples were inoculated separately into allantoic cavity of 9-day-old-embryos and examined for lesions 6 days PI.

Pathogenicity test

All the samples were subjected to pathogenicity test in one-day-old broiler chicks by intra-tracheal injection. The symptoms, lesions and mortality were recorded.

RESULTS AND DISCUSSION

Hemagglutination activity was recorded with all IBV positive samples and was confirmed by other tests. Complete, clear and consistent HA reactions were obtained within 5 minutes of incubation of IBV inoculated AF sensitized with 2.0% trypsin solution. Hemagglutination reactions were observed between 5 and 10 minutes of incubation at 37°C and remained stable for more than 2 hours at room temperature or at 4°C .

Table 1 shows THA activity after treatment of AF with trypsin at various concentrations. Standard reference IBV strain was IBVH-120 and five randomly selected samples were used to demonstrate the effect of different trypsin concentrations with respect to different time intervals. In the present study, it was evident that 2% final concentration of trypsin showed response within 30 minutes as compared to 1% concentration, which elicited the HA receptors on IBV at least after 3 hours of incubation.

Table 1: THA activities after treatment of AF with reagent grade trypsin at different concentrations

IBV isolates	Concentrations of trypsin (%)									
	0.00		0.25		0.50		1.00		2.00	
	30 minutes	3 hrs	30 minutes	3 hrs	30 minutes	3 hrs	30 minutes	3 hrs	30 minutes	3 hrs
Standard IBH-120	-	-	-	-	-	-	±	+	+	+
Sample # 1	-	-	-	-	-	±	+	+	+	+
Sample # 2	-	-	-	-	-	±	±	+	+	+
Sample # 3	-	-	-	-	-	-	+	+	+	+
Sample # 4	-	-	-	-	-	-	-	+	+	+
Sample # 5	-	-	-	-	-	±	±	+	+	+
Control (No virus)	-	-	-	-	-	-	-	-	-	-

- means no HA activity; ± means incomplete HA activity and + means complete HA activity

Specificity and sensitivity

Trypsin was found very specific in its ability to elicit HA activity of IBV. Table 2 shows that other non-hemagglutinating avian viruses were unaffected by trypsin activity. In addition, co-infection with other non-hemagglutinating viruses and IBV had no effect on the ability of trypsin to induce HA activity. Nevertheless, the hemagglutinating activity of NDV was not changed by trypsinization.

Table 2: Comparison of direct HA and THA on two different avian viruses with and without an IBV co-infection

Viruses	Viruses without IBV		Viruses with IBV	
	Direct HA test	THA test	Direct HA test	THA test
IBDV (Lukert)	-	-	-	+
NDV (Lasota)	+	+	+	+
No virus (-ve control)	-	-	-	+
IBV (+ve control)	-	+	-	+

Table 3 shows the sensitivities of all the tests performed to detect IBV. Trypsin-induced hemagglutination assay was 92% sensitive as compared with 76% sensitivity for agar gel precipitation test. Gross pathological lesions (curling and dwarfing) in chick embryo, intracerebral inoculation of un-weaned mice and pathogenicity test in one-day-old broiler chicks showed 79, 84 and 77% sensitivity, respectively. In the entire positive samples and known IB vaccine, THA was found superior regarding the sensitivity when AGPT and typical lesions of IBV were compared.

Animal inoculation and pathogenicity test

All the un-weaned mice injected with THA positive AF samples behaved similarly as in the mice into which known IBVH-120 was injected intracerebrally, which showed ascending paralysis leading to death within 3-4 hours, as described by Buxton and Fraser (1977). The embryos showed curling, dwarfing and clubbing of the down. Postmortem examination of the dead birds revealed pneumonic and congested lungs, sinusitis with thick nasal discharge, cloudy air sacs and crystal deposits of urates in the ureters of the birds died 6 days post-inoculation. A close correlation was established among the THA, AGPT, gross pathological embryo changes, un-weaned mice inoculation and intra-tracheal injection of one-day-old broiler chicks.

The objective of this study was to develop and standardize an economical and an easy assay for detection of IBV. Trypsin has been shown to elicit HA activity of IBV (Corbo and Cunningham, 1959). Therefore, in addition to THA, trypsin may be used for the production of stable and high-titer IBV antigen for hemagglutination inhibition (HI) assay as well. Trypsin was shown to be a strong and specific inducer of HA activity for IBV in embryos inoculated with IBV alone and when combined with several other avian viruses (Corbo and Cunningham, 1959). Although HA activity is maintained for NDV even after trypsinization but differentiation of NDV from IBV is easily accomplished (Villegas, 1998). The THA performed on vaccine as positive control was able to identify IBV infection at different 2-fold serial dilutions, as exhibited by embryo lesions. The present study indicates that the sensitivity of the THA is comparable to that of AGPT and visual interpretation of IBV infections in embryos, un-weaned mice and one-day-old chicks.

The present study shows that THA accurately identifies the presence of IBV 48 hours PI in chicken embryos and eliminates any unnecessary and additional blind passages in embryos. In addition, because all embryonic IBV lesions are not pathognomonic, the identification of IBV by the THA eliminates any guesswork in confirming IBV embryonic lesions.

Table 3: Comparison of the sensitivity of different diagnostic tests

Diagnostic tests	Vaccine (+ve control)	No virus (-ve control)	Farm No					Sensitivity (%)
			1	2	3	4	5	
THA	1	0	18	20	19	18	17	92
AGPT	1	0	15	16	14	18	13	75
Embryonic lesions	1	0	16	18	15	16	14	79
Un-weaned mice death	1	0	18	18	17	16	15	84
Day-old chicken (tracheal lesions)	1	0	17	17	15	15	13	77

On the basis of the present study, it can be concluded that a THA assay is specific, very sensitive, accurate, highly reproducible, simple and economical. Moreover, equally good HA activity may be obtained by sensitizing the AF with 2% trypsin, which needs only 30 minutes of incubation instead of 1% trypsin that needs at least 3 hours incubation. Therefore, the THA assay can easily be adopted in any laboratory for expeditious identification of IBV.

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