Detection of Infectious Bronchitis Virus in Allantioc Fluid by Rapid Hemagglutination Test

Short Communication

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Summary

To detect the presence of infectious bronchitis virus (IBV) in infected allantoic fluid (AF) of SPF embryonated eggs rapid hemagglutination (HA) activity after treatment with neuraminidase enzyme was used. Twenty IBV suspected materials were inoculated in SPF embryonated eggs via chorioallantoic cavity. Harvested AFs were treated with neuraminidase enzyme and the presence of IBV was detected by inducing hemagglutination of chicken red blood cells. The specificity of rapid HA test was examined with three non-hemagglutinating avian viruses including infectious bursal disease virus, infectious laryngotracheitis virus and fowl pox virus. The sensitivity of the test was compared with reversed transcriptase-polymerase chain reaction. The results showed that this test was specific and had a sensitivity of 100% for IBV detection. The results of this study indicate that HA test using neuraminidase treatment is an accurate, sensitive, specific and inexpensive test for rapid detection of IBV.

***Key words*:** infectious bronchitis virus, allantoic fluid, rapid hemagglutination, neuraminidase enzyme, RT-PCR

Introduction

Infectious bronchitis virus (IBV) belongs to the family *Coronaviridae*, genus *Coronavirus* is the ethiologic agent of Infectious bronchitis (IB). The disease is a

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major cause of economic losses in poultry industry and is a contributor of highly acute viral respiratory and urogenital disease. IB causes high morbidity in all ages and high mortality in chickens less than 6 weeks old. In addition, poor egg production with poor quality follows the disease (Cavanagh & Naqi 2003). As the IB signs are not specific, diagnostic tools are needed to identify the IBV. Traditionally, IB is identified by isolation of the virus in SPF embryonated eggs or tracheal organ culture. It can also be detected by immunochemical, molecular and serological methods (Cavanagh & Naqi 2003, Keeler *et al* 1998, Kwon *et al* 1993a, b) especially reverse transcriptase-polymerase chain reaction (RT-PCR). Some members of the family Coronaviridae agglutinate red blood cells, but the others don't have this ability due to lack of a receptor-destroying enzyme (Holmes and Lai 1996). Several experiments have been used to induce IBV HA activity by using trypsin, phospholipase-C and neuraminidase enzymes (Bingham *et al* 1975, Corbo & Cunningham 1959, Schultze *et al* 1992a, 1994, Muneer *et al* 1988). Trypsin induced HA only in Massachusetts strain, but specific antiserum did not inhibit HA activity. Phospholipase-C induced inconsistent HA activity, but this activity could be inhibited by specific antisera. The effect was observed while using only highly purified phospholipase-C preparation (Alexander and Chettle 1997). Therefore, this HA activity may have been related to contaminating enzyme rather than to phospholipase-C. Neuraminidase was shown to induce IBV HA activity by removing sialic acid from viral surface (Schultze *et al* 1992a, b, 1994). The receptor determinant of IBV for attachment to cells is α 2, 3-linked N-acetylneuraminic acid (sialic acid). Neuraminidase removes sialic acid of spike glycoprotein, which is responsible for RBC attachment.

The object of this study was to set up and optimize a rapid, accurate, sensitive, specific and inexpensive test for detection of IBV based on observation of HA activity induced with neuraminidase enzyme.

Materials and Methods

Virus isolation. Tracheas, cecal tonsils and kidneys were collected from twenty commercial chicken flocks suspected to IB. The materials were homogenized to make 10% w/v suspension in tryptose phosphate broth (TPB) pH7.0-7.2 containing 10,000IU/ml penicillin, 10,000µg/ml streptomycin, and 250IU amphotericin B/ml. Homogenized materials were centrifuged at 1000*g* for 15min. The supernatant fluid was inoculated at 0.2ml into 10-day-old SPF embryonated chicken eggs (Valo, Lohmann, and Cuxhaven, Germany) via chorioallantoic cavity. Inoculated eggs were incubated at 37°C and allantoic fluids (AFs) were harvested 48-72h postinoculation (Momayez *et al* 2002). In order to be sure that the samples were not contaminated with hemagglutinating viruses such as Newcastle disease and avian influenza virus, harvested AFs were tested for the lack of HA activity after each passage, before treating with neuraminidase.

Neuraminidase enzyme treatment. Neuraminidase enzyme type V from *Clostridium perfringens* (Sigma, St. Louis, MO) was used. Different concentrations of neuraminidase, 2, 1, 0.5 and 0.25U/ml, were prepared from working solution (10U/ml) using PBS (pH7.2) as diluent. 25µl of each concentration was mix with 250µl harvested AFs on ice. All samples were held at 37°C for 15-120min with 15-min intervals and then were placed at 4°C for 5min.

Rapid HA test. 25µl of treated AFs were mixed with 25µl of 5% suspension of chicken red blood cells. HA reaction was read within 1min. Clear and consistent HA was considered as positive reaction. Strain M41 was tested as standard.

Specificity and sensitivity. To determine the specificity of the rapid HA test, some avian viruses including infectious bursal disease virus (IBVD), infectious laryngotracheitis virus (ILTV) and fowl pox virus (FPV) were inoculated separately into 10 to11-day-old embryos via chorioallantoic membrane (CAM). Infected CAMs were harvested and homogenized. Each harvested material was clarified by centrifuge after three times of freezing and thawing. The supernatant fluid was

treated with different concentrations of neuraminidase enzyme, as mentioned before then HA rapid test was done. The sensitivity of the rapid HA test was compared with RT-PCR. The method described by Kwon *et al* (1993a, b) was used to amplify a 1720bp PCR product.

Results and Discussion

The results of HA activity of standard 41 strain and tested samples for different concentrations of neuraminidase enzyme are shown in table 1. Clear and consistent HA observed after 30min of incubation period with 1unit/ml of neuraminidase. Out of the 12 samples, which were positive by this test 9 (75%) samples showed HA activity after the first passage and 3 (25%) samples required a second passage.

Table 1. *Results of rapid HA test of M41 standard strain and IBV suspected samples after treatment with different concentrations of enzyme*

Neuraminidase concentration (U/ml)									
Sample	**0.25**	**0.5**	**1.0**	**2.0**	**Sample**	**0.25**	**0.5**	**1.0**	**2.0**
1	±	+	+	+	12	–	–	–	–
2	±	±	+	+	13	–	±	+	+
3	±	+	+	+	14	–	±	+	+
4	–	–	–	–	15	±	+	+	+
5	±	+	+	+	16	–	–	–	–
6	±	+	+	+	17	–	+	+	+
7	–	–	–	–	18	–	–	–	–
8	±	+	+	+	19	–	±	+	+
9	–	–	–	–	20	–	–	–	–
10	–	–	–	–	M41	±	+	+	+
11	±	±	+	+					

–: no HA activity; ±: incomplete HA activity; +: complete HA activity

Specificity and sensitivity. Neuraminidase was specific for IBV HA activity induction in which IBDV, ILTV, and FPV that are non-hemagglutinating avian viruses were not affected by neuraminidase treatment to induce HA activity. The 12 rapid HA positive samples were subjected to RT-PCR as well as the 8 rapid HA

negative samples. As shown in figure 1 all of the positive samples are detected by RT-PCR with a visible band at 1720bp. The rapid HA negative samples were found to be negative by RT-PCR (data are not shown). Although, a 100% correlation is observed between the two tests but compared with RT-PCR, HA test is inexpensive and can be used easily in all laboratories. On the other hand, RT-PCR might be unable to detect IBV in the case of new strains due to new mutation whereas changes to HA activity seems to be unlikely in theses cases.

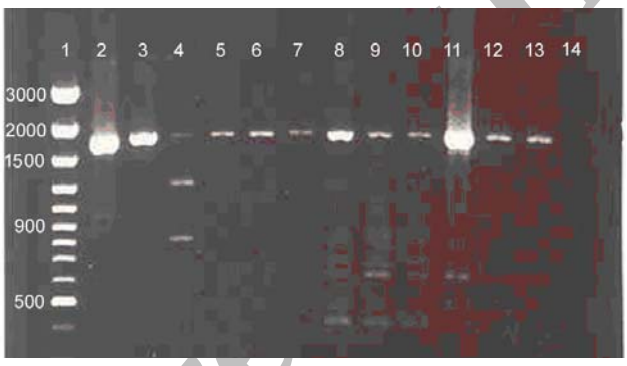

Figure1. *The RT-PCR results of 12 rapid HA positive samples. Lane 1, 100bp DNA ladder; lanes 2 to 13, IBV positive samples with 1720bp PCR product; lane 14, negative control*

The results of our study show that HA test using neuraminidase treatment is an accurate, sensitive and specific test for IBV detection. It is also inexpensive and simple, and can be used for rapid detection of IBV, even in non-equipped laboratories. This rapid test can be applied 48h postinoculation and compared with virus isolation using SPF embryonated egg that needs at least 3-5 blind passages, additional blind passages are not required. In cell culture as the other virus isolation method, in general, adaptation of IBV strains is necessary for replication of virus and induction of cytopathic effect. Chicken organ culture is another system for virus isolation in which no adaptation of field strains is required for growth and induction of ciliostasis which can usually be observed by low-power microscopy 3-4 days after inoculation. But, however, ciliostasis can also be induced by many other agents

the presence of IBV in field samples must be confirmed by an IBV-specific test. In total, the mentioned virus isolation methods can be time consuming and costly.

The embryo lesions caused by IBV are usually observes 5-7 days after inoculation and are not pathognomonic for IBV identification as well as the lesions in other virus isolation methods (McMartin 1993) therefore electron microscopic examination or IBV antigen detection methods are necessary for confirmation. In this regard, rapid HA test has some advantages over other IBV antigen detection methods.

IFA is a relatively inexpensive and rapid technique but interpretation of the fluorescence may be complicated by non-specific reactions (De Wit *et al* 1995). Monoclonal antibody (Mab) reacting only with one or small number of epitope (s) of the IBV antigen, results in a well-defined, reproducible, and specific product. But a disadvantage of using Mab is that a mutation of only one nucleotide, resulting in a different amino acid, causes false negative results in detection of IBV (Koch *et al* 1991). Although, rapid HA test does not have the disadvantages but RT-PCR, Mab and IFA in addition to detect IBV antigens can be used for virus serotyping. The results of our study are similar to the results of a previous study described by Villegas *et al* (2000). Further works are necessary to produce antigen for HI test with neuraminidase, for virus serotyping.

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