Original Article

Unveiling Indirect ELISA Test against Nucleoprotein of H9N2 Comparing With Hemagglutination Inhibition Test

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ABSTRACT

Influenza is an acute and highly contagious respiratory disease caused by an RNA virus belonging to the Orthomyxoviridae family. The virus has the capacity to infect both birds and mammals. Avian influenza is an infection or a syndrome caused by type A influenza viruses. The reservoir of this disease is defined as aquatic and migratory birds, and there is a possibility of this disease occurring in any region. Influenza can be transmitted through contact with contaminated surfaces. Some strains, such as the Asian H9N2 strain, have been observed to cause respiratory diseases in people in Asia. Therefore, this study aims to diagnose the disease in infected poultry with greater speed and ease by screening them with nucleoprotein of H9N2, thus preventing outbreaks. An indirect ELISA test was developed using the nucleoprotein of the H9N2 A/Chicken/Iran/259/2014 virus, with a molecular weight of 60 kilodaltons, which was separated from the virus by the electroelution method with the use of the monoclonal antibody against nucleoprotein serving as the standard. Subsequently, the results of the indirect ELISA test and the hemagglutination inhibition tests were compared using 300 serum samples from birds. The findings of this study illustrated the correlation between the indirect ELISA test and the hemagglutination inhibition test when analyzed together. A Spearman's correlation coefficient indicated that there was a significant and strong positive relationship between the two variables (ρ =0.901, $p < .001$, N = 300). The indirect ELISA test showed a sensitivity of 90% and a specificity of 92%. Since the disease with mild symptoms can make the diagnosis difficult, we need to control and quickly identify the avian influenza virus. Our indirect Elisa test could help detect a wide range of strains by utilizing a conserved antigen as well as being able to be used for screening more suspected samples in a time efficient manner as compared to the golden standard test, hemagglutination inhibition.

Keywords: Indirect ELISA TEST, nucleoprotein, monoclonal antibody, hemagglutination inhibition test, Influenza

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1. Introduction

Avian influenza (AI) represents one of the most important respiratory and infectious diseases with the capacity for rapid dissemination. Avian influenza viruses are classified within the Orthomyxoviridae family, which envelop negative-strand RNA viruses. The viruses are divided into three types (A, B, and C) based on the antigenic indicators present in the nucleoprotein (NP) and protein matrix (MP). Several different subtypes of Influenza type A viruses can be distinguished based on the antigenic epitopes of the virus' two main surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). A total of 11 subtypes of the NA protein and 18 subtypes of the HA protein have been identified to date (1). A number of influenza A subtypes. Have been responsible for seasonal epidemics or sporadic zoonotic infections, which have successfully infected mammals and humans (1,2). The H5, H7, and H9N2 subtypes have been identified as having significant pathogenic and economic importance for the poultry industry (3). H9N2 is classified as a low pathogenic avian influenza (LPAI), while H5 and H7 are highly pathogenic viruses or highly pathogenic avian influenza (HPAI) (4). There are several methods for the diagnosis of avian influenza (AI) infection. Virus isolation (VI) in eggs or cell cultures represents the gold standard diagnosis tests (5). However, applying these methods is challenging, because they are not flexible for a high amount of demand, and are costly and time-consuming. Three other tests are commonly employed for the identification of antibodies against AIV: hemagglutination inhibition (HI), neuraminidase inhibition (NI) test, and agar gel precipitation (AGP) (6-8). The HI and NI assays are cost-effective and are applied as standard tests in the majority of laboratories. However, these tests have lengthy processing times and require control reference reagents. Additionally, the AGP test requires the presence of significant quantities of both antigens and antibodies to facilitate the formation of the precipitation lines. Therefore, enzyme-linked immunosorbent assay (ELISA) could be a suitable alternative for the detection of antibodies against the influenza virus, which is more sensitive than the HI, NI, and AGP tests (9,10). Unlike surface glycoproteins and membrane matrix proteins, nucleoprotein (NP) is a highly protected antigen in all types of influenza viruses (11, 12). Therefore, it is the best candidate for rapid identification using serological tests. The purpose of this study is to develop a rapid and easy diagnostic ELISA against NP antigens.

2. Materials and Methods

2.1. Preparation of Virus

The virus (IRAN/772/1998 (H9N2)) was obtained from the poultry virus disease diagnosis department of Razi Vaccine and Serum Research Institute.

2.2. Electrophoresis

Gel electrophoresis was conducted following the standard SDS-PAGE method (13). A gel matrix, typically composed of polyacrylamide, was prepared in a vertical apparatus.The sample containing the virus to be separated was loaded into wells at one end of the gel.An electric current was applied across the gel, creating a positive and negative electrode.When the electric current was applied, the molecules in the sample moved through the gel towards the oppositely charged electrode.Smaller and more negatively charged molecules moved faster through the gel than larger or more positively charged molecules, leading to separation based on size and charge.After electrophoresis was completed, the separated molecules visualized by staining with dyes. The 10% SDS-PAGE gel, containing 4% stacking gel and a 10% resolving gel was prepared. Subsequently, the gel was stained with Coomassie Blue staining. After that, Native-PAGE electrophoresis was performed in accordance with the methodology outlined by Nowakowski and Nowakowski, Wobig (14). In this test, the sample was prepared in sample buffer without β-mercaptoethanol, SDS, and heating.

2.3. Electroelution

The sharp band of Native electrophoresis gel at 60 kD was excised from the gel using a sharp disposable blade and eluted in elution buffer containing 25 mM Tris–HCl, pH 8.3, 192 mM glycine, 0.1% (w/v) SDS by using an electroeluter (Bio-Rad, Madrid, Spain). The proteins were subjected to Electroelution for three hours at room temperature. To remove SDS from the prepared sample, the elution process was continued for approximately two hours using a fresh elution buffer without SDS. Subsequently, the protein solution obtained from the elution was purified using polyethylene glycol (PEG). The protein concentration was then measured through the Lowry protein assay method (15).

2.4. Serum Samples

A total of 300 chicken sera were received from the Poultry Research Department of Razi Vaccine and Serum Research Institute. These sera were subsequently measured by the HI test in that department, which was considered to be the gold standard. The results obtained by that laboratory were considered the gold standard for true positive and true negative measurements. Subsequently, the samples were tested by using the designed ELISA system.

2.5. ELISA

A checkerboard titration was conducted for all reagents with the objective of optimizing the ELISA (16) and Monoclonal antibody utilized as a standard (18) against the Np antigen from our previous study. The immunoassay plates were coated with purified NP antigen in a carbonate-bicarbonate buffer (0.05 M, pH 9.6) and incubated at 4°C overnight. Subsequently, washing the plate with PBST (containing 0/05% Tween 20) was conducted four times and then unbound sites in the wells were blocked by adding 250 µl per well of 5% skim milk to PBST and incubated at 37°C for an one and a half hours on a shaker. After three washes with wash buffer,

test serum samples, positive control serum, and negative control serum were added to the wells as duplicates and incubated for one and a half hours. After washing with PBST, anti -chicken HRP conjugate IgG antibodies were added to the wells and incubated at 37ºC for one hour and 15 minutes. After the final wash, 100µl BM blue Roche substrates were added and the plates were kept in the dark at room temperature for 20 minutes. Color development was terminated by adding 50µl of 0.1M sulfuric acid to each well. Absorbance was measured at 450 nm in an ELISA reader.

2.6. Hemagglutination Inhibition Assay

The HI assay was performed in accordance with the OIE manual OIE (17). The assay was based on the ability of certain viruses, such as influenza virus, to cause red blood cells to clump together (hemagglutination) in the presence of specific antibodies. The antibodies can inhibit this clumping activity.A series of dilution of antibody-containing serum were prepared and each dilution was mixed with a standardized amount of virus that had the ability to agglutinate red blood cells. Then, a standardized amount of red blood cells was added to the mixture.If the antibodies in the serum were able to neutralize the virus, they would prevent the virus from agglutinating the red blood cells, resulting in the inhibition of hemagglutination. The highest dilution of serum that prevented hemagglutination was called the titer of the antibodies in the sample;a high titer indicated a strong immune response to the virus, while a low titer indicated a weaker immune response.The results of the HIA can help determine the immunity to a specific virus, such as influenza, and can be used to assess vaccine effectiveness or previous exposure to the virus. The highest serum dilution used to express the HI titer completely inhibited 4 hemagglutinin units (HAUs) of the virus. If there was inhibition at a serum dilution of 1:16 or greater against 4 HAUs of the virus, the HI titer was considered to be positive. The whole particle of the H9N2 virus was used to measure antibody titers in H9-positive chickens.

2.7. Statistical Analysis

Statistical analysis was performed using SPSS version 16 software. In this research, the Kolmogorov-Smirnov test was used to evaluate the normality of the data distribution of each variable. Then, the *Spearman* correlation was used to find the relationship between the results of ELISA and HI.

3. Results

3.1. RF Measurement of Virus Bands to Determine NP Protein Location

To observe the protein bands from the influenza virus suspension, electrophoresis was performed. According to our previous study (16, 18) and the result obtained from the calculation of RF, we found the NP band on electrophoresis gel 10%, which was about 60 kD. (Figure 1& 2).

$$RF = \frac{\text{Distance moved by an analyte}}{\text{Distance moved by solvent}} = 0.53$$

3.2. ELISA Results

The results obtained from the indirect ELISA test of a monoclonal antibody given to the 300 OD serum sample were compared with the results obtained from the HI results (Table 1). Furthermore, the mean and standard deviation of the positive and negative controls were obtained and the cut-off value was calculated based on the formula of Lardeux, Torrico (19) (Table 2).

3.3. Checking the Repeatability

To analyze the repeatability of our developed assay, 4 serum samples were tested within one run (inter-assay) and in separate runs (intra-assay). The indirect ELISA showed a coefficient of variation (CV%) with an intra-assay variability of 7.25 percent and an inter-assay variability of 8.25%. The results demonstrated the reproducibility of the tests in diagnostic settings.

3.4. The Diagnostic Sensitivity, Specificity

In general, from the test of 300 serum samples that were measured, the results were as follows:

Number of true positive = 163

Number of true negative = 137

The number of false positives = 12

The number of false negatives = 18

According to the definition of the sensitivity and specificity equation, it was calculated as follows:

Number of true positives + number of false negatives/number of true positives = sensitivity

Number of false positives + number of true negatives/number of true negatives = specificity

90% = sensitivity

92% = specificity

3.5. HI Results

This test was performed to quantitatively evaluate and measure the H9N2 antigen level. The dilution was made by diluting from the dilution of the H9N2 antigen. The dilution of the last well in which the red blood cells did not agglutinate, was the hemagglutination titer of the H9N2 antigen. The other samples were measured in the same steps as explained and shown in Figure 3.

3.6. Correlation Coefficients Results Between HI and ELISA

First, the normality of tests was obtained by Kolmogorov-Smirnov, which was $0<0.05$, then the correlation coefficients were calculated and the result is shown in Figure 4 and Table 3. A Spearman's correlation showed that there was a significant and strong positive relationship between the two tests ($\rho = 0.903$, $p < .001$, $N = 300$).

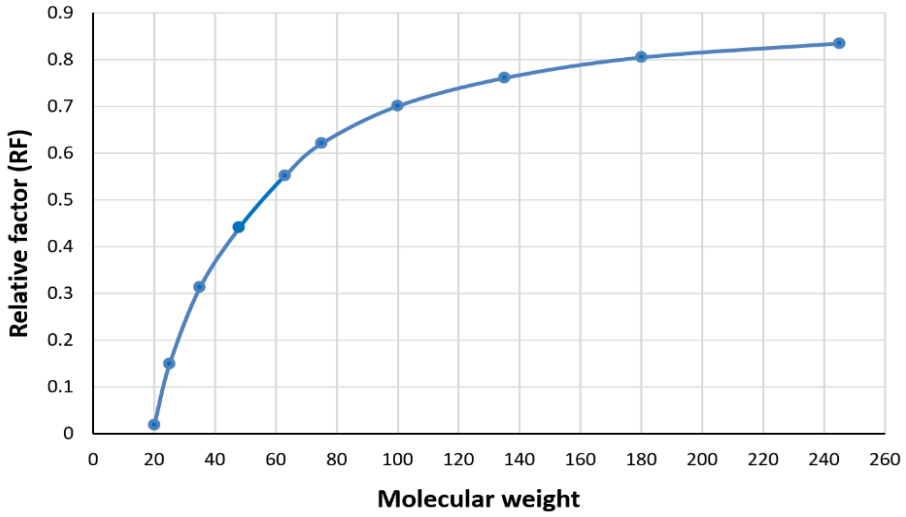

Figure 1. The curve of RF and molecular weight for the marker in gel, 10%

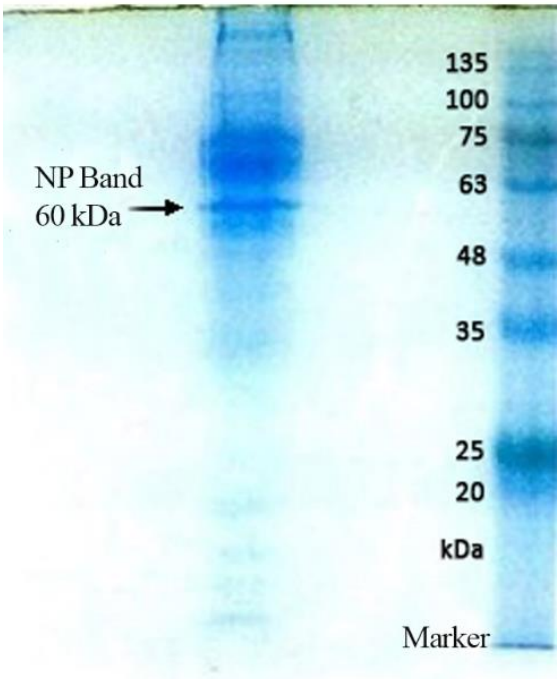

Figure 2. SDS-PAGE electrophoresis, H9N2 with 10% gel (Coomassie blue staining)

Table 1. Monoclonal antibody results and units for each serum absorbance

Mono anti-Np dilutions	OD	OD Range	Unit
Crude	3.215	≥3	6
1/2.5	2.158	≥2	5
1/5	1.697	1.5- 2	4
1/10	1.492	1-1.5	3
1/20	1.025	0.9-1	2
1/40	0.897	0.8-0.9	1
1/80	0.536	<0.8	0

Table 2. Mean and standard deviation of positive and negative controls, cut-off range

Positive control	OD (450nm)	Negative control	OD (450nm)
1	1.914	1	0.675
2	2.033	2	0.928
3	1.944	3	0.495
4	2.386	4	0.574
5	2.782	5	0.709
6	2.438	6	0.689
7	1.981	7	0.518
8	1.894	8	0.537
9	1.944	9	0.657
10	2.505	10	0.645
Mean Pos.	2.182	Mean Neg.	0.643
SD pos.	0.3	SD neg.	0.12
Cut-off (Pos.)	1.282	Cut-off (Neg.)	1.003
1.003 < Doubtful < 1.282			

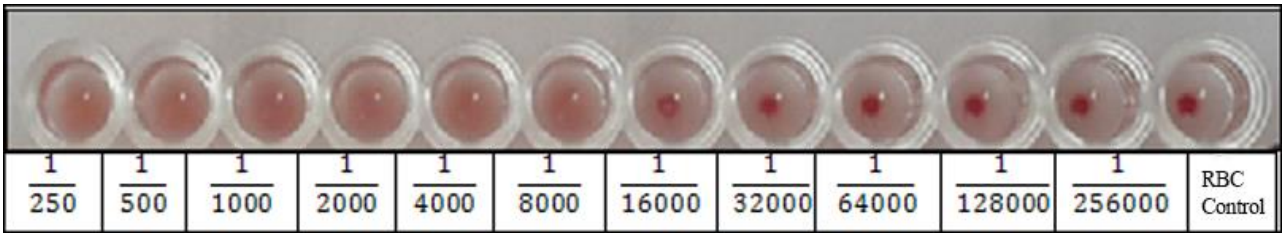

Figure 3. Dilution and determination of hemagglutination titer

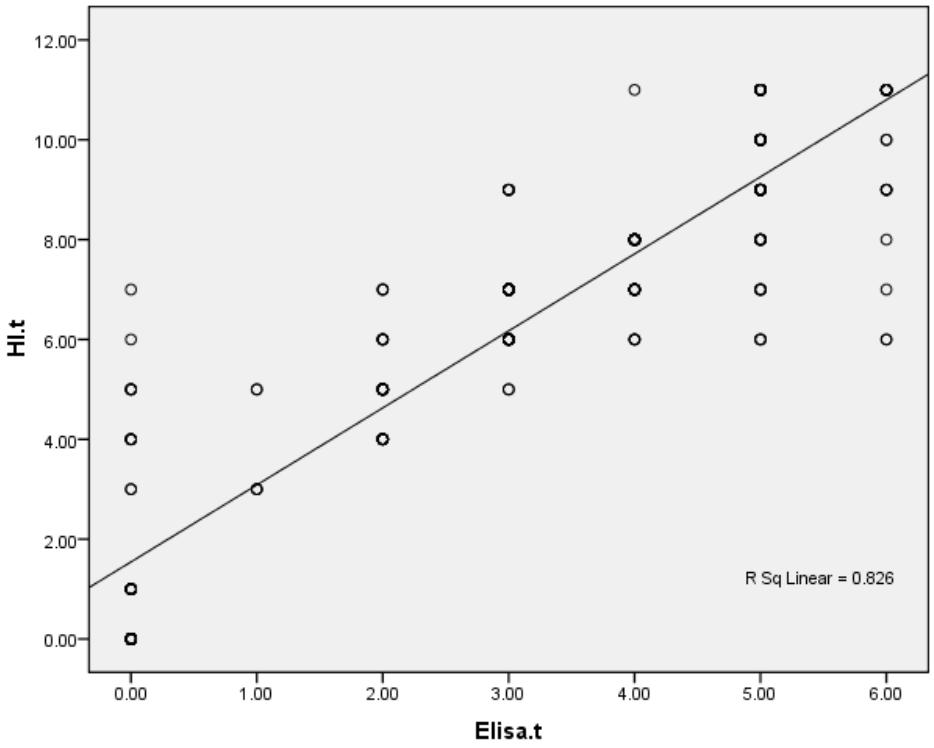

Figure 4. Graph of correlation between HI and Elisa

4. Discussion

H9N2 caused human respiratory illness in Hong Kong and mainland China between 1999 and 2003 (20-22). The glycoproteins of the Hong Kong H9N2 viruses may help spread the infection to humans, because H9N2 isolated from birds in Hong Kong had receptor specificity comparable to human H3N2 viruses (23) and mutations similar to human H2N2 and H3N2 viruses. Active serological surveillance is necessary for the prevention and management of H9 outbreaks. For the detection and screening suspected birds in aviculture, it is recommended that the labor-intensive HI assay be replaced with a straightforward ELISA assay that is more reliable, cost-effective, and does not require any special expertise. Although the HI test is the "gold standard" assay for screening IAVs, it has some drawbacks compared to the ELISA. HI can be difficult to select a comparable viral antigen if the animals from which serum samples are taken do not have a clear history of infection. Utilizing a biological safety cabinet is required because of the zoonotic potential of IAVs and the fact that all procedures in HI utilize live virus antigens. In contrast to the HI test, the NP indirect ELISA developed in our work can be easily automated for widespread monitoring of sera samples obtained from various animal species. The established ELISA has a biosafety rating because it can be utilized in a standard laboratory without the use of a biological safety cabinet. In the present study, the development and validation of the NP indirect ELISA was discussed, using the H9N2 nucleoprotein as a coated antigen, unlike the study of de Boer, Back (24) that designed a sandwich-blocking ELISA by using NP mAb as a coating of the ELISA plates.We also used NP mAb as a standard. This NP mAb was generated from mice that had been immunized with the H9N2 nucleoprotein and its immunogenicity was evaluated in the previous studies (16, 18). We were able to detect antibodies produced against NP using the NP indirect ELISA. The HI test for H9 antibody detection and the H9 indirect ELISA established in this study showed higher agreement. However, the antibody titers detected by the HI test were higher than those detected by the NP indirect ELISA because the antibodies measured by the HI test were against whole virus particles, whereas the antibodies measured in the ELISA test were against NP only. The ability of the developed NP indirect ELISA to identify the internal protein is an appealing feature because, despite the antigenic heterogeneity in the surface glycoproteins of AIV, especially haemagglutinin (HA) and neuraminidase (NA), the internal antigenic determinants on the nucleoprotein (NP) are typically constant among various strains/subtypes. Because of this characteristic, this protein is more suitable for a serological test that can identify antibodies against all AIV subtypes (25-27). The NP indirect ELISA has the potential to monitor AIV antibodies in a variety of avian and mammalian species due to the conservation feature of NP (28). In areas where the disease is endemic and vaccination is used to prevent the virus from spreading, the assay might also be used to monitor the immunization status of domestic poultry. In conclusion this study demonstarted that our indirect ELISA against the NP antigen can be utilized as an effective substitute for the HI assay for the serological identification of serum samples obtained from animals infected with AIV viruses.In addition, NP as a antigen coating is a conserved antigen among all subtypes, this indirect ELISA has an innately greater ability to detect AIV antibodies in serum samples obtained from various birds pre-exposed to AIV viruses. This test is an approach that has promise for widespread epidemiological screening and monitoring of the immune system’s response to AIV viruses.

Acknowledgment
Not applicable.

Authors' Contribution
Conceptualization: R.M
Data curation: F.G & M.H
Formal analysis: F.G & M.H
Methodology: R.M & F.G
Software: M.H
Validation: R.M
Investigation: R.M & F.G
Writing –Review and Editing: M.H. R.M & F.G

Ethics
This article does not require IRB/IACUC approval because there are no human and animal participants and the prepared samples were obtained from another department.

Conflict of Interest
No potential conflicts of interest relevant to this article have been reported.

Data Availability
The data that support the findings of this study are available on request from the corresponding author.

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