Serological and Molecular Assays for Detection of Avian Influenza Virus in Broiler Chickens Flocks of Fars Province-Iran

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Abstract: H9N2 Avian Influenza Virus (AIV) is endemic in many Eurasian countries. Since 1998, this virus is pathogen of poultry industry in Iran. The aim of this study was to serological and molecular assays of H9N2 AIVs exposure of broiler chickens by the heamagglutination inhibition test (HI), Enzyme Linked Immunosorbent Assay (ELISA) test and RT-PCR technique in Fras province between November 2010 and March 2011. In the present study, tracheal tissues, cloacal swabs and serum samples collected from 30 broiler chicken flocks with respiratory disease symptoms in Fars province-Iran. The result of RT-PCR test showed that out of 24 flocks, for H9N2 AIVs were positive. The overall 300 serum samples were examined by HI and ELISA tests. In these tests, H7N7 and H5N1 AIV antibody (Ab) titers were negative in all flocks. Also, seroprevalence of H9 AIV subtype was positive in 263 and 274 serum samples by HI and ELISA test, respectively. The difference of Ab titers related to avian’s immune system, age and many other factors. The results revealed the important role of H9N2 AIV in respiratory disease outbreak in commercial broiler farms of Fars province. In addition, the infectious flocks with high degree of mortality had high Ab titers against H9N2 AIV and it is show that low protective effects of Ab.

Key words: Avian influenza virus, Elisa, H9N2, Iran

INTRODUCTION

Influenza is a highly contagious, acute illness that has afflicted humans, animals and birds since ancient times. Influenza viruses are part of the Orthomyxoviridae family and are grouped into types A, B and C according to antigenic characteristics of the core proteins (Hadipour, 2010). Type A Influenza viruses are further divide into subtypes based on H and N antigens, some of these subtypes are highly or lowly virulent in poultry (Woo and Park, 2008). Based on the pathogenicity of Avian Influenza Virus (AIV) to domestic poultry, these viruses are sub classified into two pathotype groups of Highly Pathogenic Avian Influenza (HPAI) viruses, causing rapid mortality in poultry which often approaches 100% and non-Highly Pathogenic Avian Influenza (n HPAI) viruses including Mildly Pathogenic (MP), Low Pathogenic Avian Influenza (LPAI) viruses and Non Pathogenic Avian Influenza (NPAI) viruses, causing unapparent diseases with mild respiratory sings, egg production losses and sometimes with slightly elevated mortality and increased cost for vaccination in the commercial poultry farms (Pazani et al., 2008). However when exacerbation of this influenza infection is caused by other bacterial and viral organisms or environmental conditions, severe disease with high mortality may be seen, although the viruses isolated in these cases still produce little or no disease in experimentally infected chickens (Pazani et al., 2008). During 50 years ago, HPAI viruses such as H5N1 and H7N7 had major importance in economic damages, because they could cause severe disease with high mortality. The other hands, LPAI viruses are widely distributed in wild and domestic avian species around the world and also due to a contagious mild viral disease (Hadipour, 2011). Infections with the H9N2 subtype of AIV that named as LPAI in poultry industry, especially in chickens, have been reported frequently in many Asian countries. More recently, H9N2 viruses have been reported in Middle Eastern countries and have been responsible for widespread and serious disease problems in commercial chickens in Iran, Pakistan, Saudi Arabia and United Arab Emirates (Mehrabanpour et al., 2011). This virus was first reported in 1998 in a layer farm in Tehran and also caused widespread outbreaks in commercial broiler chickens in Iran (Mosleh et al., 2009). Fars province is an active pole of the poultry industry in Iran, and where majority of broiler, layer and broiler breeder farms of Iran are located. To control infection and transmission of AIVs in the operation, an intensive vaccination program was implemented in the following years with inactivated vaccine (Zhang et al., 2008). Despite the vaccination effort and the strict bio security
Table 1: Primers used for RT-PCR reaction

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer</th>
<th>Primer sequences</th>
<th>PCR product</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>IVNP-1200F</td>
<td>5’-CAG (A/G) TACTGGGC (A/T/C) (A/G) AC</td>
<td>330 bp</td>
</tr>
<tr>
<td>AIV</td>
<td>NP-152R</td>
<td>5’-GCATTGTCTCCGAAGAAATAAG</td>
<td></td>
</tr>
<tr>
<td>AIV</td>
<td>H5-155F</td>
<td>5’-ACACATGCYCARGACATACT</td>
<td></td>
</tr>
<tr>
<td>AIV</td>
<td>H5-699R</td>
<td>5’-CTTYGRTTYAGTGTGATGT</td>
<td></td>
</tr>
<tr>
<td>AIV</td>
<td>H9-F</td>
<td>5’-CT(C/T) CACACAGA (A/G) CACAAATGG</td>
<td>488 bp</td>
</tr>
<tr>
<td>AIV</td>
<td>H9-9R</td>
<td>5’-GTCACACTTGTTGTTGT (A/G) TC</td>
<td></td>
</tr>
<tr>
<td>AIV</td>
<td>H7-12F</td>
<td>5’-GGGATACAAATGAAYACTC</td>
<td>634 bp</td>
</tr>
<tr>
<td>AIV</td>
<td>H7-645R</td>
<td>5’-CCATABARYYTRGTCTGYTC</td>
<td></td>
</tr>
</tbody>
</table>

R: A/G; Y: C/T; B: G/C (Codes for mixed bases position)

measures imploded, these viruses were still isolated from chickens (Zhang et al., 2008). The aim of this study was to serological and molecular assays of AIVs exposure of broiler chickens by the HI test, ELISA test and RT-PCR technique in Fras province, between November 2010 and March 2011.

MATERIALS AND METHODS

Collection and processing of samples: The study was carried out in 30 broiler poultry farms with respiratory problems around Shiraz city between November 2010 and March 2011. Also, all of the flocks received of AIV vaccines. The following types of samples were collected from the diseased flocks:

- Serum samples were collected from sick broiler chickens flocks with respiratory disease symptoms.
- Blood samples were collected from wing vein and kept at 4ºC for an overnight after which serum was separated by centrifugation at 5000 rpm for 2 min.
- Collected serum samples were kept in a separate vial and stored at -70ºC.
- The other hands, tracheal tissues and cloacal swabs were collected from dead and morbid birds showing signs of respiratory distress or other symptoms for laboratory investigations. In order to check bacterial and fungal contamination, swabs and tracheal tissue samples were placed into tubes containing 1 mL PBS solution (PH 7.2) and antibiotics (10.000 IU/mL penicillin, 1 mg/mL streptomycin sulphate, 1 mg/mL gentamicin sulphate) (Noroozian et al., 2007). Then, taken directly to the laboratory in a cold box and stored at -70°C until used.

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Frozen tissues were thawed, grinded and squashed in a sterile pestle and a 10% suspension with transport medium were made (Nunan et al., 2008). Homogenate was centrifuged at 3000 rpm for 15 min and supernatant was collected. Frozen cloacal swabs were thawed, vortexed and pooled from each flock and centrifuged at 3000 rpm for 15 min (Nunan et al., 2008; Noroozian et al., 2007). Supernatants were collected and filtered through 0.22 µm syringe filter (Gelman, USA). A part of filtered material was used for extraction of the DNA required for PCR identification.

Serological analysis: Hemagglutination inhibition (HI) test: This test was used for all of the serums according to methods recommended by the World Organization for Animal Health (2009) for antibody (Ab) detection against H9, H5 and H7 virus. The amount of antigen used in each well was 4HA unit for each subtype. The test serum samples were heated at 56°C for 30 min to inactivate complements. HI test was performed in 96 well round bottomed microtiter plates. After making serial dilutions of the tested serum, antigen was added, incubated for 30 min and 1% chicken erythrocyte suspension was added. The plates were left at room temperature for 30 min. At least, the maximum dilution of each serum sample causing inhibition of hemagglutination was used as endpoint. The HI titer of each serum sample was expressed as reciprocal of the serum dilution (top to bottom) (Alkhalaf, 2010).

Enzyme Linked Immunosorbent Assay (ELISA): The harvested serums were heat inactivated at 56°C for 30 min. The test serum samples screened with the competitive ELISA for the qualitative detection of antibody (Ab) to the most common and prevalent AIV in chicken serum by used ANIGEN AIV Ab ELISA kit, according to the manufacturer's instruction. The colorimetric readings of ELISA plates that coated with the antigen (Nucleoprotein) were performed by using a spectrophotometer at 450 nm.

Molecular analysis:

RNA extraction: Viral RNA was extracted from all filtered material samples using viral Gene-spin TM viral DNA/RNA extraction kit (Vetek, Korea) according to the manufacturer's instructions.

Polymerase Chain Reaction (PCR): The PCR was carried out following the standard method of reverse transcription-PCR (Lee et al., 2001). We used from specific primers based on conserved sequences of the NP gene and HA gene of avian virus (Table 1), respectively as described previously (lee et al., 2001). RT-PCR was carried out in 8 µL reaction mixture containing (dNTP, enzyme mix, reaction buffer and stabilizing buffer), 1 µL
from each forward and reverse primer, 2 μL of template RNA, 0.25 RNase inhibitor and 8 μL of RNase free water was added to make a reaction volume of 20 μL (Lee et al., 2001). The tube was than vortexes and spin for 3-5 sec and before being placed over the thermalcycler for reaction. The one step PCR condition for the amplification was: RT at 45ºC for 45 min, one cycle at 95ºC for 3 min, 40 cycles of heat denaturation at 95ºC for 30 sec, primer annealing at 55ºC for 1 min, primer extension at 72ºC for 1 min and one cycle of final extension step at 72ºC for 10 min in automated thermal cycler (Ahmed et al., 2009; Lee et al., 2001; Mehrabanpour et al., 2011). The PCR condition for the amplification of NP, H9 and H7 was the same as above, except that the annealing temperature for H5 was 58ºC (Lee et al., 2001). The RT-PCR products were checked by 1.5% agarose gel electrophoresis and analyzed under UV light (Kang et al., 2006).

RESULTS AND DISCUSSION

The result of this study, reveled that the H9N2 Avian Influenza Virus (AIV) viruses have important role in the respiratory disease outbreak in broiler flocks of Fars province. In the present research, HI, ELISA and RT-PCR tests were applied for the detection of AIV infections. At the beginning, HI test used for AIV detection and subtyping of chicken serum samples. H7N7 and H5N1 AIV Ab titres were negative in all flocks, but H9N2 AIV antibody (Ab) titers between 3 to 9 log2 were found in all of the flocks. The cut-off point for seropositivity in HI test was determined as HI Ab titers ≥5 in all vaccinated broiler flocks and titres ≤5 were considered as negative result. Thus, a total of 300 serum samples from 30 disease flocks were examined by this test and the results show that out of 263 serum samples for H9N2 were positive and non of serum samples were positive for H7N7 and H5N1 (Table 2). The other method for detection of Abs against AIV was ELISA test. In this test, PI value ≥51 were positive, thus, 274 serum samples were positive for AIV Ab between 300 serum samples (Table 2).

At last, between 30 commercial broilers flocks with a history of respiratory disease, out of 24 flocks, for AIV were positive by RT-PCR technique (Fig. 1). Further the serotyping of 24 AIV isolates showed that all of them were H9 and we didn’t find any H5 or H7 (Fig. 2).

Avian influenza is one of the highly contagious Office of International Epizootics (OIE) lists "A" diseases. It leads to high mortality in chicken, resulting in extensive losses. Seroepidemiological and molecular studies to determine the mode of transmission of the virus and the risk factor associated with infection are deemed necessary (Alkhalaf, 2010). Even for control strategic point of view, this monitoring is mandatory.

<table>
<thead>
<tr>
<th>Sample</th>
<th>kind of test</th>
<th>Tasted samples</th>
<th>Positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA</td>
<td>300</td>
<td>274</td>
<td></td>
</tr>
<tr>
<td>HI-H9</td>
<td>300</td>
<td>263</td>
<td></td>
</tr>
<tr>
<td>HI-H5</td>
<td>300</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>HI-H7</td>
<td>300</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

HI test is common and current serological test for suspension fields. The advantage of this test is low cost as compared with other diagnostic test. The competitive ELISA is an accurate test amenable to semi-automation and the rapid survey of large number of samples. Most of the examined flocks showed high level of Ab titers to AIV by ELISA technique. The findings of the current study indicate a high infectious AIV activity among commercial broilers of Fars province. In fact, AIV is responsible for the majority of respiratory disease outbreaks in vaccinated broiler flocks (Mehrabanpour et al., 2011). Generally, AIV infections and the process of vaccination cause to stimulate immune system. The intensity of avian immune’s respond is different and depends on many agents such as age, environmental factors and etc. In total, HI and ELISA tests are more subsidy than RT-PCR technique.
Also, in most of the times, these serological methods are applied in order to evaluate extreme viral infections. It is common practice in Iran, as in other countries, to vaccinate broiler flocks against AIV. Despite the use of AIV vaccines, it is common to find AIV infections in vaccinated broiler flocks (Roussan et al., 2008). Vaccinated flocks normally have Ab titers, but higher seroprevalence rate was founded in flocks with high infections and high mortality. Thus, the low protective effects of Ab have reported in this study.

Avian influenza is the major case of acute respiratory and reproductive tract infection of chickens and every year bring about high morbidity and mortality worldwide (Siddique et al., 2008). Clinically it is impossible to detect AIV’s subtypes from each other. Therefore, it is necessary to develop a diagnostic test for the rapid identification of these viruses directly from clinical specimens (Siddique et al., 2008). Further, we and other researchers concluded that molecular techniques such as RT-PCR help in rapid and accurate identification of the etiological agents responsible for an infection (Mehrabanpour et al., 2011). RT-PCR has the ability to even detect a single virus particle, whether active or inactive (Siddique et al., 2008). It also provides better detection of the virus from the clinical samples which might otherwise appear negative due to inappropriate sampling or less of infectivity during shipment (Mehrabanpour et al., 2011).

The high percentage of avian influenza reported in this study addresses the strong need for more aggressive monitoring and vaccination of the susceptible and already vaccinated poultry flocks. Our data showed that H9N2 AIVs were the most important causes of respiratory disease in broiler chicken in Iran in this period. Further studies are necessary to assess circulating strains for prepare suitable vaccine, economic losses caused by infections and co infections of these pathogens. In fact, it was concluded from the study that AIV is prevalent in various fields of Fars province (Alkhalaf, 2010).

CONCLUSION

In this study, AIV virus of nHPAI isolated from poultry chicken in broiler farm in Shiraz. The high rates of AIV infection in broiler flocks during 2010-2011 suggested that AIV are most important causes of respiratory disease in this study.

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REFERENCES


