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Short Communication

An Improved Heamagglutination Inhibition Test for Rapid Diagnosis and Stereotyping of Avian Infectious Bronchitis Virus

Johar Hussian[1], Faiz Muhammad[2], Shafqat Fatimah Rehmani[3], Aqeel Ahmad[4], Nazir Ahmad Lone[4], Moomal Bughio[5], Syed Khurram Freed[1] and Shakeel Ahmed Khan[4]*

[1]*Department of Microbiology, University of Karachi, Karachi*
[2]*Department of Microbiology, Balochistan University of Information Technology, Engineering and Management Sciences (BUITEMS), Quetta, Balochistan*
[3]*Quality Operation Lab, University of Veterinary Sciences, Lahore*
[4]*Department of Biosciences, Barrett Hodgson University, Karachi*
[5]*Poultry Research Institute, Karachi*

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JH, FM and SFR conceived and designed the study. JH, MB and NAL collected the data. SKF and FM analyzed the data. JH, FM and AA wrote the article. SAK Supervised the work.

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Infectious bronchitis Virus (IBV), Heamagglutination Inhibition test (HI), Serological test, Antigen, Phospholipase C

ABSTRACT

Infectious bronchitis virus (IBV) causes an acute, highly contagious viral respiratory disease in poultry with huge economic impact and extremely difficult to control due to its multiple serotypes. The disease could be prevented by rapid diagnosis either molecular or serological test. However, the later test is inexpensive such as heamagglutination inhibition test (HI), but IBV fail to give Heamagglutination (HA) reaction without pretreatment. Therefore, we designed this study for preparation of IBV antigen by treating with different enzymes for HA reaction. IBV local isolates were characterized by SDS-PAGE and RT-PCR. The indigenous isolate HA antigens were treated with different proteolytic enzymes trypsin, neuraminidase and phospholipase C. The prepared antigen were stored at -86°C and used for HA test. All antigen prepared by different enzyme were found to give significant HA titer up to 7 $\log^2$ During stability test antigen prepared by phospholipase C were found most stable up to six month by giving constant 7 $\log^2$ HA titer, while neuraminidase induced antigen were stable up to five months (7 $\log^2$). Trypsin treated antigen were readily lost its activity from 7 $\log^2$ to 2 $\log^2$ after two months of incubation. During specificity test all antigens showed specific effect on IBV by eliciting agglutination of RBCs while other avian viruses avian influenza (AI), new castle disease virus (NDV) and infectious bursal disease virus (IBDV) were not affected by enzymatic inductions. Therefore, the antigen prepared by phospholipase C has been found to be more effective for HI test for rapid diagnosis of IBV during infection.

Infectious bronchitis virus (IBV) belongs to family Coronaviridae, order nidoviral. The genome of IBV is single stranded positive-sense RNA which expresses structural and non-structural proteins (Gonzalez *et al*., 2003). IBV causes a major loss to the poultry industry by causing highly contagious respiratory disease and nephritis with production of low-quality eggs which led to huge economic impact (Cavanagh, 2007; Lee *et al*., 2004). During infection, IBV show similar sign and symptom to other avian viruses' infection which hindered in presumptive diagnosis and therefore, differential diagnosis became mandatory based on serological and molecular test. IBV have multiple and distinct variants within a geographic region (Callison *et al*., 2001; Gelb Jr *et al*., 2005; Ignjatovic *et al*., 2006; Valastro *et al*., 2016) which add further complexity for vaccination and different serological methods for typing of IBV (Jackwood and de Wit, 2013).

Polymerase chain reaction (PCR) (Selim *et al*., 2013) and Enzyme linked immunosorbent assay (ELISA) (Mahzounieh *et al*., 2006) are routinely used in diagnostic laboratories but these tests are expensive, AGP and virus neutralization are rarely used because they are time consuming and laborious. Choice of the test for timely diagnosis and interpretation of result is of significance for controlling the infection in commercial poultry flocks. Heamagglutination inhibition (HI) test is one of the best tools for routinely monitoring the infection during

* Corresponding author: shakeelkhan@gmail.com
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outbreak in field and vaccine response (De Wit *et al*., 1998). To adopt a successful preventive program against IBV infection it is necessary to isolate the circulating viruses in the field (Callison *et al*., 2001). However, due to the absence of neuraminidase enzyme in viral protein core, IBV do not exhibit heamagglutination activity as is the case in Newcastle disease virus (NDV) and avian influenza (AI) (Schultze *et al*., 1991). Therefore, *in-vitro* induction, by using different enzyme is desirable to enable the IBV for heamagglutinating activity (Bingham *et al*., 1975), which could be additionally used as an effective tool for rapid and inexpensive routine diagnosis of IBV in infected commercial poultry flocks (King, 1984; Ruano *et al*., 2000). The HI test by enzyme treated antigen has been used in USA, Europe and Japan and some other Asian countries (Park *et al*., 2016). The efficacy of HI test for monitoring the vaccine response has been demonstrated through commercially available HA antigen (Bayoumie and Hikal, 2015). To carry out HI by commercially available antigen is expensive approach; therefore, for rapid diagnosis of IBV, HA antigen for HI test was developed in laboratory. This may be helpful in for the evaluation of vaccine response and identification of the strain type in poultry flocks (Corbo and Cunningham, I959).

Materials and methods

For molecular detection of IBV tissue samples (Trachea, lungs) homogenate prepared, and filtered (0.22µm). The suspension was aseptically inoculated into 10 days-old embryonated eggs, and allantoic fluid (AF) was harvested after 72 hr. of incubation for detection of IBV by SDS-PAGE and RT-PCR.

For detection of IBV by SDS-PAGE, the harvested allantoic fluid was concentrated through polyethylene glycol PEG (6000) and then dialysis to remove the salt. The concentration of protein was estimated by Brad Ford protein assay as per instruction of manufacture (Bio Rad, Bradford reagent). A required amount 3.1 µg (15 µl) was loaded on SDS-PGE and run the gel at 100 volt until the tracking die run off. The poly peptide of IBV was determined after silver staining of gel.

For detection of IBV by RT-PCR, IBV RNA was extracted from the AF as per instruction of manufacture (Promega RNA isolation Kit). The RNA pellets were dissolved in RNase free water and store at 4°C for immediate use and -86°C for long storage.

RT-PCR was performed by amplification of S1 gene of 1720bp using the primer S1 oligo 5/ forward 5'TGAAAACTGAACAAAAGACA3' and S1 oligo 3/ reverse 5'CATAACTAACATAAGGGCAA3' (Kwon *et al*., 1993). RT reaction was carried out at 45°C for 1 hour. PCR were was performed by 35 cycle of denaturation at 94°C for 1 min, annealing 45°C for 2 min, polymerization 74°C for 5 min. in addition with the initial denaturation 94°C for 5 min., annealing 45°C for 2 min, polymerization 74°C for 5 min., and final extension at 74C° for 12 min.

HA antigen of IBV was prepared by treating with neuraminidase, Phospholipase C and trypsin. The AF was centrifuged at 10,000 x g for 10 min and Neuraminidase (25 µl, 1unit/ml) was added to supernatant (0.25ml). Incubated for 30 min at 30°C then transferred to 4°C for 5 min (Ruano *et al*., 2000).

For phospholipase C treatment, harvested AF (100 ml) was centrifuged at 30000 × g for 40 min and pellet was resuspended in 1 ml of 0.01M Tris-HCl, pH 6.5. The pellet was gently mixed using the tuberculin syringe with a 25 gauge needle. Phospholipase type C added (1U/ml) of virus particles and incubated at 37°C for 3 hr. (King, 1984). The prepared antigen was stored at -86°C.

For treatment with trypsin, AF (0.25ml) was treated with 50µl of 2% trypsin and incubated at 37°C for 30 min and then placed at 4°C for 5 min (Mahmood *et al*., 2004). The prepared HA antigen was store at -86°C.

For heamagglutination test, 50µl saline and 50µl of HA antigen added in each of the 1st well. These antigens were diluted using two-fold serial dilution method up to 11th well. The 12th well was used as a control. Finally, 50µl of 1% RBC suspension was added to each well. Plates were covered with the aluminum foil and incubated for 30 min at 4°C. Agglutination titer were noted.

Results

IBV was efficiently grown in embryonated eggs and the major poly profile was obtained with molecular weight i.e., GP 84, GP36, Gp31, GP28, and P51 and P 23 (Fig. 1) indicate the presence of IBV.

IBV detection was confirmed in AF after 72 h incubation by RT-PCR. The expected 1720 bp (Fig. 2) PCR product was visualized by agarose gel.

Table I. Log^2 titer of HA after treatment with proteolytic enzyme.

AF Sample	IBV titer after treatment with		
	Trypsin (2%)	Phospholipase C 1U/ml	Neuraminidase Type V (2 U/ml)
1	10^8	10^8	10^8
2	10^6	10^6	10^6
3	10^7	10^7	10^7
4	10^5	10^5	10^5
5	10^7	10^7	10^7

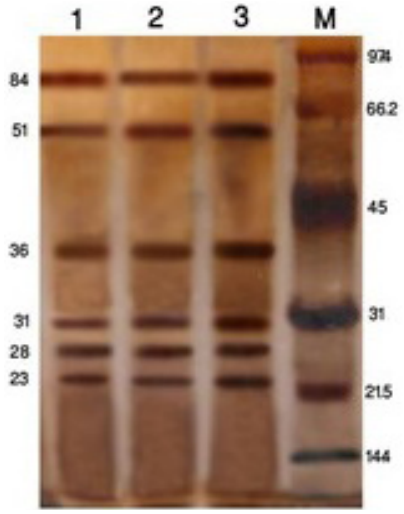

Fig. 1. The poly peptide profile of IBV four major glycoprotein and two non-glycoproteins. Lane 1, 2 and 3 IBV sample and low molecular weight marker (bio rad).

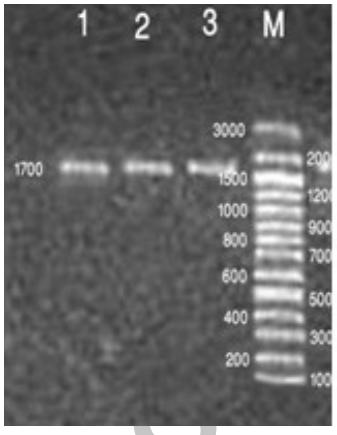

Fig. 2. RT-PCR profile of IBV S1 gene on agarose gel using primer pair New S1 oligo 5/ and degenerate 2/ wit the product size 1700 bp. Lane1, 2, 3 show amplified product of IBV S1 gene and Lane 4 DNA marker fermentas.

The effective concentration of trypsin was 2%, neuraminidase 2 U/ml for phospholipase C was 1U/ml, as mentioned in a Table I. The results revealed that IBV sample 1-5 showed similar result with all enzymes indicate all enzyme significantly effect on IBV and HA titer is not affected by any of the proteolytic enzyme.

The prepared HA antigen treated by different enzyme was stored at -86°C for six month and it was observed that the HA receptors of IBV induced by phospholipase C remained stable for up to six months while trypsin induced HA receptors started to lose its stability after two months and neuraminidase after five months of incubation.

The specificity of enzymes action, besides IBV on other avian viruses for HA reactions (as in Table II) indicated that no change of the HA activity was found in the absence or in the presence of enzyme on NDV and AI (H7), while IBV shown effective heamagglutination reaction in the presence of all three enzyme. IBDV fail to shown HA activity in the presence of enzymes. The results indicated that only HA activity of IBV could specifically induced by proteolytic enzyme trypsin, phospholipase C and neuraminidase type V.

Discussion

HI test is best choice for rapid diagnosis of IBV infection (Bayoumie and Hikal, 2015) but IBV belong to non heamagglutinating group of coronavirus (Holmes and Lai, 1996; Schultze *et al*., 1991), for that reason the HI could not be applied for rapid diagnosis during infection. For rapid diagnosis IBV HA antigen aimed to establish a rapid diagnostic HI test were prepared by treating different enzyme trypsin, neuraminidase and phospholipase C. As Neuraminidase and trypsin removes alpha 2, 3-linked N-acetylmuraminic while phospholipase C cleaves the spike protein from the surface of IBV and enables the virion to interact with RBCs (King, 1984; Ruano *et al*., 2000). All enzymes were shown significant effect of IBV to elicit the HA titer 7 $\log^2$ (Table I) which show the significant effect of all enzymes on IBV to elicit the HA activity. The efficacy, sensitivity and stability of HA antigen prepared by different enzymes were also evaluated. All antigens have shown a strong and specific inducer for heamagglutination reaction. After preparation of enzyme inducible HA antigen, the antigen was stored at -86°C. Its efficacy, sensitivity and stability were determined up to six month and then HA titer was determined periodically to check its stability and efficacy. The antigen prepared by trypsin prone to readily loses its activity from 7 $\log^2$ to 2 $\log^2$ HA titer after two months of incubation. Antigen prepared by neuraminidase was found consistency during HA reaction as reported previously (Ruano *et al*., 2000) but it's also loses its stability by giving 7$\log^2$ to 5 $\log^2$ HA titer after five months of incubation which support the previous study of significant reduction of HA titer after four months of incubation at -20°C (Park *et al*., 2016). Antigen prepared by Phospholipase C remained most stable up to six months by giving 7 $\log^2$ HA titer with reproducibility. All enzymes were shown specific effect of IBV during enzyme induction, while other selected avian viruses do not affect by treatment. Our analysis (Table II) indicated that the HA activity of AI (H7) and NDV was not affected in the absence or even in the presence of these enzymes while the HA activity of IBDV was not observed in both conditions suggests that in IBDV proteolytic enzymes trypsin, neuraminidase and phospholipase C does not effect on IBDV surface receptors.

Table II. The specificity of proteolytic enzyme that elicit the HA activity specifically on IBV while the activity of other avian viruses unchanged.

Avian Viruses	Trypsin		Phospholipase C		Neuraminidase Type V	
	Treated	Untreated	Treated	Untreated	Treaded	Untreated
NDV	++	++	++	++	++	++
AI (H7)	++	++	++	++	++	++
IBDV	--	--	--	--	--	--
IBV treated	++	--	++	--	++	--

++: Positive HA results; --: negative HA result; Nd: not done.

Various aspect of IBV HA antigen prepared by different enzyme has been discussed previously (Bingham *et al*., 1975; Corbo and Cunningham, 1959; Schultze *et al*., 1991). During the study antigen prepared by trypsin were found to readily lose its HA activity which contradict the previous study (Mahmood *et al*., 2004) for preparation of stable HA antigen by trypsin. Our observation suggest that enzyme induced HA antigen prepared by phospholipase C is an effective tool to establish a rapid diagnostic test HI for timely diagnosis of IBV during infection.

Statement of conflict of interest

The authors declare that there is no conflict of interests.

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