Serological Investigation of infectious with avian influenza (H9N2) in broilers and human population in Abhar city

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Abstract

Influenza virus H9N2 Low Pathogenicity of the viruses, it was considered as widely spread low pathogen in poultry, however there are a few reports of human infection by this virus. This study was conducted to evaluate if such infection existence in workers of slaughter house and patients in Abhar hospital. This study, was conducted in Abhar poultry houses and Abhar hospital. Two hundred samples from poultry, 100 samples from patients with non-respiratory signs, 75 samples from patients with respiratory signs and 25 samples from poultry slaughterhouse workers was taken. To prevent false positive and negative results in HI test the sera were treated by trypsin-periodate and concentrated RBC respectively, and then serums was tittered by HI test. Our results indicated 80 percent of poultry flocks, 44 percent of poultry slaughterhouse workers was positive in HI test against H9N2 influenza. The high percentage of positive cases and higher titers in slaughterhouse workers indicate that the infectivity of the virus requires close contact with birds or their droppings.

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Introduction

Influenzavirus are divided into four species of A, B, and C, based on M and NP proteins. Influenza virus A affects birds, mammals such as aspigs, horses, whales, and humans. Based on the change in HA and NAsurface glycoprotein, Influenza Avirus is classified into the 16 HA subtypes and 9 NA subtypes. All the subtypes have been isolated from birds (Swayne, 2008).

Avian influenza viruses are classified into two groups, based on pathogenicity, of low and high pathogenicity. The Compatibility of these viruses with birds and rare incidence of the disease in birds has introduced them as a natural reservoir of the virus. These viruses can break down the interspecies barrier and transmitted to mammals such as pigs, horses, seals, and humans caused the disease incidence. Among these viruses, the hyperacute influenza viruses of the H5 and H7 and a low pathogenic H9 virus are considered as new influenza pandemic candidates due to direct transmission to humans and cause disease in humans.

In 1999 and then in 2003, virus infection with A/HK/1073/99 (H9N2) and A/HK/2108/03 occurred in humans and since then the virus has been more considered as one of the candidates of new pandemic diseases.

The studies found that the internal genes of the virus are very similar to H5 N1 virus isolated from humans in 1997. Also, the H9 N2 virus that has taken the six internal genes from H5 N1 virus has been found in a wide range of Asian birds.

In a study conducted by Cameron et al. (2000) on H9 N2 virus isolated from birds of Germany, Iran, Pakistan, and Saudi Arabia during the years 1998 to 1999 it was revealed that the virus had a close relationship with the humans’ infective virus in Hong Kong (Cameron et al., 2000).

The study conducted by Karimi et al. (2005) shows the similarity of the H9 N2 virus with the virus (H9 N2) A/Quail/Hk/G1/97 (Karimi, 2005).

In a study conducted by Moosa Khani et al. on 12 cases of H9 N2 viruses in Iran, considering the similarity of the viruses to Pakistani viruses of A/Hk/1037/99 and A/quail/Hk/G1/97, it could be concluded that these viruses can infect humans too. These all indicate the extent of the outbreak of H9 N2 in Asian poultry and humans (Moosakhani et al., 2010).

The study conducted by Matrosovich et al. (2000) showed that the amino acid leucine at position 226 of the hemagglutinin protein are found specifically in human strains of H2 and H3, they also demonstrated that the conversion of glutamine to leucine at position 226 of the virus A/Hk/1037/99 (H9 N2) occurred in Hong Kong which makes identifying the receptors (2, 6) of α acid silence in humans and human infection with this virus (Matrosovich et al., 2004).

In the study conducted by Moosa Khani et al. (2010) on 12 isolated H9 N2 viruses from Iran, it was found that 10 of the 12 viruses had leucine amino acid at position 226 and the two other viruses had glutamine amino acid (Moosakhani et al., 2010).

The mentioned 10 viruses were often related to 2005, indicating a change in the direction of the virus for human infection (Karimi, 2005). So, following the amino acid changes at position 226 of virus H9 N2 and acquire the ability to identify the recipient human flu, the virus can be considered as a candidate for the development of pandemic (Matrosovich et al., 2000). According to the conducted researches on the amino acids of Iranian H9 N2 virus hemagglutinin protein breakdown, the sequence of amino acids in this region is R-x-x-R (x is a non-basic amino acid, and R is arginine) that has potential to become a sequence.

Furthermore, the studies conducted by Karimi, Torogi, and Moosa Khani et al. confirm these sequences (Karimi, 2005; Moosakhani et al., 2010). With respect to the changes of 12 amino acids in...
H9N2 viruses isolated from poultry in Iran in 2010 by the study of Moosa Khani, in case of the circulation of the virus in the Iran poultry farms, the incidence of point mutations of these viruses is possible (Moosakhani et al., 2010).

Materials and methods
A total of 200 human blood serum samples and 200 blood samples from broilers prepared for slaughtering were collected.

Human blood samples were consisted of 100 serum samples of hospitalized patients without respiratory symptoms (patients in the emergency department) was collected and 75 serum samples of patients admitted to hospital with respiratory complications and 25 serum samples were collected from slaughterhouse workers in Abhar.

The patients with respiratory symptoms and patients without respiratory symptoms were identified and separated, and blood samples were taken from them. Then, about 1 ml of the considered patients’ serum samples were obtained from the hospital laboratory in 1.5 ml tube and transmitted to microbiological lab of veterinary college of Tabriz in order to search flue antibody using HI method. The subtype antigen of commercial H9N2 was used. 20 blood samples from each broiler flock were collected (a total of 10 broiler flocks) and after transferring the samples to the laboratory, the samples were centrifuged and the serum was tested using HI method.

Serum digestion using Trypsin - Heat - Periodate Treatment method
Toremove nonspecific inhibitor of hemagglutination (α, β, γ) inhuman serum, and avoid false positive test result, hemagglutination inhibition (HI) was performed. In short, 5 volumes of trypsin were added to 1 volume of serum (15.0 ml trypsin + 3.0 ml serum), then the trypsin containing serum was inactivated at 56°C water bath for 30 min. Then, it was beencooldeto room temperature. 3 volumes of 0.01 M of meta potassium periodate (0.9 ml) were added to the serum. Then, the mixture was incubated for 15 min at room temperature. After 15 minutes, 3 volumes of 1% glycerol (0.9 ml) were added to the mixture. Then, the mixture was incubated again for 15 min at room temperature and finally, 2.5 volumes of 85% normal saline (0.75 ml) was added to the mixture until the serum was reached to a final dilution of 1:10 (Who, 2005).

Detection of non-specific agglutins in the digested serum
To avoid the false negative result of hemagglutination inhibition, the nonspecific agglutins should be detected in digesting human serum. In the case of a positive result, they must be absorbed. In short, a serial dilution of digested serum was made in a 96-hole U-shaped plate, then 1% chicken erythrocytes were added to the plate and they were incubated at 22-25°C for 30 minutes. Finally, if coagulum is formed after this period, the serum can be used for testing. However, in the case of agglutination, the nonspecific agglutinins should be absorbed in serum by condensed red blood cells (Who, 2005).

Nonspecificagglutininabsorption in the digested serum by condensed red blood cells
Twenty volumes of serum are added to 1 volume of rinsed condensed red blood cells and are mixed thoroughly. The mixture is placed at 4°C for an hour. Then, the mixture is centrifuged at 1200 RPM. The supernatant liquid was removed and tested for the presence of non-specific agglutinins. The practice will be continued until negative serum result is obtained (Who, 2005).

Statistical analysis
To study the data of the study, the T-test, ANOVA, and the chi-square test was used based on the results of the study.

It should be noted that SPSS software (version 19) was used for statistical analysis of the data.
Results

The amounts of antibody in 10 poultry flocks were evaluated and the results of the farms with flu symptoms and non-flu farms (vaccinated) were evaluated using T-test and the results are expressed in Table 1. The results of the study showed that there is a significant difference (p<0.01) between affected broiler farms and the farms without the flu.

Table 1. Mean, standard error, standard deviation, and statistical significance of antibody titers against influenza in understudied poultry flocks.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean±SE of antibody</th>
<th>SD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flocks with flu</td>
<td>5.97±0.179</td>
<td>2.26</td>
<td></td>
</tr>
<tr>
<td>Flocks without flu</td>
<td>0.85±0.192</td>
<td>1.11</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Eight of 10 flocks (80%) had symptoms of the avian influenza inhibition test also showed high titers and 2 flocks (20%) were unaffected and their antibody titer was due to vaccination which are described in Table 2.

Table 2. Percentage of infected and uninfected birds in understudied flocks.

<table>
<thead>
<tr>
<th>Group</th>
<th>Infected flocks percentage</th>
<th>Uninfected flocks percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected flocks</td>
<td>80</td>
<td>8</td>
</tr>
<tr>
<td>Uninfected flocks</td>
<td>20</td>
<td>2</td>
</tr>
</tbody>
</table>

The antibody titer in samples from slaughterhouse workers, referring to Abhar Hospital emergency ward and hospital patients in the pulmonary ward was evaluated. The results of the study showed that there is a statistically significant difference between the 3 study groups (p<0.01). Also, the results of Duncan test showed that the difference between antibody titer of slaughterhouse workers with the other two groups was significant (p<0.05).

Table 3. Mean, standard error, standard deviation, and statistical significance of antibody titers against avian influenza in the understudied human population.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean±SE</th>
<th>SD</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slaughterhouse workers</td>
<td>3.48±0.26</td>
<td>1.3</td>
<td>0.001</td>
</tr>
<tr>
<td>Emergency ward referring</td>
<td>1.31±0.11</td>
<td>1.13</td>
<td></td>
</tr>
<tr>
<td>Pulmonary ward referring</td>
<td>1.31±0.21</td>
<td>1.27</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Percentage infected human population by poultry influenza.

<table>
<thead>
<tr>
<th>Group</th>
<th>Infection percentage</th>
<th>Infected number/total samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slaughterhouse workers</td>
<td>44</td>
<td>11/25</td>
</tr>
<tr>
<td>Emergency ward patients</td>
<td>4</td>
<td>4/100</td>
</tr>
<tr>
<td>Pulmonary ward patients</td>
<td>10.66</td>
<td>8/75</td>
</tr>
</tbody>
</table>
It should be noted that all positive cases of emergency and pulmonary wards have already worked at aviculture or had poultry farm.

Discussion and conclusion

Othersimilar studies in other parts of the world have been conducted to assess the prevalence of the H9N2 virus using serological and virological techniques that conform to obtain results in this study. In a study conducted by Cheng et al. (2002) to determine the distribution of poultry influenza A (H9N2) among chicks and people in Shenzhen of China, they isolated 27 tags of H9N2 strain from poultry, but no virus was isolated from human cases. However, approximately 26% of the sera obtained from patients and 7% of chickens' sera had H9N2 antibody, and HI antibody in human serum has been isolated viruses from chickens (Cheng et al., 2002).

So, there is a close relationship between H9N2 antibodies with viruses isolated from chickens. In another study, Li et al., in 2004 investigated the outbreak of avian influenza among poultry and humans in Guangzhou, China. Their study demonstrated a positive H9N2 antibody in 12.8% of the chickens' sera and 5.1% of poultry farms and slaughterhouse workers. Also, they found that there was a close relationship between viruses isolated from chickens with antibodies against this virus in humans (Li et al., 2004).

In another study by Gou et al., in 2000, on a patient's serum in Guangzhou who was in recovery period and H9N2 virus was separated from his serum. They compare the levels of antibodies against the virus in his mother and it was found that the patient had H9N2 titers at 1:400-1:640. Also, his mother had the antibody against the same virus at 1:2 levels. It was likely that the mother has been infected with the virus...
through contact with birds or through airborne particles (Gou et al., 2000). Furthermore, Gou et al., in the study in 1999 to determine whether the H9N2 virus can infect humans, took serum and throat samples from patients with flu symptoms, and chicken, and they found that the patients were carrying the H9N2 virus. Also, they found that about 19% of them have antibodies against the H9N2 virus in their serum (Guo et al., 1999).

It is revealed, by comparing the results of these studies with the present study, that the results are similar to each other. Interestingly, in these studies, the isolated viruses from humans viruses isolated from chickens that the poultry farm or slaughterhouse workers were in close contact with them were used as antigens for the HI test, and positive HI antibody of their serum has been against these antigens. This indicates that in places such as slaughterhouses and poultry farms in which contact with the birds and their droppings is high, these viruses have a high capability to transmit and infect the humans and to compromise the body’s immune system.

The issue is similar to the human infection with H5N1 virus in which the most cases of human infection were in places where the poultry are infected with the virus and people indirect contact with poultry, became infected and sick.

Serological studies on clinic staff and poultry farms demonstrated the positivity of HI antibody titters against the H9N2 virus (Momayez, 2000).

Fig. 3. Comparison of antibody titters against influenza in the understudied human population.

In another study conducted on 200 ordinary people in shahre-Kord, it was found that about 16% of patients had positive serum HI antibodies against the H9N2 (Azizi and Aali, 2006). In these studies, the lack of understudied people’s serum digestion by periodate or the receivederadativeenzyme (RDE) to remove non-specific inhibitors of hemagglutination, caused positive results in ordinary people who had no contact with poultry. It was likely due to a false positive test result.

Fig. 3. Percentage of occurrence or non-occurrence of influenza indifferent groups of human cases.

In the present study, the slaughterhouse workers and the hospital referring sera were digested by trypsin-
periodate-heat, which the results of before and after digestion were considerable; such that most of the pre-digested sera of the control group were positive in terms of H9N2 antibody. But, all serum specimens were negative followed by digestion suggesting a false positive results and the presence of non-specific inhibitor of hemagglutination.

Surveys conducted by Cameron (2000) and Karimi (2005) show the similarity of the virus H9N2 of Iran with H9N2 (A/quail/Hk/G1/97 (virus infecting humans in Hong Kong in 1999) (Cameron et al., 2000- Karimi, 2005).

The study by Mayahi et al., (2003) showed that the mean antibody titer against influenza H9N2 in people involved in poultry industry was significantly higher compared with p < 0.01 those not involved in the poultry industry. Also, the serum antibody titers of drivers and workers of poultry transport to slaughter houses were significantly greater compared with the veterinarians (p < 0.01) (Mayahi et al., 2003). The results of the study conducted by Goudarzi et al., (2011) on people working in avicultures of the East Azerbaijan province showed that the antibody titer was negative in 79 of 96 cases (82%) and positive in 17 cases (18%) (Goudarzi et al., 2011).

Rahimian et al. (2009) found that the highest antibody titer against H9N2 subtype was in slaughterhouse workers that 19.7% of the tested sera were positive as well as in aviculture workers that 14.2% of the samples were positive. All the 29 serum samples of FluVaccine preparation staff of Razi Institute had negative antibody of subtype H9N2. Only 2% of those working in poultry clinics had positive samples (Rahimian et al., 2009).

The results of the present study showed that 44 percent of workers in the slaughterhouse had positive antibodies against influenza and the mean antibody titer was 3.48 ± 0.26. Four percent of emergency patients and only 10.66% of pulmonary ward patients had positive antibody titers against influenza and all of which had a history of direct contact with poultry or working in aviculture. The results of this study are consistent with previous studies on the incidence of avian influenza in human cases.

In a study conducted by Moosakhaniet al. (2010) on 12 cases of H9N2 viruses isolated from Iran, it was found that 10 of 12 viruses had leucine amino acid at the position of 226 (similar to human influenza viruses H2N2 and H3N2) and the other viruses had the amino acid glutamine (Moosakhaniet al., 2010).

Previous studies were indicating the changing of the virus into infect humans (Karimi, 2005). Therefore, the amino acid changes at position 226 of virus H9N2 and the ability to identify the human influenza receptor, the virus can be considered as a candidate for the development of pandemic (Matrosovich et al., 2000). So, the positive serologic tests in this study and other studies, suggest changes in the receptor binding site and the ability of the virus to infect humans.

Due to the changes in the amino acids at positions 226 and 190 of the hemagglutinin protein of H9N2 virus in Iran and specific sequences of hemagglutinin and the receptor of these viruses and their capability to change into hyperacuteviruses, as well as H9N2 virus circulating in poultry flocks and those who are in contact with poultry and occurrence some changes in infect humans, it is necessary to combat the virus.

In the present study the existence of antibodies specific for influenza A, H9N2 subtype in the serum of infected people suggests the immune system response against the virus and indicating that infection occurs in them.
People working in the poultry industry may be infected by inhalation of airborne viruses or consumption of food contaminated with viruses.

Since the symptoms of influenza A, H9N2 subtype in humans are similar to symptoms of human influenza, such as, headache, fever, chills, muscle aches, cough, sore throat, sneezing. Therefore, it is not easily distinguishable from each other.

**Conclusion**

According to the mentioned issues, slaughterhouses and avicultures workers should be vaccinated against human influenza viruses. H9N2 avian vaccines should be made by new virus strains and it must be tried to make an effective and beneficial vaccine by revising these strains, every 6 months. Also, all poultry flocks of the country must be vaccinated to prevent the circulation of the virus in the country's poultry.

Furthermore, the continuous study of the molecular prevalence of the virus in different regions should be conducted in order to take care of the virus changes in pathogenesis and human disease. The results of the present study also indicate the infection of influenza A subtype H9N2 in people who are related to the poultry industry.

Considering the probability of influenza virus mutations, health organizations of the country need to develop and implement a comprehensive program to control and eradicate the virus in the poultry industry as well as people working in the poultry industry must be constantly monitored for their health.

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