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Development of An Indirect Enzyme-linked Immunosorbent Assay (ELISA) for the detection and quantification of avian influenza A, subtype H5 using a recombinant H5 antigen expressed in sf9 insect cells

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ABSTRACT

Avian influenza (AI) is a serious infectious disease caused by negative sense single strand RNA viruses that belong to the genus influenza virus in the family Orthomyxoviridae. In the present study, we developed an indirect enzyme-linked immunosorbent assay (ELISA) employing a recombinant H5 antigen expressed in sf9 insect cells. The HA nucleotide sequence of an Iranian strain of H5N1 virus from clade 2.2, (A/chicken/Iran/53-3/2008), was obtained from Genbank. The nucleotide sequence data was codon optimized for insect cells. Using pIEx-3 vector a molecular construct, encoding a secretion signal peptide of adipokinetic hormone (AKH) and 6xHis-Tag coding sequence, upstream of the cloning site for expressing fusion recombinant HA protein with N-terminal tags, were designed and ordered for synthesis by GenScript, USA. The recombinant construct was used to transfect Sf9 insect cells in a commercial serum-free medium. The H5-His protein was purified from the supernatant of Sf9-cell cultures. Purified rH5 was analyzed using SDS-PAGE, followed by western blot assay. Using the rH5 antigen, an ELISA assay was developed. The H5-ELISA was compared with the hemagglutination inhibition (HI) test for the assessment of the specificity of the test. The H5-ELISA condition was optimized for antigen concentration, serum dilution and conjugate antibody concentration. Optimum condition for antigen concentration, conjugate antibody dilution and serum sample dilution were 100 ng/ml, 1:1000 and 1:500 dilution, respectively. Based on the optic density observed, an ELISA titer (ET) prediction equation was derived from a positive/negative (P/N) ratio standard curve. The correlation coefficient of the results in intra- and inter-assayay of the test was statistically significant ($P<0.05$). The test was specific with no cross reaction with the sera containing high titers against Newcastle disease (ND), infectious bronchitis (IB), infectious bursal disease (IBD), infectious laryngotracheitis (ILT) and avian influenza virus H9N2 The H5-ELISA developed was validated for the detection of H5 antibody in a limited number of available sera, but it has to be validated against a large numbers of known H5 positive sera. The assay has potential to be employed as a serological tool for the detection of antibody against H5 viruses in poultry and avian populations, which may be the host for several influenza virus subtypes.

Keywords: Highly Pathogenic Avian influenza, Hemagglutinin, H5N1, H5-ELISA, detection and quantification of antibodies

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INTRODUCTION

Influenza viruses are negative sense single strand RNA viruses that belong to the genus influenza virus in the family Orthomyxoviridae. viruses are classified into three types; A, B, and C. Avian influenza (AI) is caused by type A strains of influenza virus. The type A viruses are classified into subtypes based on their surface glycoprotein antigens including haemagglutinin (HA) and neuraminidase (NA) (1)

The HA is categorized into sixteen subtypes (HI, H2... H16) while NA is categorized into nine subtypes (Nl, N2 ... N9). Various combinations have been detected in avian species (2, 3).

Avian influenza viruses have been shown to exist as low pathogenic (LPAI) or high pathogenic (HPAI) biotypes based on their ability to cause severe disease in domestic galliforme birds. HPAI viruses are defined as those that kill 75% or more of 4- to 8-week-old chickens within ten days of inoculation (4). To date the avian influenza A viruses that have shown the HPAI biotype in domestic poultry are predominantly in the H5 and H7 subtype. The H5N1 HPAI viruses that evolved and resulted in the massive epizootic since 2003, not only resulted in fatalities in wild and domesticated birds, but also caused disease with a high mortality rate in humans and some other mammals (2, 5, 6)

Serological surveillance of antibodies against HPAIV is an important strategy in preventing and controlling AI. Currently, the most widespread serological diagnostic techniques for detection of antibodies are the agar gel propagation (AGP) and hemagglutination inhibition (HI) tests. The AGP test requires large quantities of antigens and antibodies to form the precipitation lines and at least 24 h for the test result. The HI assay, although is subtype specific but it is very laborious and not suitable for high throughput work flow (7).

As an alternative, enzyme-linked immunosorbent assays (ELISAs) have been developed to detect AIV antibodies in poultry as well as other species (8-12). The traditional ELISA tests normally require concentration and purification of live-virus preparations for use as antigen, which may allow dissemination of the virus. The H5-ELISA assays developed are specific, sensitive and inexpensive. They can quantitatively detect antibody titers in the flocks and individual birds and can be used in serological epidemiological investigations.

Hemagglutinin (HA) is the major surface antigenic protein against which neutralizing antibodies are produced (13, 14). Both antigenic drift and shift promote epidemics to occur. The HA protein has been expressed in a number of expression systems. Expression of this glycoprotein in some expression systems such as prokaryotic system has resulted in a product with different glycosylation status than native virus(15). Proper glycosylation is important for the processing, the functional integrity and the antigenicity of Hemagglutinin gene product (16-19). We expressed the HA protein in an insect expression system based on the Sf9 cells and baculovirus free approach that is called the InsectDirect™ System developed by Novagen company, USA. (20).

MATERIALS AND METHODS

Expression, purification and analysis of recombinant H5

The HA gene of H5N1 virus from clade 2.2, (A/chicken/Iran/53-3/2008), was codon optimized for insect cells and synthesized by GenScript, USA, based on the sequences from the NCBI influenza database (AC number: FJ445017.1). After synthesis, HA gene was cloned into pIEx-3 vector, a component of InsectDirect™ System (Novagen, USA). The pIEx-3 vector encode a secretion signal peptide of adipokinetic hormone (AKH) and 6xHis-Tag coding sequence upstream of the cloning site for expressing fusion recombinant HA protein with N-terminal tags. For influenza HA expression, plasmid DNA containing HA was used to transfect Spodoptera frugiperda (Sf9) insect cells in serum-free medium by using *Insect GeneJuice*® Transfection Reagent (Novagen, USA) and incubated at 28°C, following the instructions of the manufacturer. At 72h post transfection, culture medium from the transfected cells was collected. Several batches of H5-His protein were purified from the supernatant of Sf9-cell cultures using the Insect RoboPop™ Ni-NTA His Bind Purification Kit (Novagen, USA), following the procedure provided by the manufacturer. Briefly, Insect PopCulture® Reagent and Benzonase® Nuclease were added directly to each culture to mediate cell lysis and viscosity reduction, respectively. Ni-NTA His-Bind resin was then added to the lysate to bind the fusion protein. The affinity resin was captured, washed, and eluted using a 96-well filter plate.
Purified rHA was analyzed with a sodium dodecyl sulfate and 10% polyacrylamide gel electrophoresis (10% SDS-PAGE) and transferred onto nitrocellulose membranes (Sigma). Expression of recombinant H5-His was analyzed by

western blot using a His•Tag AP Western Reagents kit (Novagen, USA). Briefly, the membrane was blocked one hour in a Tris-buffered saline (TBS) containing 3% bovine serum albumin (BSA) at room temperature. Subsequently, the membrane was incubated at RT for 1 hr with mouse anti-6xHis monoclonal antibody (Novagen, USA) at a 1:1000 dilution in a 3% BSA-TBS buffer. The membrane was washed two times with TBS-0.05% Tween 20. After washes, the blot was incubated for 1 hr with a 1:5000 dilution of Goat Anti-Mouse IgG AP Conjugate antibody. After washing, the membrane-bound antibodies were visualized by AP-Buffer containing BCIP and NBT, following the manufacturer's recommendations.

Field sample detection.

Three field serum samples were provided by Iranian veterinary organization (IVO) and stored at -20 °C until testing. Collected sera were inactivated at 56 °C for 30 minutes before testing. Samples were tested with the optimized condition of H5-ELISA, and Hemagglutination inhibition (HI) assay.

Hemagglutination inhibition (HI)

The haemagglutination assay is a test for detecting haemagglutinating viruses such as the HPAIV. The HA technique used in this project was based on WHO methodology as described briefly below. In the HI assay, 25µl of a two-fold serial dilution of the sera in PBS were incubated in 96-well U-bottom plates at room temperature for 1h with an equal volume of 4 hemagglutinating units (HAU) of reference inactivated AIV H5 haemagglutinin antigen (H5 antigen- GD Diagnostics,VLDIA096) and antiserum (Istituto Zooprofilattico delle Venezie, OIE/FAO Refrence laboratory for AI and NDV SERUM H5N1) were supplied by the Khorasan Razavi Veterinary Administration Central Laboratory, followed by the addition of 50µl of washed chicken erythrocytes in PBS. The plates were kept for 1h, after which the haemagglutination patterns were read and HA titres were determined from the last dilution showing complete haemagglutination. For reading the HA activity the plates were tilted at an angle of approximately 45° and observed for tear-shaped streaming of the RBCs. Reciprocal of the highest dilution of the antibody showing no hemagglutination was reported as the HI titer. Samples positive to HA were further processed to detect the presence of avian influenza H5 specific antigen. Both positive and negative control antigens and antisera were used for each HI testing plate.

H5-ELISA optimization.

Different purified antigen concentrations were tested against serial dilutions of positive and negative control sera in a checkerboard titration fashion. Several twofold, serial dilutions of the H5-His antigen, ranging from 200ng to 25ng, were performed in polystyrene, flat-bottom, 96-well plates with 100mM Bicarbonate/carbonate coating buffer. Positive and negative sera were tested in triplicate by H5-ELISA in a same plate (intra-assay). They were also tested in two more separate assays (inter-assay). The two fold, serial dilutions (1:250 to 1:1000) of chicken positive standard serum and negative chicken sera were used for assay optimization. Briefly, diluted H5-His antigen was incubated with 100mM Bicarbonate/carbonate coating buffer (3.03g Na2CO3, 6.0g NaHCO3 in 1000ml distilled water, pH 9.6) at 4° C overnight. The plate was washed twice with PBS (1.16 g Na2HPO4, 0.1g KCl, 0.1g K3PO4, 4.0g NaCl in distilled water up to 500ml, pH 7.4) and subsequently blocked with PBS supplemented with 1% (w/v) bovine serum albumin (BSA) for 2h at room temperature, followed by two washes. Plates were either used immediately or stored at -20°C. The positive and negative sera were added, and then the plates were incubated for 30 min at 37 °C. The plate was then incubated with HRP-goat anti-chicken antibody conjugate (IDEXX, USA) at a 1:1000 dilution followed by three washes. The colorimetric reaction was developed with peroxidase substrate solution (IDEXX, USA) for 15 min and terminated with a stop solution (IDEXX, USA). The reactions were read at 405 nm in an ELx808 plate reader (Bio-Tek, Winooski, VT). The optical density (OD) values obtained were compared to determine the optimal antigen concentration that produced the lowest background and the best positive-control titration curve. Using the optimal antigen concentration and serum dilution, a positive chicken serum samples confirmed as positive for H5N1 influenza virus by Hemagglutination inhibition (HI) were tested by the H5-ELISA in triplicate in a plate and in three plates separately.

The H5-ELISA positive threshold

The H5-ELISA plate was coated with optimal H5 antigen concentration and sera at the optimal dilution were added. The H5-ELISA was performed as described above. Ten sera negative to AIV H5 determined by HI tests were detected and the mean OD_{650} value and standard deviation were calculated. The endpoint cut-off was established by titration as the mean OD_{650} value of the 10 negative sera plus 3 standard deviations (mean+3SD).

Specificity and Stability of the test
To determine the specificity of H5-ELISA, we used sera positive for Newcastle disease (ND), Infectious bronchitis (IB), infectious bursal disease (IBD), infectious laryngotracheitis (ILT) and avian influenza H9N2 (AIV A, H9N2) and measured the OD value in H5-ELISA.

Positive/negative ratio
Using the P/N ratio method, the standard curve was constructed (Briggs and Skeeles, 1984). Briefly, the OD_{650} value of the positive serum (P value) was divided by the OD_{650} value of the negative sera (N value). The P/N ratio was calculated for each serum sample.

Standard curve
According to Briggs and colleagues (1985), two points were recorded from each serum sample described above. One point was the P/N ratio at 1:500 dilution, and the other point was the ELISA titre (ET). Using these two points from 4 serum samples, the ETs were plotted against P/N ratios at 1:500. The prediction equation of this regression line was determined.

RESULTS

H5 protein expression and purification.
The recombinant H5-His protein was secreted into the supernatant of Sf9 cultures and purified by the Insect RoboPop™ Ni-NTA His Bind Purification Kit (Novagen, USA). The purified protein appeared as a single band with a relative molecular weight of approximately 95 kD, as estimated from migration in SDS-PAGE relative to molecular weight standards (Fig. 1, lane 1). Absence of other proteins besides purified H5-His in Coomassie-stained gels ensured proper purity of the preparation. In western blot analysis, purified H5-His was detected by the anti-6xHis monoclonal antibady (Fig. 1, lane 3).

H5-ELISA optimization.
In the protocol developed for the H5-ELISA, the optimal condition were as described below. The optimum concentration of antigen was coated in each well, incubated at 4°C overnight, washed twice with PBS and subsequently blocked with PBS supplemented with 1% (w/v) bovine serum albumin (BSA) for 2 h at room temperature, followed by two washes. Sample sera were diluted 1:500, added and incubated for 30 min. The plate was then incubated with HRP-goat anti-chicken antibody conjugate (IDEXX, USA) at a 1:1000 dilution followed by three washes. The colorimetric reaction was developed with peroxidase substrate solution (IDEXX, USA) for 15 min and terminated with a stop solution (IDEXX, USA). The reactions were read at 650nm in an ELx808 plate reader (Bio-Tek). Using the established protocol, each sample along with positive and negative samples was tested 9 times in inter- and intra-assays.

Determination of the optimal antigen concentration coated and serum dilution
To determine of the optimal concentration of the antigen and the test serum, inter- and intra-assay checkerboard titration were employed. Optimal concentration for the antigen was found to be 100ng per well (Figure2). Meanwhile, the optimal serum dilution was 1:500 (Figure4).

The ELISA positive threshold
The end-point cut-off was established by titration as the mean OD_{650} value of the 30 negative sera plus 3 standard deviations (mean + 3SD). OD_{650} values of most sera varied between 0.091 and 0.171. The mean OD_{650} value and SD were 0.131 and 0.022, respectively. Thus, the positive threshold was 0.197. A serum sample was considered positive when its OD_{650} value at optimal dilution (1:500) was greater than the positive threshold.

Specificity test
The H5-ELISA was used to detect positive sera of birds for various viral diseases. The result showed that the H5-ELISA was specific for antibodies against HPAIV H5N1 and there was no cross-reaction with positive sera for ND, IB, IBD, ILT and AI H5N1 (Table I).

Repetition test
A positive serum was detected three times by H5-ELISA in inter- and intra-assay fashion in same batch of plates and in different batches of plates. The results indicate good reproducibility of the H5-ELISA.

Standard curve

The P/N ratio of each serum was delineated (data not shown). The ET of each serum was calculated. A standard curve [y (ET) = 0.047x (P/N value at 1:500 serum dilution) + 0.040 [R^2 = 0.986] was developed using the P/N ratio at optimal dilution of each serum and the ET value (Figure 3). t-Test ($p < 0.05$) indicated significant correlation between P/N ratio and ET.

Sensitivity test

In an independent evaluation the detection sensitivities of two different H5-ELISA assays and HI test were compared on flock samples. The ELISA tests demonstrated better specificity and sensitivity than HI. Based on the constructed standard curve, we analyzed individual serum titers from collected positive sera. The ET of H5-ELISA for individual serum samples was compared with HI titer (Table II). The result showed the H5-ELISA was more sensitive than the HI test.

DISCUSSION

A highly pathogenic avian influenza (HPAI) virus of the H5N1 subtype, spreading through live bird markets in Hong Kong was recognized and confirmed by international authorities in 1997. Migration of the infected wild birds along with the movement of the infected poultry flocks contributed to the spread of a HPAI virus to several countries (21-23). In spite of the national and the international veterinary authorities to combat the disease, H5N1 infection is currently endemic in some Asian and African countries. So far, more than 60 countries have reported H5N1 HPAI outbreaks in domestic poultry or wild birds. Other than serious human fatalities, the epidemic of H5N1 resulted in tremendous economic losses in the affected countries (24).

Since 2003, clade 2 of H5N1viruses circulated in birds in China and Indonesia and spread westward during 2005-2006, to the Middle East, Europe, and Africa (2). The H5N1 viruses belonged to Clade 2 have been the main cause of human infections since late 2005 (2, 25, 26). Regarding the presentation of the infection in wild birds, the serological and virological studies showed a low prevalence in the wild waterfowl population. Meanwhile, the infected birds showed no obvious clinical signs. Also, a number of healthy wild birds were found to have neutralizing antibody to the virus indicating either subclinical infection or infection with subsequent recovery (27).

To combat the disease and to prevent rapid spread of the infection early detection is so important. It allows decision makers to respond in a timely manner. It is also important to ensure that the vaccines in use are effective at protecting infected poultry from disease and decreasing viral load that is shed into the environment. There is thus a need for concerted efforts to develop diagnostics materials, including serological assays suitable for high throughput studies. Other than national and international surveillance programs and serological profiling of poultry flocks, these tests may improve understanding of the epidemiology of H5N1 disease in wild avian population (25, 28).

Serological detection of AI is still the most important detection test. The AGP test normally measures antibody to the NP and M proteins. The main disadvantage of the AGP test is its lower sensitivity in comparison to ELISA and HI tests (29, 30). It is also need a long time to form the precipitation line. However, the most reliable methods will most likely remain the more time consuming HI and in vivo studies. The HI assay for influenza viruses, including H5N1 needs the complete virions. If the virus is not inactivated, biosecurity and biosafety are a major concerns. Thus it is important to develop serological detection assays based on recombinant antigens. The assay should have the characteristics, such as sensitivity, stability and specificity. It also has to be easy to perform and be inexpensive.

Influenza A virus has two major glycoproteins on the virus surface, HA and NA. Cleavage of the HA is essential for the ability of the virus to enter the cells. This antigen is also an important determinant for the spread of infection through the host and for the pathogenicity of the virus. HA is the major antigenic protein against which neutralizing antibodies are generated (31, 32). Multiple amino acids at the cleavage site of the Hemagglutinin is associated with virulence of avian influenza viruses (33). This study was designed and conducted to develop an ELISA assay to detect antibodies against H5 subtypes of avian influenza virus. In this study, for the sake of safety, coding sequence of the cleavage site of H5 gene of H5N1 virus was deleted. Accordingly, in this work we developed an indirect ELISA adapted to detect AIV H5 antibody based on the recombinant H5 antigen. The H5 antigen successfully reacted with H5-positive sera and did not react with sera positive to ND, IB, IBD or ILT. These results indicated that H5 antigen expressed in sf9 cells had immunological specificity and the H5-ELISA developed was highly specific.

However due to lack of access to other anti-hemagglutinin sera, it is possible that the cross reaction with other influenza viruses be observed. In this case, generated recombinant antigen can be used to develop a Competitive ELISA assay. The results of comparison among H5-ELISA and HI assay indicated that ELISA detection assays were more specific and sensitive than HI assay.

Briggs and colleagues (34) first developed a standard curve of ET versus serum P/N ratio at one dilution. In this study, we used P/N ratio regression lines. A predication equation was derived from which the H5-ELISA titre (ET) of a serum sample could be determined using one dilution. The co-relation coefficient (*r* value) between ET and P/N at 1:500 reached at 0.986, which indicated that ELISA titers of antibodies would be derived at one serum dilution. The H5-ELISA assay, which can rapidly evaluate levels of antibodies to H5-HPAIV, is suitable for detection of antibodies against H5N1 in large numbers of serum samples in a limited time. The comparison between ET and HI, supported that H5-ELISA is as specific as HI. The test also showed the sensitivity of the assay.

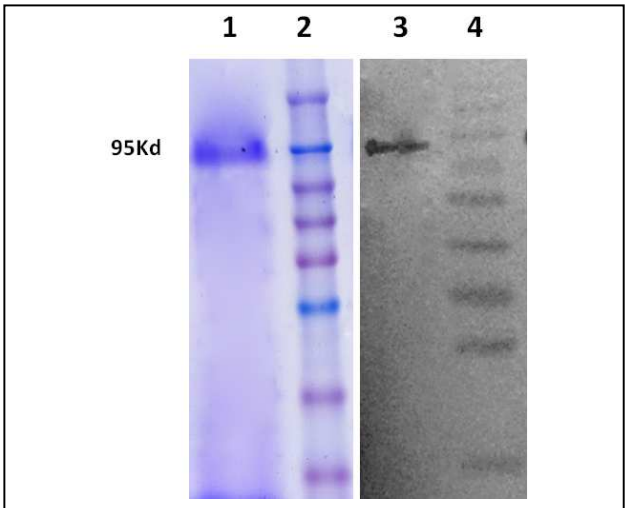

Fig. 1. Expression of H5 gene (A/chicken/Iran/53-3/2008(H5N1)) in the supernatant of SF9 cells transfected with H5-pIEx-3 vector by SDS-PAGE and western blot analysis. (Lanes 1-2) 10% SDS PAGE gel. (Lane 1) Purified H5 protein from Supernatant of SF9 cells transfected with H5-pIEx-3 vector. (Lane 2) Molecular weight marker. (Lanes 3-4) Western blot analysis with (Lane 3) purified H5 protein reacted with monoclonal antibody specific to the 6-histidine protein tag, (Lanes 4) Molecular weight marker.

In conclusion, the H5-ELISA provides an alternative and rapid serological diagnostic tool and is suitable for Highly Pathogenic Avian influenza H5 antibody screening, especially in poultry flocks and avian populations that may harbor several influenza subtypes. It can be used in the serological diagnostics and surveillance studies. This paper describes the expression of H5 subtype antigen in sf9 insect cells. This antigen was successfully employed as a recombinant antigen for development of an ELISA assay for detection of H5 antibodies in poultry and avian population.

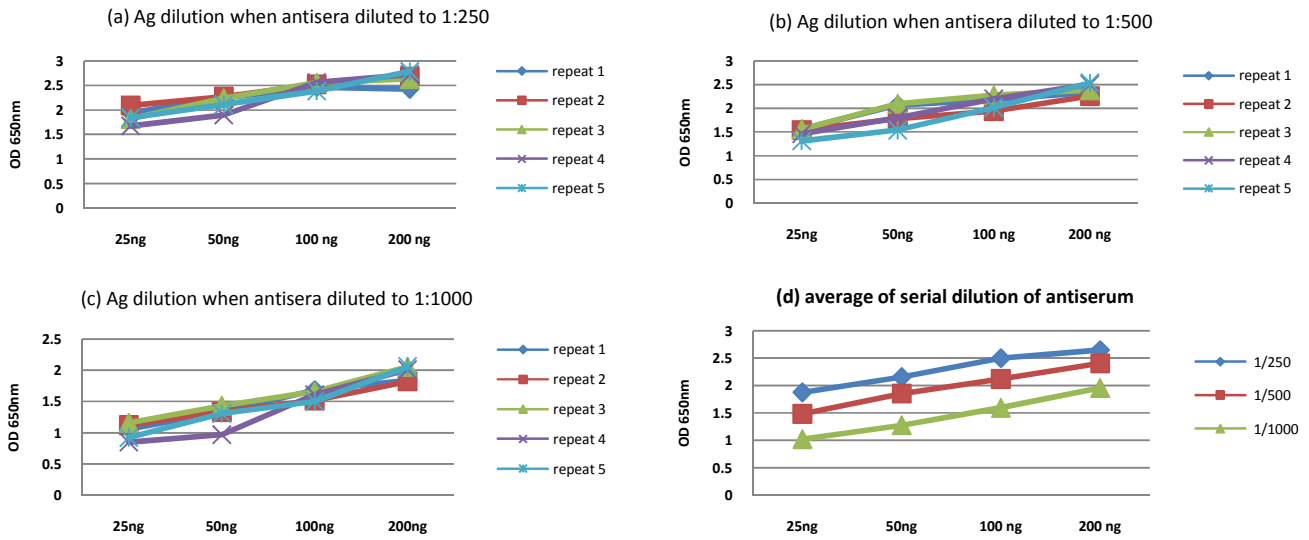

Fig. 2. Checkerboard titration of H5-antigen at different dilutions. Serum dilutions: (a): serum 1:250; (b): serum 1:500; (c): serum 1:1000; (d): average of standard positive serum in different dilutions. Results of repeats 1, 2 and 3 are belong to inter- and repeats 4 and 5 are belong to intra-assays.The standard deviation (SD) and Coefficient of variation (CV) were 0.022 and 16.8, respectively. Optimal concentration for the antigen was found to be 100ng per well.

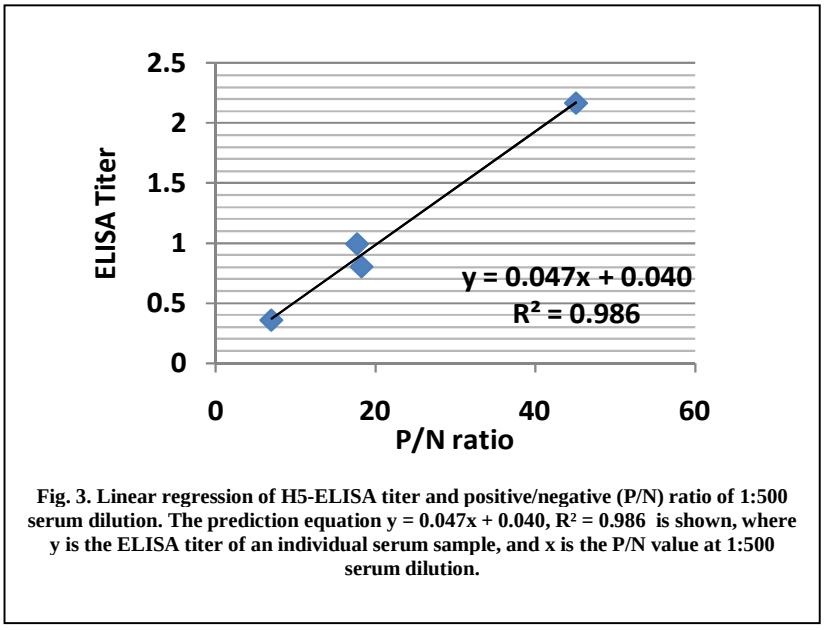

Fig. 3. Linear regression of H5-ELISA titer and positive/negative (P/N) ratio of 1:500 serum dilution. The prediction equation y = 0.047x + 0.040, R^2 = 0.986 is shown, where y is the ELISA titer of an individual serum sample, and x is the P/N value at 1:500 serum dilution.

TABLE I Result of specificity test of H5-ELISA against known positive sera for various viral diseases and known negative sera. The standard deviation (SD) is 0.022.

	Positive serum							Negative serum
	H5N1	H9N2	ND	IBD	IB	ILT	MD	H5N1
OD650 value	2.273	0.043	0.059	0.062	0.065	0.049	0.053	0.057
+/-	+	-	-	-	-	-	-	-

TABLE II The H5-ELISA assay compared with the HI test against killed antigen of H5N1 virus.The standard deviation (SD) and Coefficient of variation (CV) were 0.022 and 16.8, respectively. The detection sensitivities of two different H5-ELISA assays and HI test were compared on flock samples. Based on the constructed standard curve, we analyzed individual serum titers from collected positive sera (S1-S4). The result showed the H5-ELISA was more sensitive than the HI test.

	Serum sample titer			
	S1	S2	S3	S4
HI	1:64	1:128	1:32	1:1024
ET	0.804	0.993	0.358	2.165

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