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Full Article

Applying conserved peptides of NS1 Protein of avian influenza virus to differentiate infected from vaccinated chickens

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ABSTRACT

Avian influenza (AI) is a highly contagious disease in poultry and outbreaks can have dramatic economic and health implications. For effective disease surveillance, rapid and sensitive assays are needed to detect antibodies against AI virus (AIV) proteins. In order to support eradication efforts of avian influenza (AI) infections in poultry, the implementation of "DIVA" vaccination strategies, enabling the Differentiation of infected from Vaccinated Animals have been recommended by international organizations. A system, based on the detection of antibodies to the Non-Structural (NS1) protein of AI has been proposed to enable the detection of field exposure in vaccinated flocks, and through this detection, infected flocks may be properly managed. In this project we have used two conserved peptides of NS1 protein to develop a peptide based ELISA method. This ELISA could screen the infected and vaccinated sera due to their titer of antibody. Following experimentally infection and vaccinate of chickens, antibodies to the peptides of the NS1 protein were detected by enzyme-linked immuno sorbent assay (ELISA). These findings indicate that there is a significant difference in the viral replication in chickens, resulting in a variation in the production of antibodies to NS1, as detected by the peptide- based ELISA used. These results demonstrate the specific ELISA for anti NS1 antibodies that have diagnostic value for the poultry industries.

Keywords: Avian influenza , ELISA, NS1, Peptide, infected , vaccinated

INTRODUCTION

Influenza viruses (family *Orthomyxoviridae)* are grouped into three types, A, B and C by the *antigenicity* of the nucleoprotein and matrix proteins (Lamb & Krug 2001). Avian influenza virus, a type A virus, is subtyped according to the antigenic subtype of the hemagglutinin (H) and neuraminidase (N) proteins. With the recognition of another H subtype, there are now16Hsubtypes and 9Nsubtypes (OIE 2004) .Avian influenza virus is categorized into two groups based on their virulence. Avian influenza virus is categorized into two groups based on their virulence. Avian influenza (AI) virus poses significant threats to both animals at human health (Faucher *et al* 2004). To get AI under control according to recommendation of International organization (FAO/OIE) then eradication can be achieved by differentiation of infected from

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vaccinated animals (DIVA) strategies. Several DIVA systems have been developed although they have some limitations in the field (Capua *et al* 2002, Cattoli *et al* 2003, Lee *et al* 2004, Pasick *et al* 2004, William *et al* 2006). A promising system based on the detection of antibodies against a specific antigen, the non structural (NS1) protein of AI has been a good candidate (Tumpey *et al* 2005, William *et al* 2006, William *et al* 2007). The NS1 protein is synthesized in large amounts in infected cells but is not incorporated into the mature virions. In this study data generated in our laboratory, based on the work of Tumpey et al. indicated that following experimental infection and vaccinated with H9N2, antibodies to the conserved peptides of NS1 protein were detectable with synthetic peptide based ELISA.

MATERIALS AND METHODS

Viruses. Viruses were obtained from the repository of the International Reference Laboratory in Razi Research Inst, Iran and were typed using standard methods (CEC 1992, William *et al* 2006). They were isolated throughout the 2005–2008 H9N2 avian influenza in Iran. The viruses used in this study, which were passaged in specific pathogen free (SPF) eggs not more than twice and the median embryo infectious dose (EID50) was calculated according to the Reed and Muench formula(Reed *et al* , William *et al* 2007).

Samples. 53 chickens (White Leghorn specific pathogen free) that were 3 to 4 wk old and had been hatched and reared in isolation were infected nasally with 10^6 EID50 of the virus at days 7, 14 and 28 after first infection. 20 chickens were immunized with the commercial inactivated vaccine (10µg) one to three times by s.c. inoculation of 0.5ml of vaccine in the nape of the neck. Boosted poultry received a second inoculation 16 days after the initial vaccination. Control birds received the same volume of normal allantoic fluid emulsified in the same adjuvant (Sigma, St. Louis, Mo). The serum samples were collected from those birds and 69 infected sera from field.

Peptide. According to Tumpey et al. studies, two peptide, of NS1 protein sequences 28- GDAPFLDRRDQK-42 and 35-LRKDQKALKGRGS-49 were selected on the basis of their high degrees of antigenicity and were synthesized by Metabion, Germany.

ELISA. Maxisorp ELISA plates (NUNC, Roskilde, Denmark) were coated with the NS1 peptides (l, 2, 4, 8 µg/well) in bicarbonate buffer (pH 9.6, Fluka, Buchs, Switzerland) and incubated overnight at $4^{\circ}C$ The plates were blocked using 5% (w/v) nonfat skimmed milk(Sigma) in phosphate buffer solution plus 0.05% Tween 20 (PBST, Merk, Darmstadt, Germany), and bound antibody was detected using anti-chicken IgG conjugated to horseradish peroxidase (Sigma) serum samples were diluted 1:50 to 1:800 in PBST. Wash steps were performed using PBST. Absorbance at 450 nm was measured following the addition of a peroxidase substrate solution (BM Blue, Roche, Mannheim, Germany). Serum samples were considered positive for the peptides if they yielded an OD450 nm greater than the mean mines0.5 standard deviations for the corresponding pre-immune serum.

Statistic Analysis. Kruskal-Wallis one way analysis of variance on ranks was used to compare study groups and pairwise comparison was made by Tukey test.

RESULTS

NS1 peptides were used as antigen to develop the Indirect ELISA test for diagnosis of infected poultries. Optimization experiments were carried out to determine the most suitable dilution of serum and secondary antibody for poultry species under study. Four concentration of peptides: 1, 2, 4, 8 µg/well were used to evaluate the best optimum concentration of peptides for coating the plates, for each concentration two positive and negative sera were selected and five dilution of serum used for checker board assay. Without influencing the sensitivity of the test, the optimal dilutions determined were as follows: serum samples diluted 1:200 for all chickens in PBST, secondary antibody diluted 1:10000 in PBST and the concentration of NS1 peptide was achieved 4 µg/well

(Table 1). Using these optimized conditions ELISA test was developed to determine the antibody titer against peptide A and B in several candidates, 20 infected and 20 vaccinated sera of chickens. As shown in Figure 1 and Figure 2, the titer of antibody in infected sera in comparison with vaccinated sera shows higher titer against NS1 peptides. Statistical analysis shows all study groups were significantly different ($P<0.001$) and O.D. of infected sera was significantly higher than O.D. of vaccinated sera in both peptide A and peptide B antigen ($P<0.05$).This peptide based ELISA is able to differentiate infected poultries from vaccinated. There is not any difference between peptide A and B.

Table1. Checker board assay for optimization the titer of Sera and NS1 peptides as antigen.

Peptides Concentration	1 (µg/well)		2 (µg/well)		4 (µg/well)		8 (µg/well)	
Serum dilution	+	-	+	-	+	-	+	-
1/50	1.052	0.602	1.128	0.609	2.410	0.618	2.518	.889
1/100	0.989	0.431	1.123	0.451	2.321	0.481	2.508	0.798
1/200	0.981	0.240	1.118	0.241	2.258	0.243	2.302	0.699
1/400	0.726	0.234	0.956	0.238	1.456	0.241	1.816	0.587
1/800	0.548	0.231	0.763	0.235	1.021	0.236	1.118	0.573

DISCUSSION

Influenza presents the world with an ongoing threat of disease associated with a high morbidity and mortality .The control of AI infections in poultry is a target the scientific veterinary community must achieve in order to manage the pandemic potential, to preserve profit of the poultry industry and to guarantee food security to developing countries (Capua *et al* 2002). In this study, we have developed a Peptied base-ELISA in line with previous studies (Natsumi *et al* 2011, Tumpey *et al* 2005, Zhao *et al* 2005). This assay simultaneously measure antibodies to two highly conserved peptides of NS1 protein derived from influenza A. We have shown that this ELISA assay is able to measure antibodies directed against both peptides in a quantitative manner to detect anti-NS1 antibodies in chickens experimentally infected and would not detect antibodies in chickens vaccinated (Drummond *et al* 2008).

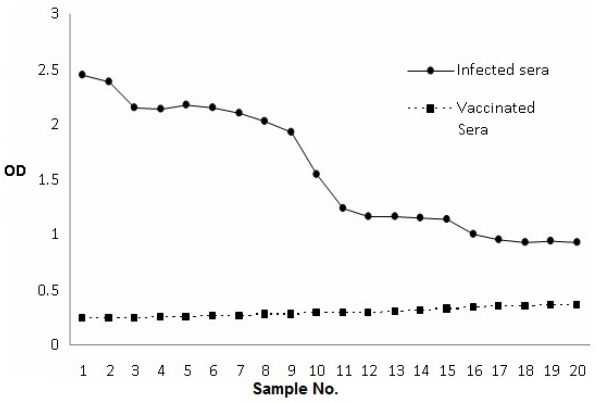

Figure 1. Obtical density of infected and vaccinated sera with NS1 peptide A as antigen in ELISA.

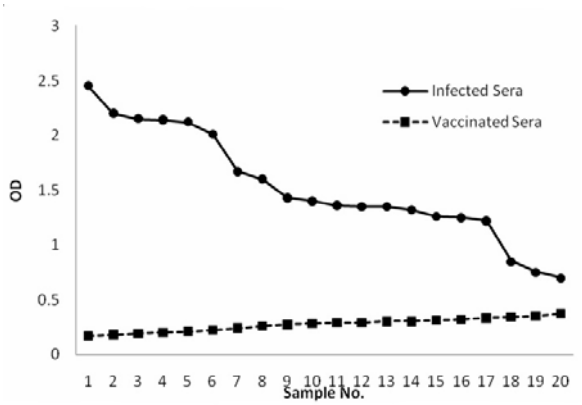

Figure 2. Obtical density of infected and vaccinated sera with NS1 peptide B as antigen in ELISA.

The NS1 protein, although a weak antigen in comparison to other influenza virus proteins, is remarkably conserved among type A influenza viruses (Buonagurio *et al* 1986, Ludwigs *et al* 1991, Suarrez *et al* 1999, Teanor *et al* 1989). Previous studies demonstrated that the NS1 protein could be detected in the sera of horses experimentally infected with the H3 subtype of influenza virus but not in the immune sera of animals immunized with inactivated viruses (Birch-Machin *et al* 1997, Ozaki *et al* 2001) The inactivated whole-virus equine vaccine used in the aforementioned studies was partially purified, thus removing residual NS1 protein that would be present in the infectious medium. This allowed the clear distinction between H3-vaccinated and H3 infected horses by using the Ns1

protein as a differential marker ((Birch-Machin *et al* 1997, Ozaki *et al* 2001). Many authorities and international agencies advocate that the DIVA strategy should be used in combination with AI vaccination (Natsumi *et al* 2011, Suarez *et al* 2007, Suarez 2005). The NS1- ELISA is able to detect highly pathogenic avian influenza virus infection in both unvaccinated and vaccinated chickens and provides a proof of concept DIVA in AI vaccine programs. A study (by Ozaki *et al* 2001) identified antibodies to the NS1 exclusively in the sera of mice infected with equine influenza viruses and not in those mice immunized with inactivated virus. These data indicated that the NS1 protein could be used for serological diagnosis to distinguish horses infected with equine influenza viruses from those immunized with inactivated vaccines. Similarly, recent work on a limited number of avian samples has indicated that antibodies to the NS1 protein could possibly be used as part of a DIVA strategy as seroconversion to antibodies against the NS1 protein was achieved in chickens and turkeys experimentally infected with different subtypes of influenza A virus. A similar reaction was not detected in birds inoculated with inactivated vaccines (Tumpey *et al* 2005). Likewise, a study by (William *et al* 2007), determined whether the truncation involved an immunogenic region of the NS1 protein a peptide spanning residues 219 aa to 230 aa was synthesized and tested in an indirect ELISA against sera obtained from turkeys experimentally infected with a virus strain known to have a full length NS1 protein. Although a part from the serological diagnoses, molecular approaches, such as real-time PCR, have been developed for diagnosis of various viral diseases, including influenza. Previous study showed validated real-time PCR assays are at least as sensitive as virus isolation and as fast as ELISA, the costs and equipments required for the assays may limit their field applications (Douglas *et al* 2009, Natsumi *et al* 2011).

In conclusion, the date from this work indicate that a Peptide-ELISA system based on the detection of antibodies to the conserved region of NS1 Protein appear, to be suitable for differentiating infected and vaccinated avian influenza poultry.

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