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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

Influenzaviruses are divided into four species of A, B, and C, based on M and NPproteins. Influenza virusAaffects birds, mammalssuch aspigs, horses, whales, andhumans. Based on thechange in HA and NAsurfaceglycoprotein, Influenza Avirusisclassified intothe16HAsubtypesand9NAsubtypes.
Allthesubtypes have beenisolatedfrombirds(Swayne, 2008).

Avianinfluenza viruses are classified into two groups, based on pathogenicity, of low and highpathogenicity.The Compatibilityof these viruses withbirdsandrareincidence of the diseaseinbirds has introduced themas anatural reservoirof thevirus. These virusescanbreak down theinterspeciesbarrierand transmitted tomammalssuch aspigs, horses, seals andhumanscaused the disease incidence. Amongthese viruses, thehyperacuteinfluenza viruses of the H5 and H7 and a low pathogenicH9virusareconsidered as new influenza pandemic candidatesdue to direct transmissionto humansandcause diseasein humans.

In 1999and then in2003, virusinfectionwithA/HK/1073/99(H9N2) andA/Hk/2108/03occurredin humans and since then the virus has been more considered as one of the candidates of new pandemic diseases.

Thestudiesfoundthatthe internalgenes of the virus are very similar toH5N1virusisolated fromhumans in 1997.Also, TheH9N2virus that has takenthesixinternalgenesfromH5N1 virushas beenfoundin awide range of Asianbirds.

In a study conducted byCameron*et al*(2000) on H9N2virusesisolated frombirds ofGermany,Iran, PakistanandSaudi Arabiaduring the years1998 to 1999it was revealed thatthe virushad a close relationship with the humans' infectivevirus in HongKong(Cameron*et al.*, 2000).

The study conducted byKarimi*et al*(2005) shows the similarityoftheH9N2virus withthe virus(H9N2) A/Quail/Hk/G1/97(Karimi, 2005).

Ina study conducted byMoosaKhani*et al* on12 cases of H9N2viruses 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 thatthese virusescaninfecthumans,too. Theseallindicate theextent of theoutbreakofH9N2in Asian poultryandhumans(Moosakhani*et al.*, 2010).

The study conducted byMatroskwitch*et al* (2000) showedthat the amino acidleucineat position of 226hemagglutininprotein are found specifically in humanstrains of H2 andH3, they also demonstrated that the conversionof glutamine toleucineat position of226of the virusA/Hk/1037/99 (H9N2)occurredinHong Kongwhichmakesidentifying the receptors(2, 6) of αacid silence in humans andhuman infectionwith this virus (Matrosovich *et al.*, 2004).

In the study conducted by MoosaKhani*et al*(2010) on 12 isolated H9N2virusesfrom Iran,it was found that10of12viruses hadleucine amino acid at position of 226 and the two other viruses hadglutamineamino acid(Moosakhani *et al.*, 2010).

The mentioned 10 viruses were oftenrelated to 2005, indicating a changein thedirectionof the virus for human infection (Karimi, 2005). So, following theamino acidchangesat position of226 ofvirusH9N2 andacquirethe ability toidentifytherecipienthuman flu, the virus canbeconsideredas acandidate for thedevelopment ofpandemic(Matrosovich*et al.*, 2000). According to the conducted researches on the amino acids of Iranian H9N2virushemagglutininproteinbreakdown,the sequence ofamino acids inthis regionisR-x-x-R (x is a non-basic amino acid, and R is arginine) that has thepotentialto becomeacutesequence.

Furthermore the studies conducted by Karimi, Torogi, and MoosaKhani*et al*confirmthesequences (Karimi, 2005- Moosakhani *et al.*, 2010). Withrespect tothe changes of12amino acids in

H9N2virusesisolated frompoultryinIranin 2010by thestudy ofMoosaKhani, in case of thecirculationofthe virus in the Iran poultry farms, the incidence ofpointmutationsofthese virusesispossible(Moosakhani *et al.*, 2010).

Materials andmethods

A total of200human bloodserum samplesand200blood samplesfrom broilers prepared for slaughteringwere collected.

Humanbloodsamples were consisted of 100serumsamples ofhospitalized patientswithoutrespiratory symptoms(patients in the emergency department) was collected and75serum samplesofpatients admitted tohospital withrespiratory complicationsand25serum sampleswere collected fromslaughterhouse workers inAbhar.

The patientswithrespiratory symptomsandpatientswithoutrespiratory symptomswereidentifiedandseparatedandblood sampleswere takenfromthem.

Then, about 1ml 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 H9N2was used. 20blood samples from each broilerflockwere collected (a total of10broilerflocks) and aftertransferringthe samples to thelaboratory,the samples were centrifugedandtheserumwastested using HI method.

Serumdigestion usingTrypsin - Heat - Periodate Treatment method

Toremovenonspecificinhibitorof hemagglutination (α, β, γ)inhuman serumandto avoidfalsepositivetest result, hemagglutination inhibition(HI)was performed. In short, 5.0volumesof trypsin were added to 1volumeofserum(15.0mltrypsin +3.0mlserum), then the trypsin containing serum was inactivated at56^{o}Cwater bathfor 30 min.Thenithas beencooledto room temperature. 3volumesof0.011 M of meta potassiumperiodate(0.9 ml) were addedto theserum.Themixturewas incubatedfor 15minat room temperature. After15minutes, 3volumesof 1%glycerol(0.9 ml) were addedto themixture. Then, the mixture was incubated again for 15minat room temperatureand finally 2.5 volumeof85% normal saline (0.75 ml) was addedto the mixture until the serum was reached toa finaldilution of 1.10 (Who, 2005).

Detection ofnon-specificagglutininsin the digested serum

Toavoid the false negative result of hemagglutination inhibition, the nonspecificagglutinins should be detected in digestinghumanserum. In the case of a positive result, they must be absorbed. In short, aserial dilution of digested serums was madeina96-hole U-shapedplate, then 1% chicken erythrocytes were added to the plate and they were incubated Aat room temperature(22-25^{o}C)for 30minutes.Finally,ifcoagulum is formed afterthisperiod, the serumcanbe usedfortesting. However,in the case of agglutination, the nonspecific agglutinins should beabsorbedinserumbycondensed redblood cells in serum(Who, 2005).

Nonspecificagglutininabsorption in the digestedserumbycondensed red blood cells

Twentyvolumes of serum are addedto1 volumeof rinsed condensed red blood cellsand are mixed thoroughly. The mixture is placed at 4^{o}C for an hour.Then the mixture is centrifugedat1200 RPM. The supernatant liquid wasremovedandtestedfor the presence ofnon-specificagglutinins.The practicewill becontinued untilnegativeserum result is obtained(Who, 2005).

Statistical analysis

To study thedata of thestudythe T- test, ANOVAandthe chi-square testwas usedbased onthe results of thestudy.

It should be notedthatSPSS software (version 19)was used for statistical analysisof the data.

Results

The amounts of antibody in 10 poultry flocks were evaluatedandthe results of the farms with flu symptomsandnon-flu farms (vaccinated) were evaluated usingT-testandthe resultsareexpressedinTable 1.The results of thestudy showedthat there is a significantdifference(p<0.01)between affected broilerfarmsand the farmswithout the flu.

Table1. Mean, standard error, standard deviation, and statistical significance of antibody titersagainstinfluenzain understudied poultryflocks.

Group	Mean±SE of antibody	SD	P value
Flocks with flu	5.97±0.179	2.26	
Flocks without flu	0.85±0.192	1.11	0.001

Eight of10flocks(80%) had symptomsofthe avian influenzain thehemagglutination inhibitiontestsalsoshowedhightitersand2flocks(20%) were unaffected and their antibody titer was due to vaccination whicharedescribedin Table 2.

Table2. Percentage of infected and uninfected bids in understudiedflocks.

Group	Infected flocks percentage	Uninfected flocks percentage
Infected flocks	80	8
Uninfected flocks	20	2

Theantibody titerin samplesfromslaughterhouse workers, refferings toAbhar Hospitalemergency wardandhospitalpatients inthepulmonary wardwas evaluated.The results of thestudy showed that there isa statisticallysignificantdifferencebetween the3study groups(p <0.01). Also, the results of Duncan testshowed that thedifferencebetweenantibody titer of slaughterhouse workerswiththe other two groupswas significant(p <0.05).

The resultsshowed that11out of 25samples (44%) obtained fromtheslaughterhouse workershad positive antibody titersagainstinfluenzaand4 out of100 samples obtained from admitted reffering to theemergency ward(4%) werepositive,and 8 out of 75 samples (10.66%) of thepulmonarywardpatients had positive influenzatiterswhicharedescribedin Table3.

Table3. Mean, standard error, standard deviation, and statistical significance of antibody titersagainstavianinfluenzainthe understudied human population.

Group	Mean±SE	SD	P Value
Slaughterhouse workers	3.48±0.26^{*b}	13	0.001
Emergency ward referring	1.31±0.11^{a}	1.13	
Pulmonary ward referring	1.31±0.21^{a}	1.27	

Table3. Percentage infected human population by poultry influenza.

Group	Infection percentage	Infected number/total samples
Slaughterhouse workers	44	11/25
Emergency ward patients	4	4/100
pulmonary ward patients	10.66	8/75

It should be noted that all positive casesofemergency andpulmonary wards have already worked ataviculture or had poultry farm.

Discussion andconclusion

Othersimilarstudiesin other parts ofthe world have been conducted toassess the prevalenceoftheH9N2virususingserologicalandvirological technique sthat conform toobtain results in thisstudy. In a study conducted by Cheng*et al* (2002) todetermine the distributionof poultryinfluenzaA (H9N2)amongchicksandpeopleinShenzhenofChina, they isolated 27 tags of H9N2strainfrompoultry,but noviruswasisolatedfromhuman cases.However,approximately26% ofthe seraobtained frompatientsand7% of chickensera had H9N2 antibody, and HI antibodyinhuman serumhas beenfrom isolatedviruses from chickens (Cheng *et al.*, 2002).

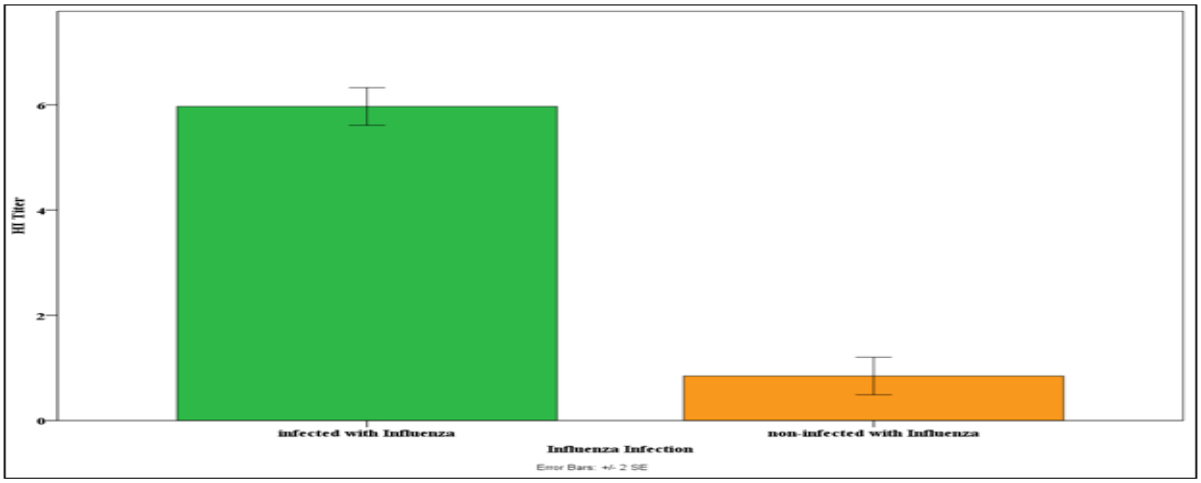

Fig. 1. Comparison ofantibody titer meanagainstinfluenzain understudied flocks.

So, there is aclose relationship between H9N2 antibodies withvirusesisolated fromchickens.In another study, Li *et al.*, in 2004investigated theoutbreak ofavian influenzaamong poultryandhumansinGuangzhou, China. Their study demonstrated a positiveH9N2antibodyin12.8% ofthe chickens'seraand 5.1 % of poultry farms and slaughterhouse workers. Also, they found that there was a close relationship betweenvirusesisolated fromchickenswithantibodiesagainst this virusin humans (Li *et al.*, 2004).

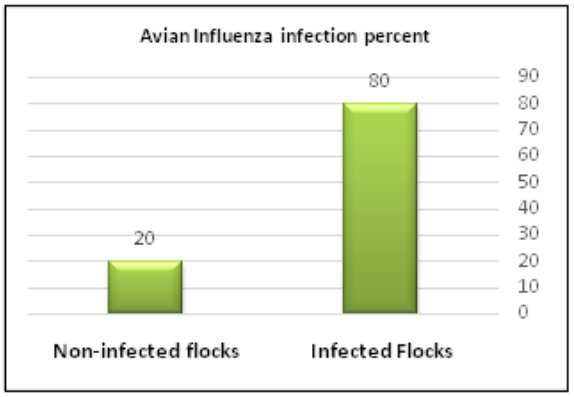

Fig. 2. Percentage of occurrenceor non-occurrence of influenzainunderstudied herds.

Inanother studybyGou*et al.*,in 2000,on a patient's serum inGuangzhou who was in recovery period and H9N2 viruswasseparatedfromhis serum. They compare thelevels of antibodiesagainst thevirus in hismother andit was found thatthepatient had H9N2 titers at1.400-1.640.Also, his mother had the antibodyagainstthe samevirusat 1.2 levels. It was likely thatthemother has been infected with the virus

through contact withbirdsor throughairborne particles(Gou*et al.*, 2000). Furthermore, Guo*et al.*,in thestudyin 1999to determinewhether theH9N2viruscaninfect humans, took serum and throatsamplesfrompatients withflu symptoms,and chicken, and they found thatthe patientswerecarrying theH9N2virus.Also, they found thatabout 19% of them have antibodiesagainst theH9N2virus intheirserum(Guo *et al.*, 1999).

It is revealed, by comparing theresults ofthesestudies withthepresent study,thatthe resultsare similarto each other.Interestingly,in thesestudiestheisolated virusesfromhumanvirusesisolated fromchickens that the poultry farm or slaughterhouse workers were in close contact withthemwere used asantigensfor theHI test, and positiveHIantibody of their serum has been against these antigens.Thisindicatesthatin placessuch asslaughterhousesand poultry farmsin which contact with thebirds andtheirdroppings is high, these viruses have a high capability to transmit andinfectthe humansandtocompromise thebody's immune system.

Theissue is similar to thehuman infectionwithH5N1 virus in which the most casesof humaninfection werein placeswhere thepoultry areinfected with the virusandpeopleindirect contactwithpoultry, became infected and sick.

Serological studiesonclinicstaffandpoultryfarmsdemonstrated thepositivity ofHIantibody titersagainsttheH9N2virus(Momayez, 2000).

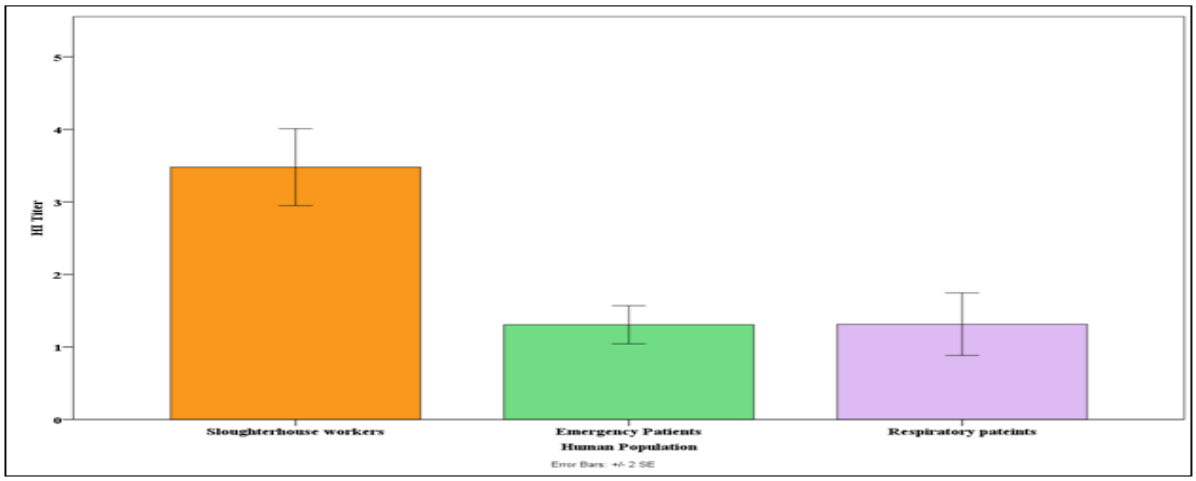

Fig. 3. Comparison ofantibody titersagainstinfluenzainthe understudied human population.

Inanotherstudy conducted on 200 ordinary people inshahre-Kord, it was found thatabout 16% of patients hadpositiveserumHIantibodiesagainsttheH9N2 (Azizi and Aali, 2006). In these studies, the lack of understudied people's serum digestion by periodate or thereceiverdegradativeenzyme (RDE)to removenon-specificinhibitors ofhemagglutination, caused positive resultsinordinary peoplewhohadno contact withpoultry.It was likelydue toa false positivetestresult.

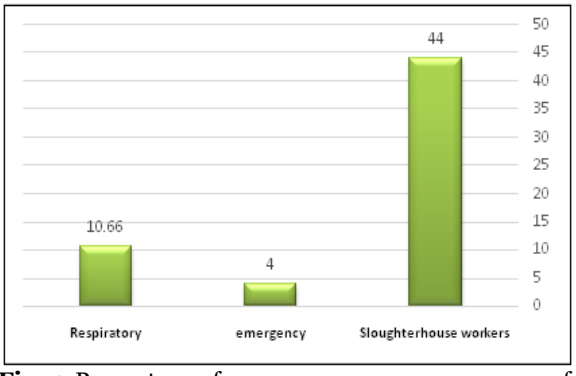

Fig. 3. Percentage of occurrenceor non-occurrence of influenza indifferent groups of humancases.

Inthe present study, the slaughterhouse workers and the hospital refferingssera weredigestedbytrypsin-

periodate-heat, which the resultsofbefore andafterdigestionwere considerable; such that most of the pre-digested sera of the control group were positive in terms of H9N2antibody. But, all serumspecimens were negative followed by digestion suggesting a false positiveresults and the presence ofnon-specificinhibitorsofhemagglutination.

Surveys conducted byCameron(2000) andKarimi(2005) show the similarityofthevirusH9N2 of Iran with H9N2 (A/quail/Hk/G1/97 (virus infectinghumans inHongKong in1999) (Cameron *et al.*, 2000- Karimi, 2005).

The study byMayahi*et al.*,(2003) showed that the meanantibody titeragainstinfluenzaH9N2in people involved inpoultry industrywas significantly higher compared with($p < 0.01$)those not involved in the poultry industry. Also, theserumantibody titersof drivers and workers ofpoultrytransporttoslaughterhouses weresignificantlygreater compared with the veterinarians($p<0.01$) (Mayahi *et al.*, 2003).
Theresults of the study conducted by Goudarzi*et al.*,(2011) on thepeople workingin avicultures of the East Azerbaijan provinceshowed that theantibody titer was negative in 79of96 cases(82%) andpositivein 17 cases(18%) (Goudarzi *et al.*, 2011).

Rahimian*et al*(2009) found that thehighestantibodytitersagainstH9N2 subtypewas inslaughterhouse workersthat19.7% of the testedserawere positive as well as in aviculture workers that 14.2% of the samples were positive.All the 29 serum samples ofFluVaccine preparation staffofRaziInstitutehad negativeantibody of subtypeH9N2.Only2%of those working inpoultryclinicshadpositivesamples (Rahimian *et al.*, 2009).

The results of the present studyshowedthat44percentofworkersin theslaughterhouse had positiveantibodiesagainstinfluenza and the meanantibody titerwas 3.48 ± 0.26. Four percentof emergency patients and only 10.66% of pulmonary ward patients hadpositive antibodytiters againstinfluenza andall of which whom hada history ofdirect contactwithpoultryorworking in aviculture. The resultsofthisstudyare consistentwithprevious studieson theincidenceavian influenza inhuman cases.

In a study conducted by Moosakhani*et al*(2010) on12 cases of H9N2virusesisolated from Iran,it was found that10of12viruses had leucine amino acid at the position of226(similar tohumaninfluenza virusesH2N2andH3N2)and2otherviruseshad theamino acidglutamine(Moosakhani*et al.*, 2010).

Previous studies was indicating the changing of the virusesto infecthumans (Karimi, 2005). Therefore, theamino acidchangesat position226of virusH9N2andthe abilityto identify the human influenza receptor, the viruscan beconsideredas acandidate for thedevelopment ofpandemic(Matrosovich*et al.*, 2000). So, thepositiveserologic testsinthis studyandother studies, suggestchanges in thereceptorbinding siteandthe abilityof the virustoinfecthuman.

Duetothe changes inthe amino acidsat positions226and 190 of thehemagglutininproteinofH9N2virus in Iran andspecificsequences of hemagglutininfracturezonesof these virusesand their capability to change into hyperacuteviruses. As well as H9N2viruscirculatinginpoultry flocksandthose whoare in contactwithpoultryand occurrence some changestoinfecthumans, it is necessary tocombat thevirus.

In thepresent study the existence ofantibodiesspecific for influenza A, H9N2 subtypein the serumofinfected people suggests the immune system responseagainstthe virus and indicating that infection occursinthem.

People working in thepoultry industrymaybe infected by inhalation ofairbornevirusesortheconsumption offoodcontaminated with viruses.

Sincethe symptoms ofinfluenza A,H9N2subtype in humansaresimilar to the symptoms of human influenza,such as, headache, fever, chills, muscle aches, cough, sore throat,sneezing,isthereforenoteasilydistinguishablefro meach other.

Conclusion

According to thementioned issues, slaughterhouses andavicultures workersshould bevaccinatedagainsthumaninfluenzaviruses in order to betteridentify and controlthe virusto prevent the confusion between human and avian influenza viruses.H9N2avianVaccineshould be made by new virusstrainsandit must be tried to make an effective and beneficial vaccine byrevisingthese 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 continuousstudy ofthe molecular prevalenceof the virusindifferent regions should be conducted in order to take care of thevirus changes topathogenesis andhumandisease. Theresults ofthe present study also indicate the infection of influenzaAsubtypeH9N2in people who are related to the poultry industry.

Considering the probability of influenza virus mutations, health organizations of the country need to develop andimplement a comprehensive programtocontroland eradicatethevirusinthe poultry industry as well as people working inthe poultry industrymust be constantlymonitored for theirhealth.

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