High Titers of Hemagglutination Inhibition Antibodies against 2009 H1N1 Influenza Virus in Southern Iran

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ABSTRACT

Background: Pandemic flu had at least two waves in Iran. Knowing how many of the general population were already exposed to this infection has a major impact on national preventive measures. As of December 30, 2009, a total of 3672 confirmed cases of human infection with a novel Influenza A (2009 H1N1) virus had been reported in Iran with 140 deaths. **Objective:** In this study we aim to measure, as a pilot study, the seroprevalence of positive antibody titer (humoral immunity) against 2009 H1N1 virus in Iranian population in Shiraz, Southern Iran. **Methods:** Through cluster random sampling of families residing in Shiraz, 2553 subjects were selected and after a medical interview blood samples were taken and checked for polyclonal antibody against 2009 H1N1 antigen using hemagglutination inhibition assay. An antibody titer of more than 1:40 dilution was considered positive. Data were analyzed considering the demographic characteristics of the population and were compared among different age groups. **Results:** 1504 (58.91%) samples were tested positive for the presence of polyclonal antibody against 2009 H1N1 virus. The prevalence of positive titers were significantly higher in 60 to 64 years old group and significantly lower in 20 to 24 years old group ($p<0.05$). Data did not differ based on other demographic characteristics or the history of flu like illnesses in the past 6 months. **Conclusion:** High seroprevalence of antibody against 2009 H1N1 in the sera of our subjects describes either a high level of pre-existing immunity against H1N1 in Iranian population or a high rate of asymptomatic infection in our area compared to other countries.

Keywords: Flu Vaccine, **H1N1, Hemagglutination Inhibition, Humoral Immunity**

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INTRODUCTION

With the first reports of unusual respiratory illness in Mexico and the United States by the end of April 2009, a novel influenza A (2009 H1N1) virus has spread globally among humans. This virus consists of specific combinations of gene segments from both North American and Eurasian swine lineages. It has antigenic distinction from seasonal human influenza A and has not been identified till now in either swine or human populations (1).

With the worldwide progression of the disease, World Health Organization (WHO) officially declared it as influenza pandemic on June 11, 2009 (2). As of December 30, 2009, a total of 3672 confirmed cases of human infection with a novel influenza A (2009 H1N1) virus has been reported in Iran with 140 deaths (3).

The swine originated influenza virus (S-OIV) hemagglutinin differs genetically and antigenically from hemagglutinins of contemporary human seasonal H1N1 influenza viruses. At the beginning of the pandemic around the world, there was a fear that little protective immune memory exists in the general human population. This concern was confirmed by surveys demonstrating that the neutralizing antibodies against S-OIV can be found almost exclusively in those born before 1957, probably because of their exposure to H1N1 influenza strains that did not circulate after that time (4). Also regardless of significant mortality rate in Mexico and USA at the beginning of the disease progression, pandemic flu has caused mild symptoms, with mortality rate remaining at 0.45% (5).

Hemagglutination Inhibition (HI) assay has long been proposed as a standard method for detection of antibody against influenza virus antigen. Influenza virus agglutinates erythrocytes through the interaction of the virus surface glycoprotein, the hemagglutinine (HA), with receptors on the surface of the erythrocytes. If viral particles are sufficient in quantity, the interaction of HA protein with erythrocytes will form a complete network of linked erythrocytes and will prevent them from precipitation.

The basis of hemagglutination assay is agglutination of erythrocytes and inhibition of the agglutination reaction by HA subtype specific antisera is the basis of the hemagglutination inhibition (HI) assay (6-8).

At the beginning of policy making for vaccination strategy, little was known in Iran about the level of pre-existing antibodies and immunity against the novel 2009 H1N1 virus in the population which are considered to be the most important determining factors for the susceptibility of the community to S-OIV. In this study, we aimed to measure, as a pilot study, the seroprevalence of the antibody titer (humoral immunity) against 2009 H1N1 virus using HI assay in an Iranian population in Shiraz, southern Iran.

MATERIALS AND METHODS

Subjects and Sample Collection. Shiraz is the center of Fars province in southern Iran with a population of 1,300,000 in urban area and 600,000 in the rural part. For determining the seroprevalence of the protective antibodies in the community against 2009 H1N1 in this city, we conducted a cross sectional study in December 2009 prior to the beginning of a vaccination program against this novel virus.

Random clustered household family members, who lived either in urban or rural areas of Shiraz, were selected. After explaining the purpose of the study and filling the consent forms, 2553 subjects were accepted to participate in this survey. Each individual

was then interviewed by a trained interviewer using a standard questionnaire regarding such demographic data as age, gender, residence and history of any sign or symptom of flu like illnesses in the past 6 months, history of vaccination against seasonal influenza or the history of any chronic medical condition.

The pattern of age distribution of the family members was matched completely with the age distribution of the community. Finally blood samples were drawn from each individual, kept on ice during transport to the laboratory, and centrifuged within 4 hours. Sera were separated and stored at -70˚C until tested. The study was conducted after the approval of the institutional ethical board review and the signed informed consent of each participant.

Antigen Preparation. The influenza virus was isolated from throat swabs of patients for whom H1N1 infection was previously confirmed by polymerase chain reaction (PCR). Madin-Darby Canine Kidney (MDCK) cell monolayer was grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MI) supplemented with 10% fetal bovine serum (FBS) (Gibco, Gaithersburg, MD), 100 unit/ml penicillin G and 100µg/ml streptomycin and infected with the pharyngeal specimen and incubated at 34°C for 4 to 5 days (9). Upon development of cytopathic effect, viral presence was shown by hemmagglutination assay and real time PCR using specific 2009 H1N1 flu primer Taqman® Probes (Flu A, swine Flu A) (CDC protocol of real time RTPCR for H1N1 influenza A).

Hemagglutination Inhibition Assay (HI). In this study HI assay was carried out according to Suing method (10). Briefly, the patients' sera were inactivated at 56°C for 30 minutes, nonspecific inhibitors (11) and autohemagglutinins were then removed. Doubling dilutions of sera (resulting in dilutions from 1:10 to 1:1,280) were made in 96 V shaped microplates. Twenty five microliters of a solution containing four HA units were added to each sera dilution and incubated at 37˚C for one hour. Then 50 microliters of guinea pig erythrocytes (0.5% v/v) were added to each well, and the results were recorded after one hour at 4˚C. The HA titer of each serum sample was determined to be the inverse of the last dilution where cells were not agglutinated. The antibody titer of 1:40 or more was considered positive as reported in other studies (12-14)

Statistical analysis. Categorical variables (sex, type of symptoms and age group) are described as count and percentages. The Fisher Exact test was used to compare the independent groups. When Categorical variables were described as proportions, χ^2 test and Fisher Exact test were used for comparison.

A two-sided p value less than 0.05 was considered statistically significant. To account for multiple comparisons performed in the analysis, p values were adjusted using permutational tests. All data were analyzed using SPSS 15, PC version.

RESULTS

Participation Rate and Demographic Data. The study was performed in December 2009 in Shiraz and out of 2730 participants from 1 month to 87 years of age, interviewed for the evidence of flu like illness during the past 6 months, where applicable. 2553 (91%) had their sera tested for the presence of 2009 H1N1 novel virus antibody. Although all 2553 participants were tested for the presence of antibody, there were some missing demographic data due to incomplete filling of the questionnaires as described in Table 1. The percentage of participants whose samples were tested did not

vary by sex or education index. 37.4% (957) of the participants were male and out of 1603 women who enrolled in this study, 41 (2.5%) were pregnant. 1952 (76.3%) cases were living in urban parts of Shiraz and the rest lived in rural areas. The complete demographic data of the participants is shown in Table 1. Only 96 (3.8%) subjects had a history of travelling abroad in the past 6 months out of which 61 travelled to either Iraq or Saudi Arabia (SA).

Table 1. Summary of demographic data of the participants.

Demographic characteristics	Number (%)
Residence	
- Urban	- 1952 (76.3%)
- Rural	- 608 (23.8%)
Sex	
- male	- 957 (37.4%)
pregnancy	41 (2.5% of females)
History of chronic illness	602 (23.5%)
- Asthma	- 56 (2.1%)
- Respiratory illness	- 27 (1%)
- Diabetes	- 83 (3.2%)
- HIV infection	- 1 (0.03%)
- Immune deficiency	- 4 (0.1%)
- Others	- 431 (16.8%)
Age	Mean: 33.13 ± 18.41 Min: 1 Max: 87
Level of education	
- Illiterate	- 289 (11.3%)
- high school	- 1916 (75.1%)
- university graduate	- 348 (13.6%)
History of seasonal flu vaccination	179 (7%)
History of flu like illness in the last 6 months	1405 (54.9%)
History of Tamiflu (Oseltamivir) ingestion	13 (0.5%)
History of travelling abroad (last 6 months)	87 (3.4%)

We also gathered information regarding the prior history of seasonal influenza vaccination. 179 (7%) of the cases had a history of receiving at least one dose of seasonal influenza virus vaccine which in some cases dated back to the year 2004. Of these 179 subjects, 153 were vaccinated for the first time during the year 2009.

Regarding the history of flu like illness signs and symptoms, 1405 (54.9%) participants had experienced flu like symptoms such as fever, sore throat, cough, myalgia, etc. during the past 6 months. However, only 13 (0.5%) cases had received Tamiflu for their symptoms as a treatment for pandemic flu. The most common complaints of those with a history of flu like illness during the course of the disease was sore throat (38.2%), cough (32.4%) and fever (28.3%), respectively. Diarrhea (3.4%) and vomiting (2.5%) were the least prevalent symptoms.

Also 41 pregnant women and 602 subjects with chronic health conditions such as respiratory, renal or liver illnesses, cancer, diabetes, etc. were enrolled in our survey. Among these patients, the overall percent of positive antibody was roughly 59.4% which was close to the percentage in the general population of the study (58.91%).

Hemagglutination Inhibition Assay. As mentioned earlier, after performing the hemagglutination inhibition assay on the sera, a 1:40 titer of antibody against 2009 H1N1 virus was considered as the cut off point and therefore titers equal or greater than 40

were considered positive. Overall 1504 (58.91%) of the samples were tested positive for the antibody against 2009 H1N1 virus. Comparison of the rate of positive titers between those with a history of flu like illness in the past 6 months and those with no history of such symptoms showed no statistically significant difference (p=0.06). The prevalence rates of positive (>1:40) titer in the above groups were 58% (811) and 61.1% (697), respectively. The serum titers had no statistically significant differences based on sex, level of education or other demographic data.

All subjects were categorized into 12 groups based on their age. The results of the prevalence of positive titers of HA inhibition assay in each age group is presented in Table 2 and Figure 1.

Table 2. Age distribution of serum antibody titers against 2009 H1N1 virus after application of hemagglutination inhibition assay.

Hemagglutination Inhibition Assay results

Age Groups	Mean age (years)	Positive titer (>1:40) N (%)	Suspicious (borderline) N (%)	Negative titer (<1:40) N (%)	Total number of participants in age groups N (% of total participants)
0-23 months	1.00±0.46	14 (60.87%)	-	9 (39.1%)	23 (0.9%)
2-4 years	3.40±1.13	77 (57.04%)	10 (7.4%)	48 (35.6%)	135 (5.3%)
5-9 years	7.55±1.16	72 (55.81%)	6 (4.7%)	51 (39.5%)	129 (5.1%)
10-14 years	11.91±1.40	77 (52.74%)	13 (8.9%)	56 (38.4%)	146 (5.7%)
15-19 years	17.27±1.36	118 (64.84%)	14 (7.7%)	50 (27.5%)	182 (7.1%)
20-24 years	22.21±1.49	128 (47.76%)	19 (7.1%)	121 (45.1%)	268 (10.5%)
25-29 years	26.90±1.36	167 (58.39%)	19 (6.6%)	100 (35%)	286 (11.2%)
30-39 years	34.34±2.91	312 (60.58%)	34 (6.6%)	169 (32.8%)	515 (20.2%)
40-49 years	44.00±2.91	208 (59.43%)	20 (5.7%)	122 (34.9%)	350 (13.7%)
50-59 years	53.78±2.86	155 (59.16%)	26 (9.9%)	81 (30.9%)	262 (10.3%)
60-64 years	61.46±1.52	73 (78.49%)	6 (6.5%)	14 (15.1%)	93 (3.6%)
> 65 years	71.32±5.01	103 (62.80%)	10 (6.1%)	51 (31.1%)	164 (6.4%)
Total	33.13±18.41	1504 (58.91%)	177 (6.9%)	872 (34.2%)	2553 (100%)

Prevalence of serum positivity in 20 to 24 years old group was significantly lower than other age groups ($p<0.05$). Serum positivity was significantly higher in 60 to 64 years old group (p=0.001) compared to the other age groups.

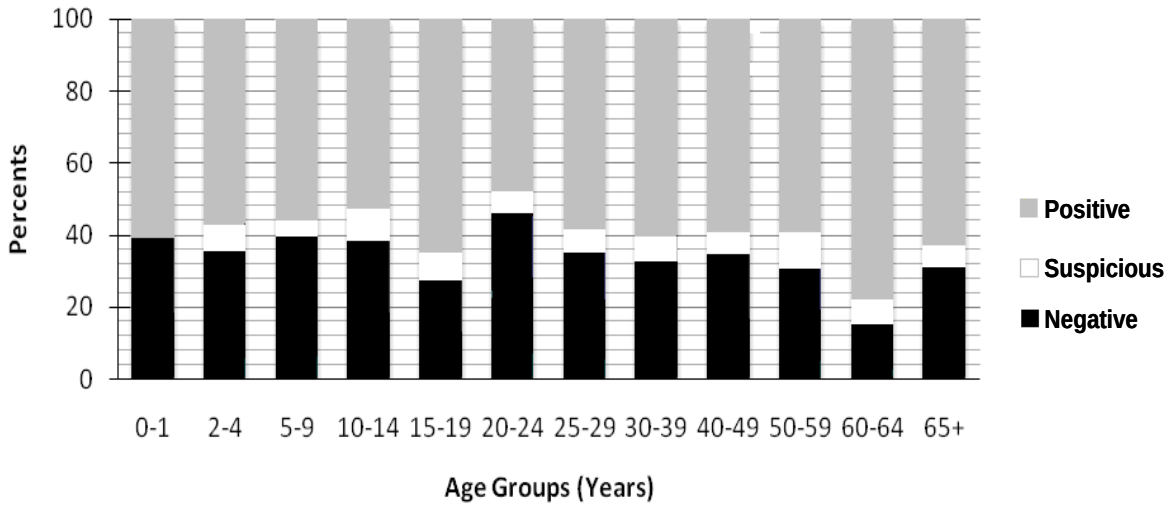

Figure 1. Age distribution of 2553 subjects used in Shiraz sero-epidemiology study for Influenza A (H1N1) virus according to serum positivity (titer ≥ 1:40) for 2009 H1N1 virus.

The detailed information regarding the prevalence of different serum titers are listed in Figure 2.

The total of 2553 participants in the study group were categorized into 12 age groups consisting of: 23 cases in 0-1, 135 cases in 2-4, 129 cases in 5-9, 146 cases in 10-14, 182 cases in 15-19, 268 cases in 20-24, 286 cases in 25-29, 515 cases in 30-39, 350 cases in 40-49, 262 cases in 50-59, 93 cases in 60-64 and 164 cases in >65 years of age. The total numbers in some categories are different from each other due to some missing data in the questionnaires.

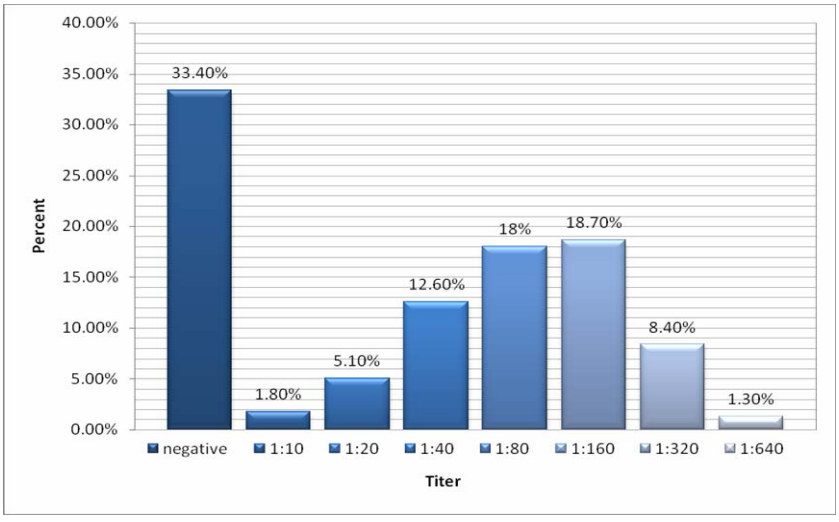

Figure 2. Quantification of serum antibody titers against 2009 H1N1 virus in an Iranian population. A total of 2553 sera were tested using hemagglutination inhibition assay; titers equal or more than 1:40 were considered positive for antibody against 2009 H1N1 virus.

DISCUSSION

Since the outbreak of H1N1 influenza was considered pandemic, there has been debate whether it should be considered as a major health issue leading to high rates of mortality and morbidity; furthermore there has been concerns regarding the need for identification of the target groups for H1N1 virus vaccination (15). According to WHO latest data on H1N1 influenza, at least 13554 deaths related to 2009 H1N1 pandemic have been reported and nearly 883 of them were reported from WHO Regional Office for the Eastern Mediterranean (EMRO) (16).

The report of the first confirmed cases of H1N1 flu in Iran has recently been published (17). Based on this study a total of 2662 cases of H1N1 flu were confirmed until November 2009, with 58 deaths. The results of this investigation revealed a rapid increase in the number of cases diagnosed as H1N1 flu in the consecutive months since the appearance of the disease in mid October. This raises another issue which is if H1N1 epidemic should be considered as a major health burden in Iran and if a concise and nationwide vaccination strategy for the Iranian population has to be tailored.

Based on the latest guidelines (18,19) and in the presence of resource limitations, priority for the vaccination against H1N1 virus should be considered for high risk groups in the following order: health care medical workers, pregnant women, individuals older than 6 months with severe chronic medical conditions such as lung disease or renal impairment etc., healthy individuals aged 15 to 49 years, healthy children, healthy adults aged 50 to 64 years, and healthy adults older than 65 years. The mechanisms of protection against H1N1 influenza by vaccination are thought to be of humoral immunity and antibody formation against H1N1 virus (20).

A number of investigators have supported these claims by the measurement of the humoral immunity status of their population and the pre-existing antibody against H1N1. The results have revealed that while individuals older than 60 years had high levels of antibody against pandemic flu, probably due to previous exposures to this type of virus (such as 1918 pandemic flu), the younger population had a low or a negative titer of antibody, posing their sensitivity to H1N1 infection. This necessitates the vaccination of younger groups and children (21-23).

Hancock et al., (21) conducted a study to evaluate the immune status of different age groups against H1N1 influenza. While 39 (34%) unvaccinated subjects older than 60 years had high titers (more than 1:40) of antibody against 2009 H1N1 virus, only 4 (4%) unvaccinated cases younger than 30 years had positive (1:40) titers of the antibody. Another report published by the Center for Disease Control and Prevention (CDC) demonstrated that 33% of those older than 60 years had pre-existing neutralizing antibody against 2009 H1N1 virus in USA (12).

These findings are in contrast to our results which revealed that 68.5% (176 out of 257) of the cases older than 60 years had a positive (1:40) titer against 2009 H1N1 virus and overall 58.91% (1504 subjects out of 2553) had a 1:40 positive titer of antibody against 2009 H1N1 virus compared to the overall 11% of positive serum samples in the study done in USA (12). This high seroprevalence of positive antibody titers in Iranian subjects is more notable when compared to the results of a survey in a Chinese population showing that out of 4043 subjects with no prior history of vaccination for Influenza virus, only 1.7% (70 serum samples) had positive (1:40) titers against this virus (13). The authors concluded that due to high prevalence of seronegative persons for H1N1 virus

in China, repeated vaccination is required for partial immunity against 2009 H1N1 virus.

Altogether, data analysis of the pre-existing antibody against 2009 H1N1 virus suggests that a high level of immunity against 2009 H1N1 virus exists in Iranian population even in those with chronic illnesses. Not only the prevalence of immunity was high in the elderly but also similar results were observed in the younger groups and even children.

There was no pre-existing cross-reactive antibody against 2009 H1N1 virus in children of 6 months to 9 years in the investigation of Hancock et al., (21), positive antibody titers were found in 60.87%, 57.04%, 55.81% of cases in the age groups of 1 to 23 months, 2 to 5 years and 5 to 9 years, respectively. The high seroprevalence of positive titers of the antibody against H1N1 virus in our samples was also observed in other age groups either in elderly and adults or in younger groups or in children as shown in Figure 1. The prevalence of positive titers was significantly higher in 60 to 64 years old group (78.49%) compared to other groups ($p=0.001$) which agrees with similar findings by other investigators (11-13). Yet the percentage of positivity was interestingly higher in our findings compared to those of the same age group in similar studies. Seropositivity rate in the age group of over 65 years was significantly lower than the 60-64-year old group. Previous endemics have caused positivity in sera of over 60 year old individuals worldwide, however, the reason for higher prevalence of the antibody in 60 to 64 years old group compared to that of the older ones is not fully understood. Weaker immune system and decreased immune memory of the elderly may contribute to lower positivity rates in over 65 year old group.

On the other hand the lowest rate of positive hemagglutination inhibition assay was observed in the younger (20-24 years) age group (47.76%) which was significantly lower than other age groups ($p<0.05$). However, there was no statistically significant difference in the prevalence of positive titers among other age groups. Overall, even the lowest seroprevalence of positive titers in our population was meaningfully higher than those of other groups (12,13).

Additionally, even in those subjects with underlying diseases that categorized them as high risk individuals for H1N1 infection, the positive titer was seen in as high as 59.4%.

This high level of positive antibody titer in the society and in different age groups which was of polyclonal B-cell nature can be interpreted by one of the following theories:

One possibility is that 2009 H1N1 virus has caused considerable number of asymptomatic infections in the population. Considering the fact that the prevalence of positive titers ($\geq$1:40) did not differ in those with a history of flu like illness in the past 6 months and those with no history of any flu like illness symptom (58% compared to 61.1%, respectively with a p value greater than 0.06), it can be hypothesized that asymptomatic infection with 2009 H1N1 virus or repeated previous encounters with a similar antigen might have played a role in high seroprevalence of 2009 H1N1 antibody in the sera of participants and therefore seroconversion after asymptomatic infection has produced a high prevalence of positive hemagglutination inhibition assay.

However, higher rates of seropositivity in infants (0-23 month age group) cannot be simply elucidated by the presumption of prior asymptomatic infection. Although asymptomatic infections can occur in this age group, usually there is a little chance that an infant or a newborn will be infected with influenza virus and develop seroprotection in such a short period. The high prevalence of seroprotection in infants in our study can also be explained by a possible maternal transfer of IgG against influenza virus during fetal life leading to the immunity in newborns and infants and a subsequent seroprotec-

tion. This is further supported by the fact that a high prevalence of seropositivity was observed in other age groups including women in a child bearing age. However, the certainty of this claim should be further tested.

It may also be hypothesized that there might be some form of innate or pre-existing immunity in the Iranian population that has resulted in such a high rate of seroprotection in all age groups and this also might be due to cross reactivity of the measured antibody to other forms of antibodies against influenza A viruses that might have been previously epidemic in Iran and has led to a significant protection in all age groups. However, all of these claims remain hypothetical and they should be investigated further although putting such theories into test might not be easy or even possible.

Furthermore, it should be kept in mind that the type of virus genome that was used was the one that caused the pandemic in Iran and might differ slightly in genomic structure from those in other parts of the world.

It is also noteworthy that even though the polyclonal antibody that was measured and interacted with H1N1 virus is not specific for this serotype and surely has cross reactivity with other antibodies, yet the antibody creates at least some form of protection and cross-reactivity against the pandemic flu virus in individuals. It is well established that the humoral immunity and antibody titer against H1N1 virus correlates directly with a reduction of mortality and morbidity caused by pandemic flu (20). In fact serum antibody titer of 1:40 (used as the cut off point in this survey) is proven to be associated with a minimum of 50% decrease in the risk of influenza infection and the related mortality rates (14). Based on this fact and the results of the present study, it can be hypothesized that pandemic flu might really not be as dangerous as it was previously thought, at least in Iranian population, due to a high level of effective antibody titer (1:40) against 2009 H1N1 virus in the sera of different age groups. The appropriateness of setting the cutoff point at 1:40 antibody titer for the determination of seropositivity and protection in this study was also supported by other similar investigations (12-14,24). The fact that we observe a fewer number of hospitalized cases of pandemic flu than what was expected based on worldwide estimates supports this theory. Based on the latest modeling for pandemic flu, it is predicted that the number of hospitalized cases due to pandemic flu will be at least 0.5% to 1.5% of the outpatient presentations of 2009 H1N1 flu in the community (25,26). The current numbers of hospitalization and case fatalities in Iran are far less than what was predicted by these models (17). On the other hand, due to the limited availability of H1N1 vaccine worldwide, countries have chosen different strategies on vaccination. For example, Chinese government has targeted only 5% of its population for the first phase of vaccination, while Canada has planned a nationwide program to cover 100% of its population (27). In our country there are debates regarding identification of target groups for vaccination. Considering the high prevalence of pre-existing immunity in our population on one hand (approximately overall 60% positive titer against H1N1 virus) and the limited resources for providing the vaccine on the other hand, mass vaccination against H1N1 virus does not seem reasonable or cost-effective. Even the need for vaccination of high risk groups such as those with chronic underlying illnesses or the elderly or even young adults and children should be re-evaluated since they all demonstrated high levels of positive titers against 2009 H1N1 virus in our investigation. Therefore, the existing guidelines for targeting high risk groups for vaccination do not apply to our community. Yet, in order to be able to design an accurate strategy for vaccination or the management of a pandemic

flu, more multicentric studies with larger sample sizes are needed to support this hypothesis.

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