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Original Article

Development of a Nano-ELISA system for the rapid and sensitive detection of H9N2 avian influenza

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ABSTRACT

Influenza is one of the most important viral diseases that is common among the birds and the mammals and is caused by specific viruses that belong to the Orthomyxoviridae family. Migratory aquatic birds are the reservoir of the disease and there is a likelihood of the disease in any region. There are different methods for detecting the avian influenza, but by the point of detection rates, the ELISA may be one of the most important current methods. In this work we synthesized Gold nanoparticles and conjugated it with rabbit-anti-chicken IgG-HRP. An ELISA test was done to compare the bioactivity of Au-anti-chicken HRP with anti-chicken HRP in order to detect the antibody against the H9N2 subtype of avian influenza virus. Using 133 field chicken sera, the sensitivity of nano-ELISA as compared to traditional ELISA was calculated to be 100%, whereas the specificity was 92%. This method was significantly more sensitive than the traditional ELISA and didn't require extra costs**.** It can therefore be concluded that the AuNP-HRP conjugate can be applicable in immune analysis procedure where a more confident result is required.

Keywords: Avian influenza, ELISA, Gold nanoparticle

INTRODUCTION

Influenza is a highly contagious disease that exists among humans and animals. Its virus is a member of the Orthomyxoviridae family and is classified into three types of A, B and C according to the differences in the characteristics of the matrix and nucleoproteins. (Fouchier et al., 2005; Loeffelholz, 2010). The influenza A virus based on its two surface glycoprotein antigens including hemagglutinin (HA) and neuraminidase (NA), can be divided into different subtypes. (Jia et al., 2009a). So far, 16 HA subtypes (H1, H2, … H16) and 9 NA subtypes (N1, N2, … N9) have been identified and different combinations of these subtypes have been detected in avian species (Webster and Hulse, 2004; Alexander, 2007). The first detection of H9N2 subtype of avian influenza A virus was in the United States in 1996. (Ge et al., 2009). Then this subtype was isolated from domestic pigs in Hong Kong in 1998 and two strains of this virus

obtained from Human for the first time in 1999 (Uyeki et al., 2002). The evidence of human infection by H9N2 subtype of avian influenza A virus in Hong Kong and China indicated that these viruses have ability to transmit directly from birds to humans (Guan et al., 1999; Peiris et al., 1999). Reports of humans infected by H9N2 virus in recent years in different parts of Iran, showed that these viruses can alter their structure and they may cause pandemics in human populations. (Homayounimehr et al., 2010). ELISA is a biochemical method that uses a solid-phase to diagnose the presence of compounds like antibody or antigen in a liquid sample based on antibody-antigen immunoreaction (Engvall and Perlmann, 1971). Due to its facility, high sensitivity and low cost, ELISA has been known as an appropriate experimental assay for serological diagnosis. (Wu et al., 2007; Brown et al., 2009). Because of the limitations of other serological assays, different enzyme-linked immunosorbent assays (ELISAs) have been developed for diagnosis of antibody against influenza A viruses (Shafer et al., 1998; Starick et al., 2006; Wu et al., 2007). Gold nanoparticles due to their high chemical stability, unique structure and size-dependent optical properties, can be used to increase sensitivity in diagnostic probes. These nanoparticles have a high surface area to volume ratio and physicochemical characteristics that because of these features have ability to bind to DNA, enzymes, antibodies and other biomolecules. Probes made of gold nanoparticles, have higher sensitivity, good durability and lower expenditure (Jia et al., 2009b). The optical properties of these nanoparticles have no effect on the properties of biomolecules attached to them (Tansil and Gao, 2006). Secondary conjugated antibody has an important role in immunology tests like ELISA, western blot and dot blot. Most of the conjugated antibodies attach to the horse radish peroxidase (HRP) enzyme which reacts with the substrate and produces a product with special optical absorption (Kumar et al., 2008). In this study a novel nano-ELISA was developed by combining colloidal gold nanoparticles with rabbit anti-chicken IgG-HRP to detect antibody against influenza virus in chicken sera and the Au-anti-chicken HRP (nano-ELISA) was compared with anti-chicken HRP (traditional ELISA). We found that using gold nanoparticles in traditional ELISA creates a multi-enzyme system that not only amplifies the optical signal, but also increases the sensitivity and decreases the detection limit. This procedure is as simple as that of traditional ELISA and doesn't require extra cost.

MATERIALS AND METHODS

Chemicals and instruments. H9N2 influenza virus and 133 field chicken sera were obtained from Department of Poultry Diseases of Razi Vaccine & Serum Research Institute, rabbit anti-chicken IgG-HRP as secondary antibody and bovine serum Albumin (BSA), were purchased from sigma-Aldrich (USA), BM blue OPD Enzyme substrate was purchased from Roche (Germany), 96 well ELISA polystyrene microplate were from Nunc (Denmark). well absorbencies were read with a microplate reader (BIO-RAD,USA). Chloroauric acid (HAuCl4) and three sodium citrate (C6H5O7Na3) were obtained from Sigma-Aldrich (USA).

Solutions and buffers. The coating buffer consisted of 0.05 M carbonate/bicarbonate buffer (pH 9.6). the phosphate buffer saline (PBS) consisted of 0.01 M disodium phosphate, 0.01M mono sodium phosphate and 0.01 M NaCl (pH 7.4).washing and dilution buffer (PBST) consist of 0.01 M PBS with added 0.05% (v/v) tween 20, blocking buffer solution consist of PBST solution with added 5% (w/v) skim milk.

Synthesis of Gold nanoparticles. Gold nanoparticles were synthesized according to Turkevich method (Turkevich et al., 1951). Synthesizing Gold nanoparticles based on the reduction of tetra chloroauric acid by sodium citrate at special concentrations and high temperature (around 80 - 100 °C), causes the gold to reduce from Au^{+3} ($HAuCl_4$) to Au^0, with a change in color from light yellow to dark red indicating the formation of gold nanoparticles. In the synthesis, Citrate has a dual role of reducing and stabilizer agent of gold nanoparticles. Gold nanoparticles

were characterized by using transmission electron microscopy (TEM) in order to measure the size.

Preparation of gold nanoparticle bio-probe. The rabbit-anti-chicken IgG-HRP conjugation with gold nanoparticles prepared according to the previously published procedure with slight modification (Ambrosi *et al* 2010) The pH of AuNP solution was adjusting to pH 7 with NaOH 0.01 M. 12 µl of rabbit-anti-chicken IgG HRP solution at a concentration of 200 µg/ml was adjusted to 100 µl by adding 88 µl of Distilled water and then added to 1ml of the AuNP solution, then the mixture was stirred for 30 min and to remove the extra antibody, added to micro tube and centrifuged at 15000 g for 15 min. the clear supernatant was removed and the precipitated gold nanoconjugate were resuspended in 1 ml of PBST (pH7.4) and stored at 4 °C for use.

Standardization of Antigen coating concentration and optimum dilution of gold nanobioconjugate. A checkerboard titration was developed to determine the optimal Ag coating concentration and the optimal gold nanobioconjugate dilution. For better comparison of two tests (traditional ELISA and nano ELISA), serum samples were used at a constant dilution, as in commercial kit (IDEXX, USA). In this test, each row of wells was coated with the various concentrations of H9N2 Ag (0.5, 1, 2 and 5 µg/ml) and the known positive and negative sera in a constant dilution at 1:300 was used. Also gold nanobioconjugate was prepared in three specified dilutions (1:2, 1:4, 1:8) and was added to wells. The best Ag coating concentration and the optimal gold nanobioconjugate dilution were determined based on maximum differences between the optical density (OD) of positive and negative serum.

Nano-ELISA test. The enzyme immune assay based on gold nanoparticles was performed in rigid poly styrene microplates with 96 wells. Each well was coated with 100µl of Ag (1 µg/ml) that diluted in carbonate/bicarbonate buffer (pH 9.6) and incubated over night at 4 °C. the plate was washed 3 times with PBST buffer (including 0.01 M PBS and 0.05% tween 20).The remaining sites in the well were blocked by 250µl per well of blocking buffer (5% skim milk in PBST) after incubation for 90 min at 37 °C, fluid in plate was dumped and each well washed 3 times with PBST buffer. Sera were diluted 1:300 in dilution buffer (0.01 M PBS and 0.05% between 20) and 100 µl of serum samples were added in to each well and incubated for 75 min at 37 °C. Then the plate was washed 5 times with washing buffer (PBST). 100 µl of gold nanobioconjugate in 1:8 dilution was added in to each well and incubated for 75 min at 37 °C. After washing as described above with PBST, 100 µl of BM blue OPD enzyme substrate was added to each well and the plate was incubated at room temperature for 9 min in dark. The substrate reaction was stopped with added 50 µl of stop solution (1 M H_2SO_4) and the optical density value was read at 450 nm with a 96 well ELISA reader.

Data analysis and statistics. Statistical analysis was performed with the sigma plot 12 software package. Comparisons of groups due to the Non-normally distributed data were assessed by Mann-Whitney U test. P values less than 0.001 were considered as statistically significant.

RESULTS

The gold nanoparticle which synthesized has the highest absorbance in 520 nm and it was characterized by TEM, the size was around 15-20 nm (Figure1). the highest assay sensitivity was achieved when the Au complex prepared at pH 7.

Optimum antigen concentration and gold nanobioconjugate dilution. To determine optimal concentration of the antigen and optimal dilution of gold nanobioconjugate solution, a checkerboard titration was developed using different concentrations of antigen and three different dilution of gold nanobioconjugate solution. Optimal concentration for the antigen was found to be 1µg/ml meanwhile, the optimal gold nanobioconjugate dilution was 1:8.

Determination of nano-ELISA cut-off value. The cut-off is normally used for the best discrimination between positive and negative samples. Using 1 µg/ml of H9N2 antigen the mean OD value of 12 positive

control serum samples at 1:300 dilution was 0.949. The cut off for chicken sera (mean OD of 12 positive control sera- 1/2 standard deviation) was set at 0.886[0.949 – 1/2 (0.152)]. Therefore, any sample showing an OD of 0.886 or more was considered positive for avian influenza.

Evaluation of nano-ELISA as compared to traditional ELISA. Among 133 serum samples in this study, according to the determined cut off, 69 of these samples (51.87%) were positive and 64 of them (48.12%) were negative. By comparing these results to the results from testing the same samples obtained by commercial kit (IDEXX) without nanoparticles, the sensitivity and specificity according to the following formulas were 100% and 92%, respectively (Table 1).

Table 1. Evaluation of nano ELISA to detect antibodies in chicken sera as compared to traditional ELISA

	Traditional ELISA		Total
	Positive	Negative	
Nano ELISA			
Positive	63	6	69
Negative	-	64	64
Total	63	70	133

Sensitivity=TP/TP+FN ×100, where, TP (true positive) is the number of sera positive by traditional ELISA, FN (false negative) the number of sera positive by traditional ELISA but negative by nano ELISA. Specificity=TN/TN+FP×100, where TN (true negaitive) is the number of sera negative by traditional ELISA, FP (false positive) the number of sera negative by traditional ELISA but positive by nano ELISA. Number of positive and negative sera by both nano-ELISA and traditional ELISA test. The sensitivity and specificity of nano ELISA as relative to the traditional ELISA were calculated to be 100% and 92% respectively. Precision of the developed method was calculated. Intra assay precision was assessed by running of three serum samples for four times on the same day. Acceptable value with average coefficient of variability (%CV) about 2.02% were obtained (Table 2). Also inter assay precision was assessed by running the same three serum samples on four different days and Acceptable value with average coefficient of variability (CV%) about 5.09 % were obtained (Table 3).

Table 2. Intra assay precision of the Nano ELISA method

Serum(N=3)	Mean(OD)	SD	CV%
1	0.63	0.01	1.57
2	0.68	0.01	2.05
3	0.81	0.02	2.46

Coefficient of variation= (SD/mean) × 100%

Table 3. Inter assay precision of the Nano ELISA method.

Serum(N=3)	Mean(OD)	SD	CV%
1	1.28	0.056	4.37
2	1.27	0.074	5.82
3	0.765	0.039	5.09

Coefficient of variation= (SD/mean) × 100%

The results of the difference between the optical absorbance of sera with Au-anti-chicken HRP and sera with Anti-chicken HRP are shown in Figure 2. Statistical differences between groups were found by Mann-Whitney U test ($P < 0.001$). The first group (positive sera with Au-anti- chicken HRP) that is located in the higher region, shows higher optical absorbance than the second group (positive sera with anti-chicken HRP) and also the third group (negative sera with Au-anti-chicken HRP) has a higher optical absorbance in comparison to the other group (negative sera with anti-chicken HRP). Also the negative sera with Au-anti-chicken HRP compared to those of the other negative sera showed less variation. So these results confirmed that the use of gold nanoparticles in traditional ELISA can be enhance the optical signal. Figure 3 shows the mean of optical absorbance of positive and negative serum samples with anti-chicken HRP and Au-anti-chicken HRP. The mean of optical signals in the positive and negative sera with gold nanoparticles is more than the positive and negative sera without gold nanoparticles.

DISCUSSION

In this study we developed an indirect ELISA based on gold nanoparticles for the rapid and sensitive etection of Antibody against H9N2 virus in chicken sera. A schematic representation of the steps used to perform the nano-ELISA test is shown in figure 4. Gold nanoparticles due to their high surface area can be used to design probes to enhance the optical signal by immobilizing several types of biomolecules on their surface (Ambrosi et al., 2010; Liu et al., 2010; Peng et al., 2013). We proved the notion that gold nanoparticles can be used to improve the traditional ELISA test in order to achieve higher sensitivity and amplify optical signal. When Au nanobioconjugate was used, higher signals were recorded and these results demonstrated that the IgG-HRP loaded on the surface of AuNP have signal amplification function. In nano-ELISA method, detection limit is getting much lower than the classical ELISA due to use of the gold nanoparticles (Jia et al., 2009b; Ambrosi et al., 2010). The detection sensitivity of commercial kit (IDEXX, USA) is 10 µg/ml, whereas the detection limit obtained by using nano-ELISA method was 1 µg/ml, which was 10 times less than the traditional ELISA. So with the lower concentration of antigen in nano ELISA method, the presence of antibody in serum samples can be detected. The use of gold nanoparticles as signal amplifier not only increases the sensitivity, but also reduces the incubation time of substrate, so that in this study Incubation time with BM blue OPD (substrate) reduced from 20 min to 9 min in order to develop the color in comparison to traditional ELISA. Since small amounts of gold nanobioconjugate solution is needed in each ELISA test, the remaining solution is reserved and used for several tests. (Ambrosi et al., 2010; Hoang et al., 2012). The prepared Solution of colloidal gold nanobioconjugate in this work was stable for 40 days at 4 °C and its stability allowed to be used in several ELISA tests without other stabilizing agents. To improve the efficiency of the secondary conjugated antibody, we used gold nanoparticles as a carrier of the signaling antibody against avian influenza antigen in serum samples. Our data and other research results confirm that using gold nanoparticles as the multi enzyme carrier in traditional ELISA can considerably increase the sensitivity and decrease the detection limit (10 times less than the traditional ELISA). This method is facile and its assay procedure is the same as that of the traditional ELISA and doesn't require extra cost. We propose this novel method can be extended to detecting other subtype of avian influenza virus.

In this work we presented results of the novel indirect ELISA based on gold nanoparticles for sensitive and early detection of Antibody against H9N2 virus in chicken sera. Our test proved that the use of gold nanoparticles leads to better performance and increases the sensitivity. This facile and sensitive method has potential application to recover the sensitivity of traditional ELISA methods to detect the Antibody against H9N2 avian influenza virus.

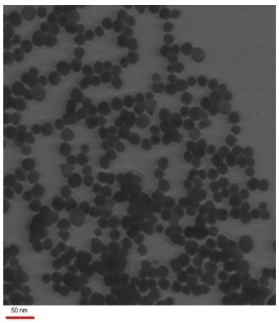

Figure1. Transmission electron microscopy image of gold nanoparticles.

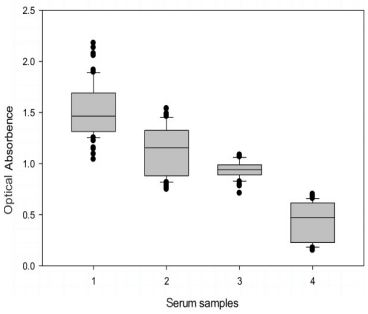

Figure 2. Diagram of Mann-Whitney U test. Pair wise comparisons between groups were performed by Mann-Whitney test and in all groups, the difference was significant ($p < 0.001$). 1. Au-anti-chicken HRP + / 2. Anti-chicken HRP + / 3. Au-anti-chicken HRP - / 4. Anti-chicken HRP -.

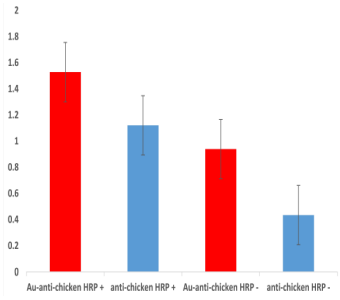

Figure 3. The mean of optical absorbance at 450 nm of positive and negative serums with Au-anti chicken HRP (Red columns) and positive and negative serums with Anti-chicken HRP (Blue columns).

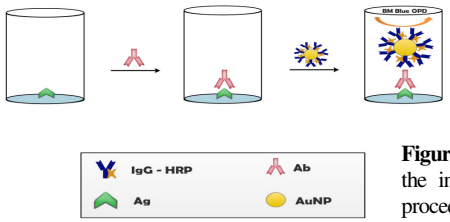

Figure 4. Schematic of the indirect-type ELISA procedure with the application of AuNPs as the multi enzyme carrier.

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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References

Alexander, D.J., 2007. An overview of the epidemiology of avian influenza. Vaccine 25, 5637-5644.

Ambrosi, A., Airo, F., Merkoci, A., 2010. Enhanced gold nanoparticle based ELISA for a breast cancer biomarker. Anal Chem 82, 1151-1156.

Brown, J.D., Stallknecht, D.E., Berghaus, R.D., Luttrell, M.P., Velek, K., Kistler, W., Costa, T., Yabsley, M.J., Swayne, D., 2009. Evaluation of a commercial blocking enzyme-linked immunosorbent assay to detect avian influenza virus antibodies in multiple experimentally infected avian species. Clin Vaccine Immunol 16, 824-829.

Engvall, E., Perlmann, P., 1971. Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. Immunochemistry 8, 871-874.

Fouchier, R.A., Munster, V., Wallensten, A., Bestebroer, T.M., Herfst, S., Smith, D., Rimmelzwaan, G.F., Olsen, B., Osterhaus, A.D., 2005. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J Virol 79, 2814-2822.

Ge, F.-F., Zhou, J.-P., Liu, J., Wang, J., Zhang, W.-Y., Sheng, L.-P., Xu, F., Ju, H.-B., Sun, Q.-Y., Liu, P.-H., 2009. Genetic Evolution of H9 Subtype Influenza Viruses from Live Poultry Markets in Shanghai, China. Journal of Clinical Microbiology 47, 3294-3300.

Guan, Y., Shortridge, K.F., Krauss, S., Webster, R.G., 1999. Molecular characterization of H9N2 influenza viruses: were they the donors of the "internal" genes of H5N1 viruses in Hong Kong? Proc Natl Acad Sci U S A 96, 9363-9367.

Hoang, H., Phan, T.H., Conrad, U., Chu, H.H., 2012. Production of antibody labeled gold nanoparticles for influenza virus H5N1 diagnosis kit development. Nanosci Nanotechnol 3, 45017.

Homayounimehr, A.R., Dadras, H., Shoushtari, A., Pourbakhsh, S.A., 2010. Sequence and phylogenetic analysis of the haemagglutinin genes of H9N2 avian influenza viruses isolated from commercial chickens in Iran. Trop Anim Health Prod 42, 1291-1297.

Jia, C.P., Zhong, X.Q., Hua, B., Liu, M.Y., Jing, F.X., Lou, X.H., Yao, S.H., Xiang, J.Q., Jin, Q.H., Zhao, J.L., 2009a. Nano-ELISA for highly sensitive protein detection. Biosens Bioelectron 24, 2836-2841.

Jia, N., de Vlas, S.J., Liu, Y.X., Zhang, J.S., Zhan, L., Dang, R.L., Ma, Y.H., Wang, X.J., Liu, T., Yang, G.P., Wen, Q.L., Richardus, J.H., Lu, S., Cao, W.C., 2009b. Serological reports of human infections of H7 and H9 avian influenza viruses in northern China. J Clin Virol 44, 225-229.

Kumar, S., Aaron, J., Sokolov, K., 2008. Directional conjugation of antibodies to nanoparticles for synthesis of multiplexed optical contrast agents with both delivery and targeting moieties. Nat Protoc 3, 314-320.

Liu, M., Jia, C., Huang, Y., Lou, X., Yao, S., Jin, Q., Zhao, J., Xiang, J., 2010. Highly sensitive protein detection using enzyme-labeled gold nanoparticle probes. Analyst 135, 327-331.

Loeffelholz, M.J., 2010. Avian influenza A H5N1 virus. Clinics Lab Med 30, 1-20.

Peiris, M., Yuen, K.Y., Leung, C.W., Chan, K.H., Ip, P.L., Lai, R.W., Orr, W.K., Shortridge, K.F., 1999. Human infection with influenza H9N2. Lancet 354, 916-917.

Peng, C.-F., Duan, X.-H., Pan, Q.-L., Liu, L.-Q., Xue, F., 2013. Ultrasensitive Nano-ELISA for Detecting Sulfadimethoxine in Chicken Tissue. Journal of Chemistry 2013, 5.

Shafer, A.L., Katz, J.B., Eernisse, K.A., 1998. Development and validation of a competitive enzyme-linked immunosorbent assay for detection of type A influenza antibodies in avian sera. Avian Dis 42, 28-34.

Starick, E., Werner, O., Schirrmeier, H., Kollner, B., Riebe, R., Mundt, E., 2006. Establishment of a competitive ELISA (cELISA) system for the detection of influenza A virus nucleoprotein antibodies and its application to field sera from different species. J Vet Med B Infect Dis Vet Public Health 53, 370-375.

Tansil, N.C., Gao, Z., 2006. Nanoparticles in biomolecular detection. Nano Today 1, 28-37.

Uyeki, T.M., Chong, Y.H., Katz, J.M., Lim, W., Ho, Y.Y., Wang, S.S., Tsang, T.H., Au, W.W., Chan, S.C., Rowe, T., Hu-Primmer, J., Bell, J.C., Thompson, W.W., Bridges, C.B., Cox, N.J., Mak, K.H., Fukuda, K., 2002. Lack of evidence for human-to-human transmission of avian influenza A (H9N2) viruses in Hong Kong, China 1999. Emerg Infect Dis 8, 154-159.

Webster, R.G., Hulse, D.J., 2004. Microbial adaptation and change: avian influenza. Rev Sci Tech 23, 453-465.

Wu, R., Hu, S., Xiao, Y., Li, Z., Shi, D., Bi, D., 2007. Development of indirect enzyme-linked immunosorbent assay with nucleoprotein as antigen for detection and quantification of antibodies against avian influenza virus. Vet Res Commun 31, 631-641.