Assessment of a rapid immunochromatographic assay for the detection of avian influenza viruses

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Abstract

Rapid spreading of the low pathogenic avian influenza virus (AIV) caused by the H9N2 subtype and the highly pathogenic AIV caused by H5N1 have caused serious economic losses in the poultry industries of Asia. Therefore, the early detection of AIVs is crucial for the control of the disease. In the present study, the applicability of a rapid immunochromatographic (RIC) assay, which specifically detected type A antigens of AIVs, was evaluated. This assay detected H9N2 viruses at $10^{3.2}$ ELD_{50}/ml and H5, H7 and H9 antigens at 128 HA titers, but did not react with other respiratory viruses. The assessment of cloacal swab samples prepared from 1 to 10 d post-inoculation (PI) revealed that the first positive samples were detectable on day 2 and 3 PI, and the last positive samples were detectable on day 10 and 9 PI, by the virus isolation (VI) and RIC assays, respectively. Collectively, the relative specificity, sensitivity, positive predictive value, negative predictive value, accuracy and correlation rate of the RIC and VI assays, were 100%, 71.5%, 100%, 78.5%, 0.86, and 0.98, respectively. There was also a good correlation (€ > 0.81) between the results of the haemagglutination (HI), VI and RIC assays of cloacal/tracheal swab samples that were obtained from broiler flocks involved with viral respiratory diseases. Overall, RIC showed a low sensitivity and high specificity for the rapid diagnosis of H9N2 isolates in both experimental and clinical infections.

Introduction

Avian influenza (AI) is a highly contagious disease caused by type A influenza virus, a member of the family Orthomyxoiridae. Avian influenza viruses (AIVs) are classified on the basis of surface hemagglutinin (HA) and neuraminidase (NA) glycoproteins. Sixteen HA (H1• H16) and nine NA subtypes (N1•N9) have been identified. There are also two pathotypes, including the highly pathogenic AI (HPAI) and low pathogenic AI (LPAI) (Alexander, 2007). The infection with H5 and H7 subtypes of HPAI resulted in high mortality and economic losses of the poultry industry worldwide. In the last decade, LPAI virus infections were caused mainly by the H9 subtype, in addition to other subtypes, such as H1, H6, and H12 (Alexander, 2007; Banet al., 2003; Homme and Easterday, 1970). The H9N2 viruses have gained the ability to cause a marked reduction in egg production and severe respiratory distress in chickens; this is accompanied with high morbidity and mortality when the respiratory infection is exacerbated by other bacterial and viral infections, such as Mycoplasma galisepticum, Escherichia coli, Staphilococcus aureus, Haemophilus paragallinarum, IBV and NDV (Aamir et al., 2007; Banet al., 2003; Kishidaet al., 2004; Naeemet al., 1999; Vasfi Marandiet al., 2003). In contrast to the H5 and H7 viruses, H9 viruses still exist only as LPAI viruses.

Since the H9N2 subtype was discovered in turkeys in Wisconsin, USA in 1966 (Homme and Easterday, 1970), this virus has become prevalent in domestic poultry in many Asian countries (China, Korea, Hong Kong and Pakistan) and the Middle East (United Arab Emirates, Iran, Saudi Arabia and Israel) (Aamir et al., 2007; Alexander, 2007; Butt et al., 2005; Guan et al., 2000; Naeem et al., 1999). The H9 subtype of the AIVs also poses the threat of zoonotic infection (Butt et al., 2005; Guan et al., 2000; Lin et al., 2000). This subtype has been considered to be one of the candidates for the next potential pandemic (Shaw et al., 2002; Shortridge, 1992). Hence, the rapid identification of the virus has important clinical, economic and epidemiological implications.

In Iran, the H9N2 subtype was first isolated from chickens in the province of Tehran in 1998, which

subsequently spread to all provinces of the country (Vasfi Marandi and Bozorgmehri Fard, 2002). During the outbreak, most broiler and layer flocks were infected with H9N2, and showed clinical symptoms such as mild respiratory signs, edema around the eyes, diarrhea, a severe drop in egg production, soft-shelled eggs, and a 5 to 20 % mortality rate; sometimes a mortality of up to 65% was reported in cases of complicated respiratory infections (Vasfi Marandi et al., 2003). Since the H9N2 outbreak in 1998, it has become the foremost threatening and epidemic poultry disease in Iran, together with IBV. In response to the circulation of these viruses in the poultry industry, an extensive vaccination program against the H9N2 subtype is currently practiced in order to reduce their impact (Vasfi Marandi et al., 2002). However, H9N2 viruses have not been eradicated either in Iran or other infected Asian and middle Eastern countries, and the infection currently remains in a panzootic state (Alexander, 2007; Guan et al., 2000).

The diagnosis of AI disease based on clinical signs alone may be inaccurate, because the symptoms of AI are similar to those caused by other respiratory infectious agents. Therefore, the laboratory diagnosis of AI disease is crucial in the accurate detection of AIV infections. Various laboratory methods are currently available for the detection, monitoring and surveillance of the H9 subtype of AIVs. The antibody-based detection of AIV includes conventional agar-gel immunodiffusion (AGI), hemagglutination inhibition (HI) and ELISA methods (Julkunen et al., 1985; Peng et al., 2007). Antigen or gene-based detection methods for AIV include virus isolation (VI), immunofluorescence (IF), immunoperoxidase (IP), RT-PCR and real time RT-PCR tests (Alexander, 2007; Chaharaein et al., 2006; Noroozian et al., 2007). However, these assays are both laborious and time-consuming. Therefore, novel methods for the rapid detection of AIVs will be clinically, economically and epidemiologically important.

The rapid immunochromatographic (RIC) assay is a sensitive, specific and rapid test that has been widely used for the diagnosis of drug residues (Wang et al., 2007), pharmaceuticals in fish (Wang and Zhang, 2006) and many contagious diseases in humans (Andreea et al., 2004), large and small animals (Kameyama et al., 2006). It is based on the detection of specific antigens or antibodies (Cui et al., 2008; Peng et al., 2007). This technique has been recently developed by several researchers for the rapid detection and surveillance of type A AIV (Andreea et al., 2004; Woolcock and Cardona, 2005), and the H9 (Fedorko and Nelson, 2006; Peng et al., 2008) and H5 (Fedorko and Nelson, 2006; Tsuda et al., 2007) AIV subtypes. The aim of this study was to compare the use of a rapid RIC assay with the VI technique for the detection of the H9N2 subtype of AIV in cloacal/tracheal swab samples in both experimental and field infections.

Materials and Methods

Viruses, antigens and diagnostic kit

The reference strain of A/chicken/Iran/ZMT-101/98 (H9N2) as a LPAI virus was used for the experimental infection of chickens. Reference antigens of H5N1, H5N3, H7N3, H7N7 and H9N2 subtypes were obtained from Weybridge; UK. Other respiratory diseases vaccine viruses, including Newcastle disease virus (NDV), infectious bronchitis virus (IBV), infectious laryngotracheitis virus (ILTV), and fowl pox virus (FPV), were obtained from the Razi Institute, Iran. The type A specific antigen detecting RIC assay was purchased from Anigen Co. (Korea).

Experimental chicken infection

A total of 20 days-old Ross broiler chickens were housed in animal experimental facilities at the Faculty of Veterinary Medicine, University of Tehran; those with no antibodies to AI on day 28 were used in the experimental infection experiment. The chickens were divided in two 10-bird groups as the test and control groups. The chickens within the test group were inoculated on day 28 via the oculonasal route with 0.2 ml of infectious allantoic fluid that contained 2 x $10^{7.5}$ ELD_{50} of the ZMT-101 virus strain. The control group was inoculated with virus-free allantoic fluid. Their cloacal swab samples were collected from day 1 to 10 post-inoculation (PI) and pretreated by dipping the swabs into 1 ml distilled water in centrifuge tubes with gentle stirring and extrusion. After the samples were allowed to settle for approximately 2 min, their supernatants were collected for the RIC assay and the remaining suspensions from each tube were stored separately at -70°C for the VI assay. The presence of H9-specific antibodies in the collected sera samples of 43-day-old chickens, were detected by the HI test in U bottle 96-well microtiter plates, as described previously (Vasfi Marandi et al., 2003).

Rapid immunochromatographic assay

The rapid immunochromatographic assay (RIC) was performed according to the instructions of the manufacturer. Briefly, the test detects type A specific antigens in a test device that contains a sample well connected to reading wells on a membrane strip (Figure 1). Each swab sample was mixed with a specimen diluent that contained buffered saline, detergent, a mucolytic agent, and preservative. Then, 0.20 ml of each sample was transferred by pipette into the middle of the test well of the device. A positive test was indicated by two purple bands in the reading well, one in the test (T) region and another in the control (C)

region. A negative test was indicated by only one purple band in the C region. The absence of a purple band in the C region represented an invalid test. Test readings were performed and recorded after 10 min of incubation at room temperature.

Diagnosis of H9N2 infection in the field

The RIC assay was applied in the diagnosis of field infections with the H9N2 subtype. A total of 60 tracheal/cloacal swabs and 60 serum samples were examined from six broiler chickens flocks suffering from respiratory infections. The samples were pretreated as described above. Then, a volume of 500 µl of the pretreated sample was transferred to the specimen diluents of the kit for the RIC assay and the remaining volume was stored separately at -70°C for the VI assay.

Avian influenza virus isolation

VI was performed according to the standard procedure (Vasfi Marandi and Bozorgmehri Fard, 2002). Briefly, a volume of 0.2 ml of the each pretreated sample was inoculated into the allantoic cavity of 10-day-old embryonated chicken eggs (ECEs) from healthy mycoplasma-free raised chickens. Each sample was inoculated into four eggs and incubated at 37°C for up to 6 d. Eggs were candled daily and embryos that died within 24 h were discarded. All other eggs with live or dying embryos were transferred to a temperature of 4°C for further investigation. Allantoic fluids were harvested, clarified by low speed centrifugation, and their viral content was determined with the use of the HA test: 25 µl allantoic fluid was mixed with an equal volume of 0.75% (vol/vol) chicken red blood cells in phosphate-buffered saline (PBS) in 96-well microtiter plates.

Sensitivity and specificity of the RIC assay

To determine virus titration, the allantoic fluid of A/chicken/Iran/ZMT-101/98 (H9N2) were serially diluted in PBS from titer $10^{8.2}$ to $10^{0.2}$ ELD_{50}/ml and inoculated into ECEs using the procedure described previously (Vasfi Marandi et al ., 2002) The same serial dilutions were simultaneously used to detect the sensitivity of the RIC assay. In order to determine the specificity of the strip, the harvested samples from 16-day-old ECEs were inoculated with sterile PBS and those that contained other respiratory viruses, including NDV ($10^{8.2}$ ELD_{50}/ml), IBV (10^{8} EID_{50}/ml, ILTV ($10^{7.4}$ EID_{50}/ml), and FPV ($10^{7.4}$ EID_{50}/ml). Furthermore, the standard antigens of the H5, H7 and H9 subtypes of AIV were tested by the RIC assay. The specificity, sensitivity, positive predictive value, negative predictive value, accuracy and correlation rate of the RIC assay was compared with those of the VI assay, and the significance of differences were analyzed statistically.

Results

Sensitivity and specificity of the RIC assay

To determine the sensitivity of the RIC, the allantoic fluid that contained the H9N2 subtype were serially diluted and tested by the RIC assay (Figure 1). When the allantoic fluid was diluted from $10^{8.2}$ to $10^{0.2}$ ELD_{50}/ml, two clear purple bands at the test (T) and control (C) lines were observed at the $10^{3.2}$ ELD_{50}/ml dilution, after which the density of the test line gradually declined.

In order to determine the specificity of the RIC, the allantoic fluid that contained the H9N2 subtype at $10^{8.2}$ ELD_{50}/ml, together with the reference antigens of the H5, H7 and H9 subtypes at 128 HA and other respiratory viruses, were examined by the RIC assay. All samples that contained other respiratory viruses showed one strong band on the control (C) line, whereas only the allantoic fluid of the H9N2 strain and the reference antigens of the H5, H7 and H9 subtypes displayed an additional band on the test (T) line of strips.

Comparison of the RIC, VI and HI tests

To compare the detection of H9N2 by the RIC, VI and HI tests, a total of 100 cloacal swab samples taken between 1 and 10 d post-inoculation (PI) were collected and tested. Furthermore, another collection of 30 samples that were collected on 1, 5 and 10 d PI, were tested as the control group. A total of 35 out of 100 samples in the test group were positive in the RIC assay, whereas 51 out of 100 samples were negative in the VI

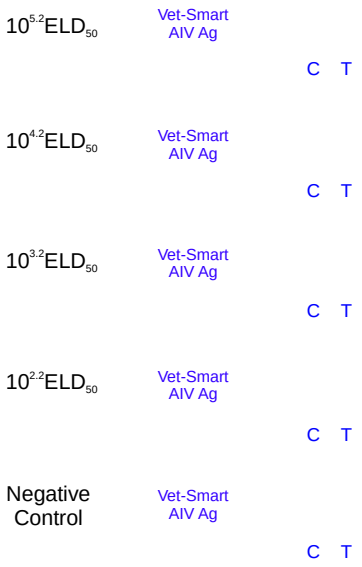

Figure 1: An example of the sensitivity of RIC assay strips using serial dilutions of allantoic fluid containing the ZMT-101 strain of avian influenza. Two clear purple bands were observed at the test (T) and control (C) lines of five different dilutions from $10^{5.2}$ to $10^{2.2}$ ELD_{50}/ml.

Table 1: Comparison of the RIC assay for the detection of the H9N2 subtype of AIV in cloacal swab samples from experimentally-infected chickens with VI and HI tests.

assay. The first positive samples were detected on days 2 and 3, and the last positive samples were detected on days 10 and 9, by the VI and RIC assays, respectively. The peak of virus shedding or isolation in both the VI and RIC assays were 5-7 d PI. None of the tested samples in control group was positive in both tests. The HI antibodies against H9N2 antigen were appeared on day 4, with a log titer of 1.5, and reached to peak on day 8 PI with average titer of 7.5 (Table 1).

Table 3: Comparison of the RIC assay with virus isolation (VI) as a golden test in detecting the H9N2 subtype of AIV in cloacal swab samples following infection of chickens with the A/Chicken/Iran/ZMT-101/98 strain.

Diagnosis of H9N2 infection in the field

To assess the applicability of the RIC assay for the diagnosis of H9N2 infections at the farm level, five birds from each of six broiler flocks that showed respiratory symptoms of viral diseases, with ages between 22-38 d, were selected and their cloacal/tracheal swabs and sera samples tested (Table 2). Half of the broiler flocks examined by the RIC assay were positive for the type A strain. A flock at 36-day-old was found to be negative when their tracheal swabs tested, whereas their cloacal samples were positive.In general, all of the flocks that were positive on the RIC assay were positive in both the VI and HI tests ($\kappa > 0.81$).

Table 2: Application of the type A specific strip for the detection of the H9N2 subtype of AIV in cloacal and tracheal swab samples prepared from broiler chickens flocks with respiratory infections.

Statistical analysis

A total number of 35 out of 100 samples collected from challenged group were definitively positive, and 51 samples were definitively negative in both RIC and VI tests, respectively. Fourteen false negative samples in the RIC assay were found to be positive after the VI test, whereas none of the samples that were positive with RIC were found to be negative in VI. In other words, the number of false positive samples was zero with the RIC method (Table 3). The relative specificity, sensitivity, positive predictive value, negative predictive value, accuracy and correlation rate of the RIC, compared with VI, as analyzed statistically, were 100%, 71.5%, 100%, 78.5%, 0.86, and 0.98, respectively

Discussion

The continued global spread of Asian H5N1 and H9N2 viruses since 1997, with consequent huge losses to the poultry industry worldwide, has resulted in the commitment of significant resources toward improving and enhancing AIV detection tools (Aamir et al., 2007; Fedorko and Nelson, 2006; Guan et al., 2000; Kawaoka et al., 1988). Epidemiological studies in Iran and other countries have shown that LPAIs caused by the H9N2 subtype have become endemic in commercial and backyard poultry (Alexander, 2007; Guan et al., 2000; Vasfi Marandi et al., 2003). Furthermore, infection with H9N2 induces a rapid drop in egg production, decrease in hatchability in breeder flocks and mild respiratory infection, and high mortality if complicated with respiratory infection in broiler chickens. Therefore, early on-site detection of H9N2 infection with a rapid, simple and easily-operated tool will be significant in the surveillance of the spread of influenza viruses.

Analysis of the sensitivity of the RIC assay showed that the strips could detect the Iranian reference H9N2 strain at the level of 10^{2} ELD_{50} /ml within 10 min at room temperature. Characterization of its specificity revealed that it could detect H9N2, H5N1, H5N2, H7N3, H7N7 and H9N2 inactivated antigens. Furthermore, the RIC assay reacted neither with allantoic/chorioallantoic fluids that were harvested from eggs inoculated with other respiratory viruses including NDV, IBV, FPV, and ILTV, nor with non-inoculated eggs. These results demonstrated that, in terms of both sensitivity and specificity under laboratory conditions, the RIC assay may be a valuable

and accurate test for the rapid screening of type A AIVs.

In order to show the applicability of the RIC in the detection of H9N2 viruses, an experimental infection was carried out in broiler chickens. The first positive samples were detected on day 2 and 3 PI, and the last positive samples were detected on day 10 and 9 PI, in VI and RIC assays, respectively. This apparent discrepancy could be due to the low sensitivity of the RIC assay when compared with VI. The HI antibodies against H9N2 antigen first appeared on day 4, with a $\log_2$ titer of 1.5, and reached a peak on day 8 PI with an average titer of 7.5. This suggests that the RIC assay can detect H9N2 infection 5 d earlier than the HI test. The peak of virus shedding in both the RIC and VI tests were 5-7 d PI. This similarity indicates the equitable sensitivity of the RIC assay in the detection of virus shedding during the acute phase of the disease. These results are in accordance with the H5 and H9 subtype specific rapid strips tests (Cui et al., 2008; Fedorko and Nelson, 2006; Peng et al., 2007; Tsuda et al., 2007; Wang and Zhan, 2006).

The Chi-squared statistical analysis of 100 swab samples with RIC and VI tests demonstrated that the relative specificity, sensitivity, positive predictive value, negative predictive value, accuracy and correlation rate of the RIC assay against VI, were 100%, 71.5%, 100%, 78.5%, 0.86, and 0.98, respectively. This is comparable with a high specificity (97.6%) and low sensitivity (88.8%) of a specific H5 RIC assay, as reported by Tsuda et al. in 2007 (Tsuda et al., 2007). This may limit the use of RIC assay kits in diagnostic field investigation, as a negative result with any of these kits would not prove that birds were completely free of AIV.

When the RIC assay was adopted for the diagnosis of field H9N2 infections of broiler flocks, there was generally a good correlation between the results of the HI, VI and RIC methods among samples from six flocks. However, no correlation was found between the results of the RIC assay using cloacal and tracheal swab samples from a 36-day-old broiler flock, and none of the tracheal samples were found to be positive. This means that at least 10 swab samples should be taken, including both cloacal and tracheal swabs, for the accurate diagnosis of H9N2 infection by the RIC assay. These results also confirmed previous results regarding the low sensitivity of the RIC assay in the detection of H9N2 isolates in both experimental and field infections.

Currently, there are several available viral and molecular tests for the detection of type A AIVs. Virus isolation in the ECEs with the use of the HA, HI, AGP, IF and IP techniques has been considered as the gold standard test (Cui et al., 2008; Noroozian et al., 2007; Peng et al., 2007). However, it is labor-intensive, time-consuming, and requires several controls that are freshly prepared for standardization, which makes them unsuitable for the rapid and on-site characterization of AIV infection. Similarly, RT-PCR and real time PCR can detect the type and subtypes of AIVs with a high sensitivity and specificity (Noroozian et al., 2007). However, these techniques require specific primers, a laboratory, skilled technicians and specialized equipment, which preclude them from the use of the rapid on-site detection of viruses in the field. In comparison with these assays, the strip evaluated in this study, can be used for the rapid on-site detection of H9 and other subtypes of AIVs. The results can be read directly by the naked eye within a few minutes of testing. This assay is easily operated and can be performed by poultry clinicians or farmers. A new diagnostic test of latex agglutination has been developed recently by Chen et al. for the rapid detection of the H5N1 AIV subtype. Its results with regards to sensitivity and specificity were comparable with those of the RIC assay (Chen et al., 2007).

Based on the results obtained in this current study, it is suggested that the RIC assay is highly specific, suitably sensitive and can accurately detect type A AIV subtypes, such as H9, H7 and H5 subtypes without crossreactivity to other viruses. This test is applicable for swab samples, including those from the cloaca and trachea, from chickens that are clinically infected with the H9N2 virus.

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