



Original Article

Trend of Changes in the Titer of Antibody against Avian Influenza Virus H9N2 during Raising Period in Vaccinated and Unvaccinated Broiler Farms in Qazvin Province, Iran: A Cohort Study

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ABSTRACT

Avian influenza virus (AIV) H9N2 is endemic in Iran and its large-scale circulation in the poultry industry of the country is devastating. This virus was first reported in the industrial poultry populations of Iran in July 1998. Some of the published studies showed that inactivated avian influenza (AI) vaccines are capable of inducing an immune response and providing protection against morbidity and mortality in different countries (Vasfi et al., 2002; Tavakkoli et al., 2011). Low pathogenicity avian influenza subtype H9N2 virus has been reported to have a zoonotic potential and widespread distribution in Iran. Therefore, water-in-oil emulsion vaccines are employed to control the disease in chickens (Nili and Asasi, 2003). This cohort study was conducted during July 2016-November 2017 in broiler chicken farms of Qazvin province, Iran to investigate the serological change trends in broiler chickens in this region. Level of immunity against the H9N2 virus was evaluated by hemagglutination inhibition assay. Fifteen farms out of thirty enrolled units used AI H9N2 killed vaccines. The minimum of mean antibody titers (MATs) was 4.54-2.42 and the maximum of MATs was 4.54+2.42 on day 3. In addition, the minimum and maximum MATs on day 50 were 0.4-0.64 and 0.4+0.064, respectively. The transfer rate of H9N2 AIV antibodies from the serum of breeders to the serum of chickens was calculated as 60.35% in our study. A significant difference was revealed between the maternal mean antibody titers (MMATs) and the MATs on day 3 ($P<0.001$). In addition, the difference between the MATs on day 3 and the MATs on day 10 was found to be significant ($P<0.01$). Moreover, MATs were significantly different between the vaccinated and unvaccinated herds on day 40 ($P<0.05$), while no significant difference was observed on days 3, 10, 20, and 30 ($P>0.05$). According to the results of this study, antibody titers in the vaccinated farms did not reach the protective level until the end of the rearing period. Most of the unvaccinated herds experienced a spurt in antibody titers due to exposure to the virus. Consequently, biosecurity measures must be implemented more seriously and strictly in broiler farms.

Keywords: Antibody titer, Broiler farms, H9N2 avian influenza virus, Iran

La Tendance des Changements dans le Titre d'Anticorps Contre le Virus de la Grippe Aviaire H9N2 durant la Période d'Élevage chez les Poulets de Chair Vaccinés et non Vaccinés de la Province de Qazvin, Iran: Une Étude de Cohorte

Résumé: Le virus de l'influenza aviaire (VIA) du sous-type H9N2 est endémique en Iran et sa circulation est dévastatrice à grande échelle dans l'industrie aviaire. Ce virus a été signalé pour la première fois dans l'aviculture industrielle iranienne en juillet 1998. Certaines études publiées ont montré que les vaccins inactivés contre IA sont capables d'induire une réponse immunitaire et de fournir une protection contre la morbidité et la mortalité dans différents pays (Vasfi et al., 2002; Tavakkoli et al., 2011). Il a été signalé que VIA H9N2, virus d'une faible pathogénicité, a un potentiel zoonotique et une distribution répandue en Iran. Par conséquent, des vaccins en émulsion eau-dans-huile sont utilisés pour contrôler la maladie chez les poulets (Nili et Asasi, 2003). Cette étude de cohorte a été étudiée de juillet 2016 à novembre 2017 parmi les élevages de poulets de chair de la province de Qazvin, en Iran, afin d'étudier les tendances des changements sérologiques chez les poulets de chair de cette région. Le test d'inhibition de l'hémagglutination a été utilisé pour évaluer le degré d'immunité contre le virus H9N2. Les vaccins tués de l'IA H9N2 ont été utilisés dans quinze fermes sur trente unités inscrites. La valeur minimale des titres moyens d'anticorps (TMAs) était de 4.54-2.42 et le maximum de TMA était de 4.54+2.42 au troisième jour. De plus, les TMAs au jour 50 étaient de 0.4 ±0.64. Dans cette étude, le taux de transmission des anticorps VIA H9N2 du sérum des reproducteurs au sérum des poulets a été estimé à 60.35%. Une différence significative existait entre les titres moyens d'anticorps maternels et les TMA au troisième jour ($P<0.001$). De plus, il y avait une différence statistiquement significative entre les TMAs des troisième et dixième jours ($P<0.01$). En outre, les TMAs étaient significativement différents entre les troupeaux vaccinés et non vaccinés au quarantième jour ($P<0.05$), tandis qu'aucune différence significative n'a été observée aux jours 3, 10, 20 et 30 ($P>0.05$). Selon nos résultats, les titres d'anticorps dans les fermes vaccinées n'ont atteint le niveau de protection qu'à la fin de la période d'élevage. La plupart des troupeaux non vaccinés ont une augmentation des titres d'anticorps en raison de l'exposition au virus. Par conséquent, les mesures de biosécurité doivent être mises en œuvre plus sérieusement et strictement dans les élevages de poulets de chair.

Mots-clés: Titre d'anticorps, Élevages de Poulets de Chair, Virus de La grippe Aviaire H9N2, Iran

INTRODUCTION

Avian influenza (AI) is one of the most considerable respiratory diseases in the poultry industry causing huge economic loss and plays an important role in public health. The AI virus (AIV) belongs to the "Orthomyxoviridae" family. Until now, only type "A" of influenza viruses have been reported to cause natural infections in birds. The AI was first reported in 1878 in Italy and highly contagious and lethal disease of poultry was described and termed as "fowl plague" two years later. In 1901, the causative agent of the disease was found to be a filterable virus, which was designated as influenza "A" virus in 1955. In addition to the highly pathogenic forms, low pathogenicity avian influenza viruses (LPAIVs) have been successively

isolated in several countries with the first virus isolation from poultry industry in Germany in 1949 [A/chicken/Germany/1949 (H10N7)] (Lupiani and Reddy, 2009; Gu et al., 2017). From the economic and pathogenic point of view, H9, H5, and H7 virus pathotypes are the most important ones in the poultry industry throughout the world. Epidemics due to H9N2 AIV have been responsible for abundant economic losses in the poultry industry of some provinces of our country during the last few decades (Vasfi et al., 2000).

The first report of H9N2 subtype AIV was during the 1960s from Wisconsin, the United States of America (Homme and Easterday, 1970; Guan et al., 1999). The virus was distributed throughout the world rapidly during 1994-1999 causing continuous viral circulation in several countries of Asia, the Middle East, and North

Africa. Moreover, this agent caused serious problems in the poultry industry of Iran, Pakistan, and Saudi Arabia since 2000. The H9N2 is still one of the most remarkable AI subtypes devastating poultry industry around the world (Guan et al., 1999; Alexander, 2000; Hadipour et al., 2011; Swayne et al., 2013; Gu et al., 2017). Recent publications have clearly demonstrated that H9N2 AIV is endemic in Iran and the large-scale circulation of this virus in different species of birds in the poultry industry of the country is devastating (Hadipour and Golchin, 2011; Fallah Mehrabadi et al., 2015; Fallah Mehrabadi et al., 2016). Despite the extensive annual H9N2 AIV vaccination campaign in the country, AI is present in broiler farms and accounts for a considerable mortality rate. In addition to the economic impact, H9N2 AIV imposes a great influence on public health due to its potential for inducing infection in humans directly (Guo et al., 1999). Moreover, this virus provides partial or even a whole set of internal genes to create new pandemic subtypes of HPAIVs, such as H5N6, H10N8, H7N9, and H5N1 subtypes (Guan et al., 2000; Kimble et al., 2011; Li et al., 2014; RahimiRad et al., 2016; Gu et al., 2017). Consequently, H9N2 AIV poses a serious threat to public health and further studies on this agent are required. With this background in mind, this study aimed to investigate the serological trends in broiler chickens in Qazvin.

MATERIAL AND METHODS

Sample Collection. This cohort study was conducted during July 2016-November 2017 in broiler chicken farms of Qazvin. The sampling frame and sampling unit were the active broiler farms and farms located in Qazvin, respectively. Thirty broiler farms were randomly selected and included in this study. In each farm, 12 blood samples were collected on days 3, 10, 20, 30, 40, and 50 of the rearing period.

Laboratory Testing. All the examinations were performed in the Razi Vaccine and Serum Research Institute (RVSRI). Immunity levels against the H9N2

virus were evaluated by hemagglutination inhibition (HI) assay. From each chicken, 1 ml of blood was taken followed by separating serum according to the guidelines of the World Organization for Animal Health (OIE, 2016). Four units of the H9N2 antigen (RVSRI, Karaj, Iran) was used to detect the presence of antibodies against this virus. Antibody titration was carried out by two-fold serial dilutions and log₂ calculations.

Data Collection and Analysis. Maternal titers of breeders as the main part of the required data were collected utilizing the questionnaires completed through interviews with farmers, veterinarians, or farm managers. The rest of the information was collected from the geographical information system (GIS) of the Iran veterinary organization (IVO). The raw data and related results were recorded in a spreadsheet. Descriptive statistics, including frequency, mean, and standard deviation were applied. Moreover, the percentage of maternal antibodies transfer was calculated from the mean antibody titers (MATs) of three-day-old chickens divided by the MAT of breeders in the related broiler breeder farms. The MATs of two different ages in one group were compared using the paired t-test. Furthermore, the MATs of different ages between the vaccinated and unvaccinated groups were compared by the repeated measure Analysis of Variance (ANOVA). $P < 0.05$ was considered statistically significant. All the statistical tests were conducted using the SPSS software version 22 and ArcGIS version 10.2 was applied for mapping.

RESULTS

Descriptive Analysis. A total of thirty broiler farms were included in this study from six cities of Qazvin province as shown in Figure 1. Seven, four, four, four, five, and six farms from Abyek, Alborz, Avaj, Buin-Zahra, Qazvin, and Takestan were selected, respectively. Fifteen farms out of 30 sampled units used AI H9N2 killed vaccines.

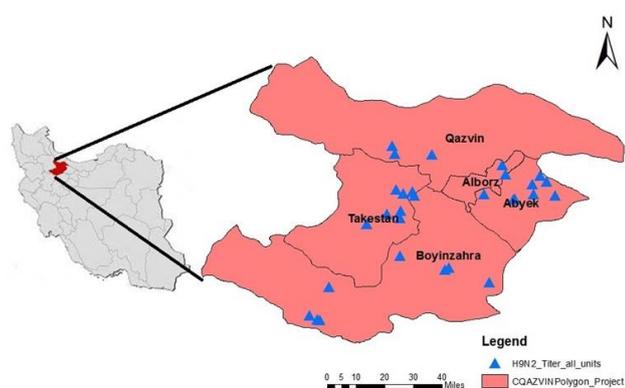


Figure 1. Spatial distributions of broiler farms in Qazvin province, Iran

Eight farms were sampled in spring, nine in summer, four in autumn, and nine in winter. Overall, 1975 birds

2.42 and the maximum of MATs was 4.54+2.42 on day 3. In addition, the minimum and maximum MATs on day 50 were 0.4-0.64 and 0.4+0.064, respectively. We found the transfer rate of H9N2 AIV antibodies from the serum of breeders to the serum of chickens as 60.35% (Table 1). In the present study, the ten unvaccinated units (67%) had at least one bird with a titer equal to or greater than four. In case this titer is regarded as a positive immune response against infection, we found ten, three, two, and one unit with at least one positive bird on days 20, 30, 40, and 50, respectively.

Analytical Results. A significant difference was revealed between the maternal mean antibody titers (MMATs) and the MATs on day 3 ($P<0.001$) (Chart 1). Moreover, our findings indicated a significant difference between the MATs on day 3 and the MATs

Table 1. H9N2 MATs in the broiler farms of Qazvin province in different ages during 2016-2017

Age of sampling	Number of sampled units		Number of sampled birds		MATs in terms of units	
	Unvaccinated	Vaccinated	Unvaccinated	Vaccinated	Unvaccinated	Vaccinated
Maternal	-	-	-	-	6.86±0.81	8.25±0.43
3-day-old chicks	15	14	175	189	6.77±0.57	3.33±1.46
10-day-old chicks	15	13	160	202	5.31±0.48	1.08±0.77
20-day-old chicks	15	14	227	214	0.42±0.22	1.64±1.45
30-day-old chicks	14	15	209	192	0.04±0.02	0.36±0.18
40-day-old chicks	13	11	139	188	0.13±0.11	0.12±0.08
50-day-old chicks	2	4	53	27	0.27±0.23	0.49±0.46
Total	74	71	963	1012	-	-

*Transfer Rate in Vaccinated Chickens = (MATs on day 3/MMATs in breeders)×100% = (3.33/8.25)×100 = 40.36%

*Transfer Rate in Unvaccinated Chickens = (MATs on day 3/MMATs in breeders)×100% = (6.77/6.86)×100 = 98.68%

were collected (Table 1), while 963 samples were taken from the unvaccinated farms. Regarding the seasons, 463, 507, 629, and 376 serum samples were collected during winter, spring, summer, and autumn, respectively. In terms of the cities, 404, 226, 306, 306, 302, and 431 serum samples were collected from Abyek, Alborz, Avaj, Buin-Zahra, Qazvin, and Takestan cities, respectively. The H9N2 AIV MATs at different ages are summarized in Table 1. The minimum of mean antibody titers (MATs) was 4.54-

on day 10 ($P<0.01$). The MATs in 10-day-old chickens were approximately half of the MATs in three-day-old chickens (Table 1). The MATs in the vaccinated farms started to increase slowly on day 20 and followed a monophasic pattern with a maximum of $> 2.5 \log_2$ on day 40 of the rearing period (i.e., about 30 days post-vaccination). However, in the unvaccinated group, HI antibodies almost always decreased continuously and the MAT was $< 0.5 \log_2$ at the age of 50 days (Chart 2).

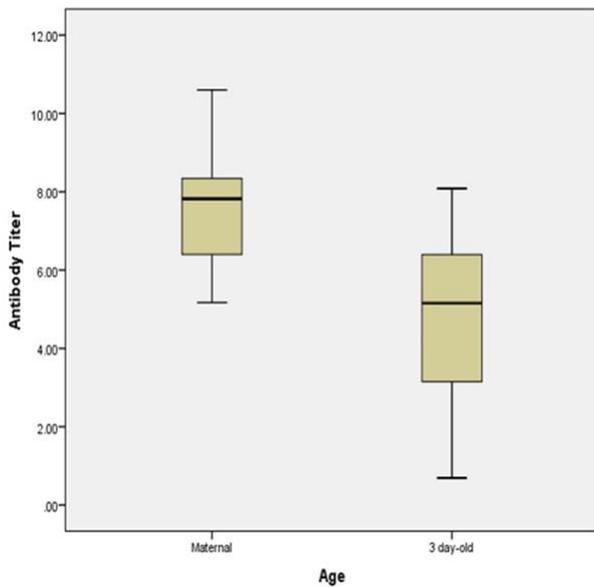


Chart 1. Distribution of the MMATs and MATs in 3-day-old chicks

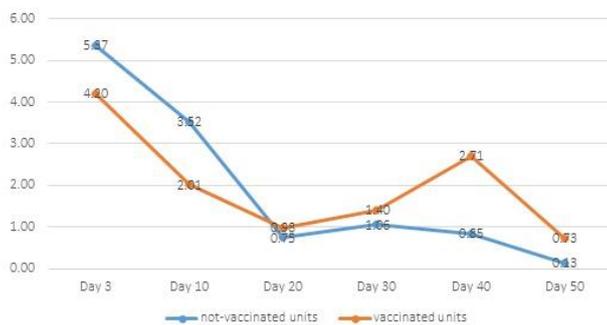


Chart 2. MATs in vaccinated and unvaccinated groups

A comparison of MATs between the vaccinated and unvaccinated herds showed a significant difference in MATs between these groups only on day 40 ($P < 0.05$). However, no significant difference was reported on days 3, 10, 20, and 30 ($P > 0.05$). Furthermore, MATs were not significantly different between day 30 (1.4 ± 2.12) and day 40 (2.7 ± 2.45) in the vaccinated group ($P > 0.05$).

DISCUSSION

Thirty epidemiological units were included in the current study. In fifteen farms, the H9N2 AI killed vaccines were prescribed for chickens at about the age of seven days. The used vaccines were in two different

monovalent and bivalent types regarding the presence or absence of Newcastle Disease Virus. According to our results, the MATs of three-day-old chickens were higher than $4 \log_2$, which is the protective titer against influenza disease (Zhang et al., 2017). We observed that the MATs diminished after day 10. The H9 ATs were detected throughout the year as the environmental condition of the country suits the persistence, transmission, and survival of LPAIV. The highest MATs were in the samples collected during November 2016. These results are in line with the studies in China (Chen et al., 2006; Fatima et al., 2017), where the highest MATs were observed in the colder months. It is believed that low temperature and humidity increase the survival rate of the virus in the environment leading to an elevated chance of viral transmission (Fatima et al., 2017). We calculated the rate of H9N2 AIV antibodies transfer to be 60.35%. In other words, the MATs in three-day-old chickens were approximately twofold lower than the MMATs (Table 1). Similar results were reported by other researchers for the transfer rate of antibodies from serum to yolk after experimental infection with another strain of LPAIV (H6N2) (Trampel et al., 2006). Another study revealed the transfer rate for antibodies against H9N2 AIV from hens to chickens as 19.5% (Gharaibeh et al., 2008). This difference may be due to various exposures to the antigen by currently in-use vaccines in the two counties or could be attributed to the higher antibody rates of breeders in our study. The MATs in ten-day-old chickens were significantly lower than the MATs in three-day-old chickens. As stated before, this rate of antibodies on day 10 cannot induce protective immunity in chickens. It is noteworthy that the reduction in MATs in the vaccinated group was the same as the other group on day 10. This indicates the lack of interference between vaccination and maternal titers at that time unlike the scenario happening about the day-old chickens. The latter point is probably due to the lower levels of antibodies on day 10. Maas et al. (2011) reported that the HI antibody titer in the sera of

14-day-old progeny chickens was approximately eightfold lower than the mean titer in the sera of the vaccinated hens. As reported in different investigations, H9N2 AIV viruses are circulating in commercial and domestic poultry in Iran (Hadipour et al., 2011; Fallah Mehrabadi et al., 2015; Fallah Mehrabadi et al., 2016; Mehrabadi et al., 2018). According to our results, ten unvaccinated farms had at least one specimen with antibody titer equal to 4 log₂ or higher since day 20, which indicates exposure to the field virus. The highest rate of exposure corresponded to day 20. Regarding monitoring the rearing period of enrolled farms, the clinical signs of influenza disease were not observed in all herds in spite of the existence of the virus. This can be attributed to the other factors that provide requirements for the occurrence of the disease and augment the mortality rate in the infected herds. The humoral response against hemagglutinin protein has the best efficacy in AI protection (Swayne, 2009) and the protective threshold of HI titer for influenza was reported as 4.5 log₂ (Zhang et al., 2017). Although ATs increased until day 40 in the vaccinated group, they did not reach the protective level at any time. A comparison of MATs between the vaccinated and unvaccinated herds showed a significant difference on day 40. In other words, although the two groups were not different before day 40, vaccination could induce an immune response and increase MATs in the vaccinated chickens, compared to the unvaccinated group at that time. Persistently, the ATs were below the protective level. The same results have been published by other authors who aimed to compare inactivated vaccines with different adjuvants. They reported a peak of HI titer equal to 4.5 log₂ on day 28 post-vaccination. These researchers stated that the inactivated vaccines of the H9N2 subtype can only induce low protective antibody titers and do not effectively eliminate virus shedding in the field. As a result, they believed that although the existing vaccines reduce the rates of morbidity and mortality in infected poultry, they do not prevent infection completely (Zhang et al., 2017). Poultry AI vaccines have a life of usage without

changing vaccine strains. In terms of this issue, one study showed the efficacy of a single vaccine in protecting chickens against the challenge of AI viruses during 1959-1997 (Spackman et al., 2003; Lee et al., 2004; Swayne, 2009). Another study in China indicated that the persistence of H9N2 AIV in a country might be due to incomplete vaccine protection and the AI vaccine must be continually updated to maintain optimal protection. In this study, the vaccinated chickens, even those with vaccine-induced HI titers of 1:1024, shed the virus after being infected with the virus. This will help the spread of the virus and help the disease to persist in the country (Sun et al., 2012). According to the results of this study, the ATs in the vaccinated farms with indigenous or foreign vaccines did not reach the protective level until the end of the rearing period. In addition, we found that some other factors have notable roles in immunogenicity. Consequently, further studies are required to investigate the role of these factors in producing antibodies. In addition to the protective titers, to produce a strong immune response and higher rates of ATs in chickens, the antigenic similarity between vaccine and field viruses is essential. Therefore, vaccination against H9N2 AIV perhaps is not effective in broiler chickens. On the other hand, most of the unvaccinated herds have seen a spurt in ATs due to virus exposure, which may lead to the occurrence of clinical disease in case the other factors are established. As a result, biosecurity measures must be implemented more seriously and strictly in broiler farms.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

Authors' Contribution

Study concept and design: Bokaie, S.; Shushtari, A.H.; Fallah Mehrabadi, M.H.; Mirzaie, K.; Peighambari, S.M.

Acquisition of data: Mirzaie, K.

Analysis and interpretation of data: Mirzaie, K.; Bokaie, S.

Drafting of the manuscript: Mirzaie, K.; Shushtari, A.H.; Bokaie, S.

Critical revision of the manuscript for important intellectual content: Fallah Mehrabadi, M.H.; Mirzaie, K.; Shushtari, A.H.; Bokaie, S.

Statistical analysis: Mirzaie, K.; Bokaie, S.

Administrative, technical, and material support: Razi institute & University of Tehran, Faculty of veterinary medicine

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