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Original Article

Preparation and diagnostic utility of a hemagglutination inhibition test antigen derived from the baculovirus-expressed hemagglutinin-neuraminidase protein gene of Newcastle disease virus

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A recombinant hemagglutinin-neuraminidase (rHN) protein from Newcastle disease virus (NDV) with hemagglutination (HA) activity was expressed in *Spodoptera frugiperda* cells using a baculovirus expression system. The rHN protein extracted from infected cells was used as an antigen in a hemagglutination inhibition (HI) test for the detection and titration of NDV-specific antibodies present in chicken sera. The rHN antigen produced high HA titers of 2^{13} per 25 μL, which were similar to those of the NDV antigen produced using chicken eggs, and it remained stable without significant loss of the HA activity for at least 12 weeks at 4°C. The rHN-based HI assay specifically detected NDV antibodies, but not the sera of other avian pathogens, with a specificity and sensitivity of 100% and 98.0%, respectively, in known positive and negative chicken sera (n = 430). Compared with an NDV-based HI assay, the rHN-based HI assay had a relative sensitivity and specificity of 96.1% and 95.5%, respectively, when applied to field chicken sera. The HI titers of the rHN-based HI assay were highly correlated with those in an NDV-based HI assay (r = 0.927). Overall, these results indicate that rHN protein provides a useful alternative to NDV antigen in HI assays.

Keywords: antibody detection, hemagglutinin-neuraminidase protein, hemagglutination inhibition, Newcastle disease virus

Introduction

Newcastle disease virus (NDV) is the only avian paramyxovirus type 1 in the genus *Avulavirus* of the family Paramyxoviridae [22]. NDV contains two surface glycoproteins, hemagglutinin-neuraminidase (HN) and fusion (F) protein. The HN protein mediates virus attachment to sialic acid-containing receptor molecules on host cell surfaces, which promotes fusion activity by F protein activation, while it also acts as a neuraminidase during virus budding via its receptor-destroying activity [6,19,35,37]. Additionally, the HN protein has the ability to agglutinate chicken red blood cells and it apparently reverses the elution of agglutinated cells. In addition to its roles in viral infectivity, the HN protein is critical to the protective immunity of vaccinated birds [10,28,29].

NDV is the causative agent of Newcastle disease (ND), which is a highly contagious and fatal viral disease characterized by respiratory, digestive, reproductive, and nervous clinical symptoms in chicken. Infection of non-immunized chickens with the highly virulent NDV may result in morbidity and mortality rates of up to 100% following introduction into a chicken flock. ND is endemic in poultry in many countries throughout Asia, the Middle East, Africa, the European Union, Central and South America, and parts of Mexico [2,3,21]. Vaccination is a control measure used in endemic regions to protect susceptible chickens from the disease, thereby containing the spread of the disease. Many diagnostic laboratories have routinely used serological tests to assess protective immunity after vaccination or for serological monitoring of virulent NDV infection among vaccinated birds [1]. The hemagglutination inhibition (HI) assay is a commonly used immunoassay for the detection of NDV antibodies in poultry in many laboratories worldwide. The antigens used in the HI assay are prepared from live whole viruses or viruses killed with formalin or beta-propiolactone after the propagation of NDV in chicken embryonated eggs [1,5]. The HI assay is based on the detection of NDV antibodies

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that block the binding of chicken RBCs to the HN protein of NDV. The expression of entire or partial recombinant HN proteins from NDV in a variety of expression systems might provide diagnostic antigens for use in the detection of NDV antibodies via enzyme-linked immunosorbent assays (ELISA) [24,38]. Recent studies also show that entire HN proteins from NDV can be expressed by recombinant baculoviruses to agglutinate chicken red blood cells, and that the hemagglutination (HA) activity can be inhibited in the presence of NDV antibodies [16,27,31]. In this study, a recombinant HN (rHN) protein from NDV was produced in insect cells that were infected using a recombinant baculovirus containing the open reading frame (ORF) of the HN protein gene from NDV. The rHN protein was used as an antigen in a HI assay to determine whether rHN protein was a suitable alternative to NDV antigen for the detection of NDV antibodies in sera.

Materials and Methods

NDV antigen

NDV antigen was prepared from allantoic fluid according to the method described by Beard *et al.* [5]. Briefly, the NDV La Sota strain was grown in specific pathogen-free (SPF) embryonated chicken eggs, which were incubated at 37°C for 4 days. The allantoic fluids from infected eggs were then pooled and clarified by centrifugation at 1,500 × g for 30 min. Next, the virus was inactivated overnight at 37°C with 0.1% (v/v) formalin, aggregated with 10% (w/v) polyethylene glycol 6,000 for 2 h at 4°C, and then precipitated by centrifugation at 8,000 × g for 30 min. The pellet was subsequently resuspended in 1/20 volume of 0.01 M phosphate buffered saline (PBS), pH 7.4, after which the inactivated NDV antigen was titrated using a microtiter hemagglutination (HA) test with chicken RBCs and stored at −70°C until use as an NDV antigen in an HI test.

Baculovirus expression of the HN protein

Viral RNA was extracted from embryonic allantoic fluid containing the NDV La Sota strain using an RNeasy Extraction Mini Kit (Qiagen, Germany). The complementary DNA of the full-length NDV HN gene was amplified using a one-step RT-PCR kit (Qiagen). The PCR primers were designed to amplify a DNA fragment measuring 1754 bp that contained the entire HN ORF of 1734 bp, *i.e.*, Xba1/HNf (5′-GC*TCTAGA*GCATGGACCGCGCCGTTAGCC-3′) and HindIII/HNr (5′-CC*AAGCTT*GGCTAGCCAGACCT GGCTTCTC-3′). As shown in Fig. 1, the amplified DNA fragment was cloned into the pCR-Blunt vector (Invitrogen, USA) and inserted into the *Xba*I and *Hind*III restriction enzyme sites of the pFastBac1 vector (Invitrogen). The recombinant plasmid containing the entire HN ORF from NDV (pFB/NDVHN) was transformed into *E. coli* DH10Bac cells (Invitrogen), which contained Bacmid (a baculovirus shuttle vector). The recombinant baculoviruses were generated by transfection of recombinant Bacmid DNA into *Spodoptera frugiperda* 9 (Sf9) cells and then plaque-purified in Sf9 cells. The expression of the HN protein from NDV by the recombinant baculovirus was confirmed using an HI assay and western immunoblotting with hyperimmune chicken anti-NDV serum.

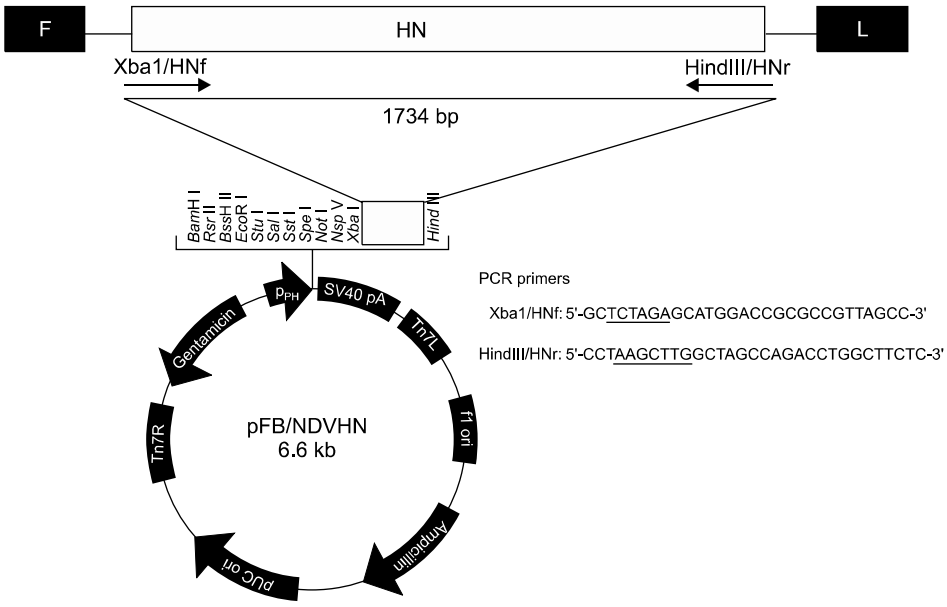

Fig. 1. Construction of an expression vector containing the entire open reading frame of the hemagglutinin-neuraminidase (HN) gene from Newcastle disease virus (NDV).

Large-scale production of the rHN protein

The rHN was prepared from Sf9 cells infected with rBac/NDV-HN. Briefly, Sf9 cells were cultured in a 175-cm^2 tissue culture flask and infected with rBac/NDVHN virus at a multiplicity of infection of 0.1 before incubating for 90 min at 27°C. Infected cells were harvested and added to 200 mL of Sf9 cells at a concentration of 2×10^6 cells mL^{-1} in a spinner flask, after which they were incubated for 4 days at 27°C and then harvested and precipitated by centrifugation at 500 × g for 20 min at 4°C. Next, the cell pellet was resuspended in 1/20 volume of 0.01 M PBS containing a protease inhibitor cocktail (Roche Molecular Biochemicals, Germany). Cells were lysed by briefly sonicating, before clarification by centrifugation at 500 × g for 20 min. The rHN protein extracted from the cell lysate was maintained at −70°C prior to use as an antigen in the HI assay.

Western immunoblotting

The rHN protein was electrophoretically separated through NuPAGE Novex Bis-Tris Gels using an Xcell *SureLock* Mini-cell (Invitrogen) according to the manufacturer's instructions. The separated polypeptides were then transferred from the gels onto nitrocellulose membranes using an Xcell II Blot Module (Invitrogen) in accordance with the manufacturer's protocols. Next, standard immunoblotting was performed using anti-NDV hyperimmune chicken serum (1 : 100 dilution) and specific antigen-antibody reactions were visualized on each membrane by applying anti-chicken IgG (H+L) conjugated with alkaline phosphatase (1 : 1,000 dilution), followed by BCIP/NBT substrate solution (Kirkegaard-Perry Laboratories, USA).

Serum samples

The HI assay used 1,221 chicken sera that were maintained at the OIE Reference Laboratory for Newcastle Disease of the Animal, Plant and Fisheries Quarantine and Inspection Agency (QIA), Korea. These sera include experimental chicken sera (n = 430) and field chicken sera (n = 791). Experimental chicken sera include experimentally vaccinated chicken sera (known positive, n = 314) and SPF chicken sera (known negative, n = 116). During 2008～2009, field chicken sera were collected from commercial broiler farms in which NDV vaccination programs had been routinely implemented. This study also used hyperimmune chicken sera to NDV, avian paramyxovirus type 2 (APMV-2), infectious bronchitis virus (IBV) and avian influenza virus (AIV), which were maintained at the OIE Reference Laboratory for Newcastle Disease at QIA, Korea.

HA and HI tests

The HA and HI tests were performed on sera in V-bottomed microwell plates according to the OIE Manual of Standard Diagnostic Tests [1]. Four HA units of NDV or rHN antigen were used and Anti-La Sota chicken serum (HI titer of 64) and SPF chicken serum were included with each test as positive and negative control sera, respectively. Back-titration of each antigen used was performed on each occasion to confirm that four HA units per well were present. Before accepting the test results, the control sera were required to be within a two-fold dilution range of their known HI titer. The HI titer was determined as the reciprocal of the highest dilution of serum that caused total inhibition of HA activity with 4 HA units of antigen. All tests were repeated in duplicate. The HI titers were expressed as the reciprocal of $\log_2$ in this study. Sera with HI titers ≥ 4 were considered as positive according to the OIE criteria [1].

Statistical analysis

The sensitivity and specificity of the rHN-based HI assay was determined relative to the NDV-based HI assay, where the degree of agreement between tests was estimated based on the kappa (κ) value and the correlation coefficient (*r*), as previously described [24,25].

Results

Expression of the entire HN protein using a recombinant baculovirus

As expected, a DNA fragment of 1754 bp that contained 1734 bp of the entire HN ORF from NDV (La Sota strain) was successfully amplified by RT-PCR and cloned into the pCR-Blunt vector. The expression vector pFB/NDVHN was constructed by insertion of the DNA fragment, which was digested with *Xba*1 and *Hind*III restriction enzymes, into the pFastBac1 vector. A recombinant baculovirus rBac/NDVHN expressing the entire HN ORF (1734 bp) was generated using Bac-to-Bac baculovirus expression systems. The rBac/NDVHN produced cytopathic effects in infected Sf9 cells. Infected cell lysates agglutinated 0.5% (v/v) chicken red blood cells strongly, while the HA activity was inhibited in the presence of hyperimmune anti-NDV serum, and no HA activity was observed in uninfected cell lysates. SDS-PAGE analysis detected a distinct protein band with an apparent molecular mass of approximately 63 kDa in the infected cell lysates, but not in the uninfected cell lysates. Western blotting showed that the protein band from infected cell lysates bound strongly to hyperimmune anti-NDV serum (Fig. 2A). These findings indicated that the band at approximately 63 kDa corresponded to the unglycosylated form of the HN protein from NDV [34].

Mass production of rHN protein

The rHN protein antigen was produced in a spinner culture with a 200-mL culture volume of Sf9 cells at a concentration of 2×10^6 cells mL^{-1}. A 10-mL volume of rHN protein antigen was finally prepared from the infected

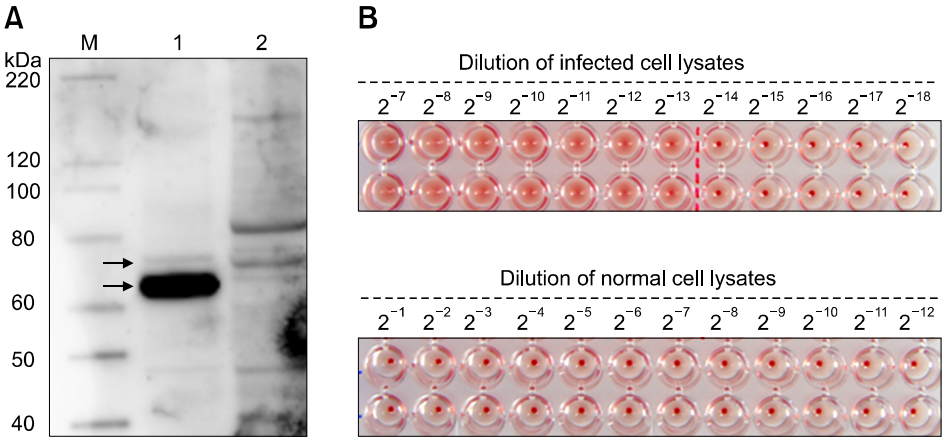

Fig. 2. Western blot analysis (A) and hemagglutination (HA) titer (B) of cell lysates from recombinant baculovirus-infected cells. The arrow in Fig. 1A indicates recombinant hemagglutinin-neuraminidase (rHN) proteins binding to NDV antibodies. The HA titer was determined as the reciprocal of the highest dilution with HA activity. M: protein molecular weight marker; Lane 1: infected cell lysate; Lane 2; normal cell lysate.

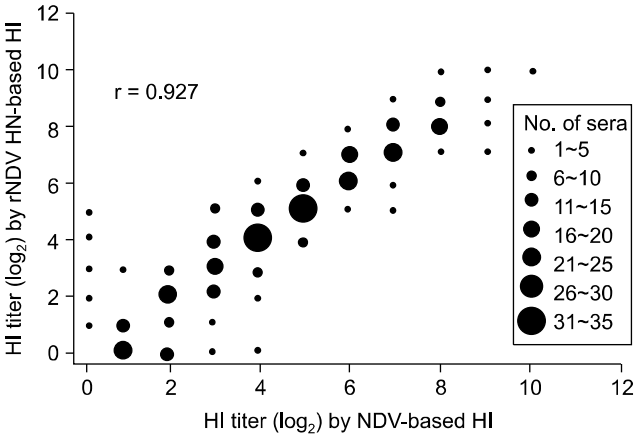

Fig. 3. Correlation between serum hemagglutination inhibition (HI) titers obtained using the rHN-based HI and NDV-based HI assays. Test sera were from healthy commercial chickens (n = 791) in broiler farms where vaccination was performed routinely.

cells. When titrated using the HI assay, the antigen concentration was 2^{13} HA units per 25-μL (Fig. 2B), indicating that the antigen could yield 20,480 mL of working solution, containing four HA units per 25-μL to be used as an HI antigen. However, lysates from normal cells exhibited no HA activity with chicken red blood cells. The same volume of inactivated NDV antigen prepared using chicken eggs contained 2^{12} HAU per 25 μL.

The stability of the rHN protein, which was prepared for use as an HI antigen, was tested with a microtiter HA test using chicken RBCs. Formalin-inactivated NDV antigen was tested under the same conditions as the control. Fig. 3 shows the HA titers of different types of HI antigen preparations during 6 months of storage at 4°C. After storage, the HA titers were compared to those obtained soon after preparation of the antigens. The rHN antigen maintained a stable HA activity for up to 6 months at 4°C, although the HA titer decreased slightly from 2^{13} to 2^{11}. The stability of the rHN antigen was similar to that of the inactivated NDV antigen (from 2^{12} to 2^{10}).

Diagnostic performance of rHN-based HI assay

The rHN-based HI assay was tested with field serum samples (n = 791) from commercial chickens that had been vaccinated using commercial live NDV vaccines and the results were compared with those obtained using an HI assay based on inactivated NDV antigen. Of the sera tested, 95.7% (757/791) were consistent with both HI assays, whereas the remaining sera (4.3%) produced results that did not agree between the rHN-based HI and the conventional NDV-based HI assay. All sera that produced differences in the two tests had HI titers of 4 when using either rHN-based HI (n = 25) or conventional NDV-based HI (n = 9). Thus, the relative sensitivity and specificity of the rHN-based HI assay were 96.1% and 95.5%, respectively, when compared with the conventional NDV-based HI assay (Table 1). The kappa value when comparing both tests was 0.90 (Table 1). The log_2-based HI titers for all sera in HI assays with rHN antigens were linearly correlated with the log_2-based HI titers obtained using the NDV antigen, while the correlation coefficient (r) of NDV versus the rHN antigen was 0.927 (Fig. 3).

Specific detection of anti-NDV antibodies using the rHN-based HI assay

To determine whether the rHN protein could be recognized by anti-NDV antibodies using the HI assay, experimental chicken sera were tested by HI assay based on rHN protein including known positive and negative chicken sera (n = 430). Known positive sera (n = 314) comprising experimentally vaccinated chicken sera were

tested using an rHN-based HI assay. In the rHN-based HI assay, 98.7% (310/314) of known positive sera were positive (*i.e.*, sensitivity of 98.7%). All known negative sera (n = 116) from unvaccinated SPF chickens were also negative based on the rHN-based HI assay (specificity of 100%). Among known positive sera, 80 randomly selected samples were titrated using the rHN-based HI assay and the results were compared with the conventional HI test (Table 2). The correlation coefficient (*r* value) for the four groups of strains (Ulster 2C, La Sota, KJW/49 and Kr005) were 0.930, 0.956, 0.960 and 0.969, respectively, upon comparison of titers using both HI tests. rHN protein was tested using the HI assay to determine whether there was any cross-reactivity with anti-sera for other pathogens, including avian paramyxovirus type 2, infectious bronchitis virus, and AIV H9N2. The HA activity of the rHN protein was not inhibited in the presence of these sera, indicating no cross-reactivity with other viral pathogens.

Discussion

This study tested whether rHN protein expressed by recombinant baculovirus was suitable for use as an antigen in an HI assay for the detection of NDV antibodies. To express the entire HN protein, a DNA fragment that contained the entire ORF of the HN protein gene was amplified, after which it was cloned into a plasmid and then incorporated into a baculovirus genome. Western immunoblotting demonstrated that the recombinant baculovirus produced an abundance of rHN protein product with a molecular weight of approximately 63 kDa, which corresponded to the unglycosylated form of HN protein from the authentic NDV. In addition, it produced a low expression level of a rHN protein product with a molecular weight of approximately 75 kDa, which corresponded to the glycosylated form of HN protein from the authentic NDV. These findings are in accordance with those of previous studies [30,31,34] and suggest that most of the rHN protein was extracted from infected cells in its unglycosylated form prior to glycosylation [30] or that the glycosylated form of rHN protein was expressed at low levels by the recombinant baculovirus in insect cells. Further study is needed to investigate how such glycosylation affects the antigenic structure of the HN protein; nevertheless, the rHN antigen prepared from insect cells in this study had high HA unit titers, and the HA activity was inhibited in the presence of NDV antibodies, indicating that the rHN protein possessed biological function (such as HA activity) of HN protein from the authentic NDV.

The rHN antigen produced in this study had several advantages over chicken egg-derived NDV antigen when used in an HI assay. The rHN antigen had a high yield of HN protein with 2^{13} HA units per 25 μL, which matched the inactivated NDV antigen prepared using the same volume of allantoic fluid from chicken eggs. Ong *et al.* [31] reported that the rHN protein produced from recombinant virus-infected cell lysates had HA titers of 2^4 HA units. The rHN protein yield reported by Ong *et al.* [31] was very low when compared with that in this study, which was probably due to differences in the virus strain used for expression of the rHN protein gene, the baculovirus expression system, or the procedure for rHN protein extraction.

The cell culture-based rHN antigen was readily prepared

Table 1. Comparison between the recombinant hemagglutinin-neuraminidase (rHN)-based hemagglutination inhibition (HI) and Newcastle disease virus (NDV)-based HI assays using field chicken serum samples

Conventional HI	rHN-based HI		
	Positive	Negative	Total
Positive	223	9	232
Negative	25	534	559
Total	248	643	791

Relative specificity = 95.5% (534/559); relative sensitivity = 96.1% (223/232); kappa value = 0.90.

Table 2. Comparison of HI antibody titers of sera samples of chicken groups vaccinated with different vaccine strains determined by the rHN-based assay and the conventional HI test

Vaccine group	Number serum tested	HI antibody titer (log_2)		Correlation (r)
		rHN antigen	NDV antigen	
Ulster2C (1)*	20	6.5 ± 1.64	6.1 ± 1.16	0.930
LaSota (II)	20	6.4 ± 2.76	6.2 ± 2.61	0.956
KJW/49 (III)	20	4.4 ± 2.76	4.5 ± 2.78	0.960
Kr005 (VII)	20	4.6 ± 3.09	4.9 ± 2.55	0.969

*Number in parenthesis represents genotype of class II NDV.

without the need to handle any biohazards or harmful chemicals such as formalin or betapropiolactone. In addition, the unpurified rHN antigen in its crude form had good stability and there was little effect on its HA activity after storage for over 12 weeks at 4°C, which was possibly due to the presence of protease inhibitors that prevented rapid degradation by cellular proteases. Thus, the method used for the high yield expression of rHN protein in this study could be beneficial for the inexpensive and simple production of cell culture-based antigen. If the supply of expensive SPF eggs is inadequate or they are not available, cell culture-based antigen could be the main alternative to NDV antigen prepared from chicken eggs.

The preparation of the NDV antigen requires the handling of infectious NDV, which can cause mild symptoms in humans such as conjunctivitis [4,7,11,13-15,26,32,33,36]. This is particularly true of virulent NDV strains, which demand virus handling in a laboratory with a biosafety level II facility. Thus, there are some biohazard risks when the NDV antigen is prepared in the laboratory. However, cell culture-based recombinant protein can be produced safely in a general laboratory without strict biosecurity.

Genotype VII antigenic variants have mainly been reported in Korea and China, where intensive vaccination of poultry has been implemented [8,18]. It has also been reported that there may be differences in measured HI titers for the same serum samples between the vaccine virus antigen and the genotype VII variant antigen [9,17], as observed in other genotypes of NDV. Such antigenic variation has led to a broad discussion of whether the vaccines have to be antigenically adjusted or not [12,20,23]. Use of genotype-matched HI antigens instead of currently available vaccine antigens would be helpful in determination of a level of protective immunity to match the circulating field strains. The use of a cell culture system in the study can facilitate the production of recombinant HN antigens that are tailor-made, as well as genotype-matched HI antigens if recombinant viruses expressing the target gene of interest are available.

The rHN-based HI assay had a specificity and sensitivity of 100% and 98.0%, respectively, when applied to known positive and negative chicken sera. The titers of sera measured by rHN-based HI assay were highly correlated with conventional HI assay, regardless of the virus genotype tested, indicating that the rHN antigen could detect NDV antibodies present in chicken sera. When compared with the NDV-based HI assay, the rHN-based HI assay had a relative sensitivity and specificity of 96.1% and 95.5%, respectively, when commercial chicken serum samples (n = 791) were tested using both HI assays. Thus, there was an agreement of 90% (kappa value = 0.90) between the rHN-based HI assay and the NDV-based HI assay. Many laboratories use HI assays on a herd basis when evaluating chicken flocks. The rHN-based HI assay could provide a reliable alternative to the NDV-based HI assay when assessing the level of humoral immune response in vaccinated chickens or infected chickens on farms. According to previous studies [24,38], ELISAs using recombinant HN antigens are sensitive, specific and accurate when compared to the standard HI test, suggesting that rHN protein used in this study can be applied to ELISA for detection of NDV-specific antibodies in chicken sera.

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