Evaluation of a Commercial ELISA Kit (IDEXX) to Differentiate AI Virus-infected Poultry from AI-Vaccinated Poultry (DIVA)

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ABSTRACT

Vaccination programs for the control of avian influenza (AI) in poultry have limitations due to the problem of differentiating between vaccinated and virus-infected birds. The use of AI vaccination in poultry would have greater worldwide acceptance if reliable tests that clearly discriminated between naturally infected and vaccinated-only animals (DIVA) were available. In this study, to differentiate avian influenza virus (AIV)-infected chickens vs. chickens immunized with inactivated avian influenza virus, a commercial ELISA Kit (IDEXX) was used. Using HI as the reference method, the sensitivity of the commercial ELISA kit was 100% and its specificity was 100%. In the present study, we demonstrated that, 3 weeks after infection or vaccination, sera from both infected and vaccinated groups were tested positive and the difference in mean optical density between vaccinated and challenged birds, as detected by a commercial ELISA kit (IDEXX), was very small. Therefore, this assay cannot distinguish infected from vaccinated poultry.

INTRODUCTION

Influenza viruses are segmented, negative-strand RNA viruses belonging to the family Orthomyxoviridae, and are divided in three types: A, B, and C – Wright *et al.* (2007). All three types of influenza virus are found in humans, but only the type A viruses are found in poultry and are referred to as avian influenza (AI) viruses.

Type A influenza viruses are divided into subtypes based on the antigenic properties of the major surface glycoproteins: hemagglutin in (HA) and neuraminidase (NA) proteins. In total, 16 HA subtypes (H1–H16) and nine neuraminidase subtypes (N1–N9) are currently described – Wright *et al.* (2007). H9N2 virus shares similar receptor binding epitopes with human influenza viruses, has a broader host range, and has been demonstrated to infect humans. In addition, H9N2 AIV infection in chickens is latent and easily overlooked, increasing its chance of infecting humans.

Although AI vaccines have been successful at providing protection against clinical signs and death among poultry – Garcia *et al.* (1998), Halvorson (2002) and Swayne (2001), they are not commonly used as part of programs for the control and eradication of LPAI or HPAI virus infections – Halvorson (2002). For a number of reasons, including the concerns with trade embargoes on poultry and poultry products, quarantine and elimination of infected flocks (i.e., stamping out or controlled marketing) are often preferred measures for controlling AI. An additional limitation is that traditional AI virus vaccines can interfere with serologic surveillance; hence, these vaccines induce antibodies that are indistinguishable from live virus infection, as determined by commercial enzyme-linked immunosorbent assays (ELISAs), and hemagglutination

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inhibition (HI) tests – Swayne (2003). Therefore, efforts have been focused on developing new serologic tools to allow differentiation of infected from vaccinated animals, commonly known as the DIVA strategy.

For international commerce of vaccinated birds, the DIVA strategy needs to not only discriminate between infected and vaccinated birds, but also to identify vaccinated birds that become infected with AIV. Several different DIVA strategies have been proposed – Suarez (2005), some of which require the use of appropriate vaccines and specific companion serologic discriminatory tests – Capua *et al.* (2004), Capua *et al.* (2003), Cattoli *et al.* (2006), Lee *et al.* (2004) and Tumpey *et al.* (2005). Such a strategy is important to provide assurance to trading partners that the poultry product is free from influenza virus contamination and can be safely traded. The DIVA strategy has been accepted by some countries in the European Union to monitor and provide assurance on the infection-free status of vaccinated poultry during an LPAI outbreak – Capua & Marangon (2003) and Suarez (2005).

One potential target for the DIVA strategy for avian influenza is the nonstructural (NS1) protein. The NS1 protein is encoded by the smallest gene segment of the influenza virus, segment 8, and it has a critical role in preventing the induction of interferon in the host to allow efficient virus replication – Garcia-Sastre *et al.* (1998). The NS1 protein is a conserved protein amongst type A influenza viruses, but it exists as two major subtypes, A and B, that differ by about 35% in nucleotide sequence – Suarez & Perdue (1999). The NS1 protein is produced during influenza virus replication in infected cells – Birch-Machin *et al.* (1997) and Tumpey *et al.* (2005), but it is not packaged into the infectious viral particle, being a true nonstructural protein. Because most AIV vaccines are primarily chemically inactivated whole virus, vaccinated animals theoretically should not have an antibody response to the NS1 protein, but infected poultry should have an NS1 antibody response because it is produced at high levels in infected cells.

This NS1 DIVA approach was first proposed for equine influenza viruses, and purified NS1 protein has been used to detect antibodies against NS1 in serum samples from ponies and horses experimentally infected with influenza virus – Birch-Machin *et al.* (1997) and Ozaki *et al.* (2001). Serologic studies on chickens and turkeys using an ELISA with AIV NS1 protein produced in *Escherichia coli* as antigen indicated that infected birds showed higher levels of NS1 antibodies as compared to vaccinated birds – Tumpey *et al.* (2005). And Zhao *et al.* (2005).

MATERIALS AND METHODS

Chickens

A total of 300 day-old broiler chicks (Ross 308) were purchased. Birds were housed in three different rooms and raised under high health conditions.

Virus

The AI viruses used in this study derived from a virus isolate (A/Chicken/Iran/AH-1/06), subtype H9N2, from a broiler chicken flock in Ahvaz, with respiratory signs and mortality. These viruses were initially isolated in 10-day-old embryonated chicken eggs (ECE). They were subtyped by standard hemagglutination-inhibition and neuraminidase-inhibition tests with polyclonal chicken antisera. The viruses were passaged in ECE and the all antoic fluids were harvested and used as stock viruses for further analysis.

Vaccines

AIV vaccine consisting of inactivated commercial AI vaccines, subtypes H9N2.

Experimental design

Birds were divided into three equal groups (1 to 3). Chicks in groups 1 were subcutaneously vaccinated (day 21) in the back of the neck with an AIV H9N2 killed vaccine. Chicks in group 2 were intranasally infected (day 21) with 0.2mL infectious all antoic fluid. Chicks in group 3 were kept as control group and did not receive vaccine or live virus. Serum was collected on day 42 and used in ELISA test design.

Commercial ELISA

A commercial ELISA assay was performed in U-bottomed 96-well microtiter plates with commercial ELISA kit) IDEXXCo.).

Hemagglutination inhibition (HI) assay

Beta procedure of hemagglutination-inhibition (HI) assays was performed in U-bottomed 96-well microtiter plates with 5% chicken erythrocytes – Spackman & Pedersen (2008).

RESULTS

Sensitivity and specificity of commercial ELISA kit (IDEXX)

In order to evaluate the specificity and sensitivity of the commercial ELISA kit (IDEXX), 268 serum samples were collected from the experimental birds, including 90 samples from birds vaccinated with the inactivated

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vaccine, 90 samples from those experimentally infected with live H9N2 AIV by intranasal inoculation, and 88 samples from control birds.All180 serum samples of vaccinated chickens and infected chickens were positive by HI. Using HI as the reference method, the sensitivity of the commercial ELISA kit was 100% and its specificity was 100%.

Results of experimental bird serum detection using a commercial ELISA kit (IDEXX)

The collected sera were assayed using a commercial ELISA kit (IDEXX).Three weeks after infection or vaccination, sera of the infected and vaccinated groups tested positive by the commercial ELISA kit (IDEXX). Sera of all control chickens were negative in this assay (Table 1-1,1-2).

Table 1-1 : Results of the serum of control and challenge group in IDEXX.

IDEXX	Status		Total
	Challenge	Control	
Positive	90	0	90
Negative	0	88	88
Total	90	88	178

Relative sensitivity=(90/90)×100=100% , Relative specificity=(88/88)×100=100% ,
Accuracy=(90+88)/178×100=100%, Kappa statistic=1,
 Positive predictive value= (90/90) ×100=100%,
Negative predictive value=(88/88) ×100=100% , McNemar Test(p>0.05)

Table 1-2: Results of the serum of control and vaccinated group in IDEXX.

IDEXX	Status		Total
	Vaccinated	Control	
Positive	90	0	90
Negative	0	88	88
Total	90	88	178

Relative sensitivity=(90/90)×100=100% , Relative specificity=(88/88)×100=100% ,
Accuracy=(90+88)/178×100=100%, Kappa statistic=1,
 Positive predictive value= (90/90) ×100=100%,
Negative predictive value=(88/88) ×100=100% , McNemar Test(p>0.05)

DISCUSSION

Because low pathogenic avian influenza (LPAI) virus infections among chickens and turkeys are often asymptomatic, the diagnosis requires serologic monitoring. Most poultry diagnostic laboratories prefer the serologic AGP test because of its simplicity and broad specificity for the detection of type A influenza virus infections among poultry. AGP antibodies recognize conserved nucleoprotein (NP) and matrix (M1) influenza virus proteins present in the sera of poultry exposed to AI virus and therefore can detect multiple subtypes of influenza A virus – Beard (1989). Other serologic tests for AI include ELISA, which also measures antibody responses to conserved internal proteins, and the subtype-specific HI assay – Office International des Epizooties (1996). However, these standard serologic tests for AI virus exposure do not differentiate between vaccinated and infected poultry when traditional vaccines are used. An improved serologic assay for AI virus infection could allow the identification of poultry that have been infected with AI virus, while it would correctly exclude animals that have been vaccinated with any subtype.

The diagnostic test described in this study used a conserved nonstructural protein that is not associated with virions, but that is expressed in large amounts in influenza virus-infected cells – Krug & Etkind (1973), the NS1 protein. Following dilution of immune sera, the NS1 ELISA was an effective tool for the screening of poultry sera to detect AI virus infection. The NS1 protein, although a weak antigen in comparison with other influenza virus proteins, is remarkably conserved among type A influenza viruses – Suarez & Perdue (1999), Buonagurio *et al.* (1986), Ludwig *et al.* (1991) and Treanor *et al.* (1989). Previous studies demonstrated that the NS1 protein can be detected in the sera of horses experimentally infected with the H3 subtype of influenza virus, but not in the sera of animals immunized with inactivated viruses – Birch-Machin *et al.* (1997) and Ozaki *et al.* (2001). The inactivated whole-virus equine vaccine used in the aforementioned studies was partially purified, thus removing residual NS1 protein that could 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) and Ozaki *et al.* (2001).

Birch-Machin *et al.* (1997) and Ozaki *et al.* (2001) described application an ELISA capable of differentiating AIV antibodies induced by vaccination from those induced by infection. Antibodies against the NS1 protein of AIV were considered the most useful marker of virus replication. Most sera of the infected chickens were positive, while all the sera of the vaccinated chickens tested negative in the NS1-ELISA as well as sera of the control chickens without previous exposure to AIV antigens either by vaccination or infection.

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The HI test has been used as a conventional method of diagnosis to detect antibodies against influenza viruses. However, the HI test does not differentiate antibodies induced by infection from those induced by vaccination. In theory, chickens immunized with an inactivated virus vaccine possess antibodies against primarily structural proteins of the virus, while chickens infected with LPAI virus produce antibodies against nonstructural AIV proteins as well. Thus, methods detecting antibodies against nonstructural AIV proteins could be used as a means of differentiation.

In the present study, we demonstrated that, by ELISA using *E. coli* expressed recombinant NS1 as antigen, anti-NS1 antibodies were detected only in the serum samples of experimentally infected chickens and not in the vaccinated chickens. This observation was also made by Tumpey *et al.* (2005). in a recent study – Tumpey *et al.* (2005). The results of the study of Tumpey *et al.* (2005) indicated that the use of a simple and specific ELISA for the sero diagnosis of infection with multiple LPAI virus subtypes can distinguish infected from vaccinated poultry. In that study, animals inoculated with inactivated gradient-purified vaccines had no sero conversion to positivity for antibodies against the NS1 protein, and animals vaccinated with commercial vaccines had low, but detectable, levels of NS1 antibodies – Tumpey *et al.* (2005). The use of a second ELISA with diluted sera identified a diagnostic test that results in seropositive for antibodies against the NS1 protein only in infected birds – Tumpey *et al.* (2005). For the field application phase of this study, serum samples were collected from vaccinated and infected poultry, diluted, and screened for anti-NS1 antibodies – Tumpey *et al.* (2005). Field sera from poultry that received commercial AI killed vaccines were found to possess antibodies against the AI virus, as measured by the standard agar gel precipitin (AGP) test, but they were negative by the NS1 ELISA – Tumpey *et al.* (2005). Conversely, diluted field sera from AI-infected poultry were positive for both AGP and NS1 antibodies. These results demonstrate the potential benefit of a simple, specific ELISA for anti-NS1 antibodies that may have diagnostic value for the poultry industries – Tumpey *et al.* (2005). This observation was also made by Zhao *et al.* in a recent study – Zhao *et al.* (2005). In study of Zhao *et al.* NS1-ELISA test was used in comparison with the HI test to detect antibodies againstNS1 in serum samples. In order to evaluate the specificity and sensitivity of the NS1-ELISA, 99 serum samples were collected from experimental chickens, including 72 samples from chickens vaccinated with an inactivated

vaccine and 27samples from chickens experimentally infected with live H9N2 AI V via subcutaneous or intramuscular injection – Zhao *et al.* (2005). All99 serum samples were positive by HI. Using theNS1-ELISA, all 27 samples from infected chickens were tested positive, and out of the 72 vaccinated samples, 68 were tested negative and there maining four samples were weakly positive. Using HI as the reference method, the sensitivity of the NS1-ELISA was 100% (27/27) and its specificity was 94.4% (68/72) – Zhao *et al.* (2005). In present study, sera of chicks experimentally infected with AIV and chicks vaccinated with a killed influenza vaccine were tested positive by the commercial ELISA kit (IDEXX). Sera of all control chickens were negative. In order to evaluate the specificity and sensitivity of the commercial ELISA kit (IDEXX), 268 serum samples were collected from the experimental chickens, including 90 samples from chickens vaccinated with an inactivated vaccine, 90 samples from chickens experimentally infected with live H9N2 AIV via intranasal inoculation, and 88 samples from chickens of the control group. The 180 serum samples of vaccinated chickens and infected chickens are all positive by HI. Using HI as the reference method, the sensitivity of the commercial ELISA kit was 100% and its specificity was 100 %. In the present study, we demonstrated that, 3 weeks after infection or vaccination, sera of both infected and vaccinated groups were tested positive and the difference in mean optical density between vaccinated and challenged birds, as detected by a commercial ELISA kit (IDEXX), was very small. Therefore, this assay cannot distinguish infected from vaccinated poultry.

Abbreviations: AI = avian influenza; AIV = avian influenza virus; bp = base pairs; BSA = bovine serum albumin; ECE = embryonated chicken eggs; ELISA = enzyme-linked immunosorbent assay; HEPA = high-efficiency particulate air; HI = hemagglutinin-inhibition; HPAI = high pathogenicity AI; IPTG = isopropyl-b-D-galactopyranoside; LPAI = low pathogenicity AI; NS1 = nonstructural protein; OD = optical density; RT-PCR = reverse transcription-polymerase chain reaction; SPF = specific-pathogen-free; TBS = Tris-buffered saline; TMB = 3,3,5,5-tetramethyl benzidine.

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