Shiraz University

IJVR

ISSN: 1728-1997 (Print)
ISSN: 2252-0589 (Online)

Vol. 21 No. 2 Ser. No. 71 2020

IRANIAN JOURNAL OF VETERINARY RESEARCH

Original Article

Comparison of autogenous and commercial H9N2 avian influenza vaccines in a challenge with recent dominant virus

Fallah Mehrabadi, M. H.[1]; Ghalyanchilangeroudi, A.[2*]; Ghafouri, S. A.[3]; Hosseini, H.[4]; Zabihi Petroudi, M. T.[2]; Modiri Hamadan, A.[2]; Rezaee, H.[2]; Motamed Chaboki, P.[2]; Vatandour, S.[5] and Shayeganmehr, A.[2]

[1]Department of Poultry Diseases, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Tehran, Iran; [2]Department of Microbiology and Immunology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran; [3]Department of Clinical Sciences, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran; [4]Department of Clinical Sciences, Faculty of Veterinary Medicine, Karaj Branch, Islamic Azad University, Karaj, Iran; [5]Department of Animal Science, College of Agriculture and Natural Resources, Qaemshahr Branch, Islamic Azad University, Qaem Shahr, Iran

[*]***Correspondence:*** *A. Ghalyanchilangeroudi, Department of Microbiology and Immunology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran. E-mail: ghalyana@ut.ac.ir*

(Received 14 Apr 2019; revised version 2 Nov 2019; accepted 24 Dec 2019)

Abstract

Background: Avian influenza (AI) caused by AI virus subtype H9N2 is a prevalent viral disease with enormous economic losses in poultry farms through significant respiratory and gastrointestinal manifestations. The degree of protection obtained from a vaccine strongly depends on the level of antigenic similarity between challenge and vaccine virus. **Aims:** The study aimed at investigating the possible effects of continuous antigenic changes occurring in circulating Iranian viruses since 1998 on the commercial vaccines outcome by using vaccine seeds from earlier outbreaks for inhibiting viral replication in target organs of broilers challenged with the recent isolate. **Methods:** Ninety broilers at one day of age were randomly allocated into 5 groups and vaccinated with autogenous or commercial vaccines (A or B). Two remaining groups consisted of challenged without vaccination and intact birds. Quantitative real time-polymerase chain reaction (qRT-PCR) was performed on the trachea and faecal samples of challenged chickens with recent H9N2 virus to determine viral load. Moreover, humoral antibodies titers were evaluated by hemagglutination inhibition (HI) assay. **Results:** There was no significant difference in H9N2 viral load in the trachea among vaccinated groups on 5 days post challenge (DPC), but on 15 DPC, the autogenous vaccine significantly lowered viral load compared to commercial vaccines ($P \leq 0.05$). No significant differences in faecal swab's viral load was observed between autogenous and commercial vaccine A, and both of them significantly inhibited viral load compared to unvaccinated group ($P \leq 0.05$). In addition, the autogenous vaccine elicited the highest HI titer. **Conclusion:** Inactivated vaccines that use isolates from previous outbreaks are no longer able to induce proper immunity against recent H9N2 viruses. It seems the time to change vaccine strains to more recent isolates that have better antigenic similarity with current circulating H9N2 viruses in the region has come.

Key words: Vaccine, H9N2, Iran, Vaccination, Viral load

Introduction

Avian influenza (AI) is one of the most prevalent viral diseases of poultry worldwide, causing huge economic losses directly through casualties and reducing productivity and also indirectly by imposing costs of prevention of AI and treatment of secondary infections. Avian influenza subtypes are divided into two categories based on pathogenicity, highly pathogenic avian influenza (HPAI), and non-highly pathogenic avian influenza (non-HPAI) (Medina and García-Sastre, 2011). Although in recent years, much attention is paid to HPAI subtypes like H5N1 and H5N8, non-HPAI subtypes still have a significant impact on commercial and backyard poultry. H9N2 is the most prevalent subtype of AI virus in many regions including Eurasia and is isolated from most AI outbreaks in terrestrial poultry (Abdel-Moneim *et al*., 2012). Even though the H9N2 subtype is a non-HPAI, it causes significant disease with respiratory manifestations, severe economic losses and even considerable mortality (Abdel-Moneim *et al*., 2012). The first reported Iranian H9N2 virus was isolated from an outbreak occurred in layer farms located in a densely populated area, Qazvin province in 1998 (Bashashati *et al*., 2013). Since then, H9N2 subtype has been spread all over the country and even is found with H5N1 and H5N8 outbreaks in some regions of Iran in recent years (Ghafouri *et al*., 2017), although H9N2 is still the only endemic AI subtype in all regions of the country (Bashashati *et al*., 2013; Malekan *et al*., 2016). All H9N2 viruses that were isolated from vaccinated or unvaccinated chickens in the past two decades belonged to non-HPAI, till now (Pazani *et al*., 2008; Ghalyanchi Langeroudi *et al*., 2013). However, the mortality rate due to Iranian isolates was reported to up to 65% in naturally infected, unvaccinated broiler flocks (Nili and Asasi,

2002).

Inactivated vaccines are routinely used to control H9N2 infection, but vaccination does not lead to fully desirable results, and outbreaks with enormous economic losses continue to occur, especially in broiler farms of Iran. Different methods have been suggested to reduce adverse effects of AI on poultry health and production and some of them, like using herbal products such as essential oils have shown significant results (Shayeganmehr *et al*., 2018), but still, the only routine way to prevent infection is to use vaccines. Humoral immunity is the main protective immunity against AI infection in birds. Both surface proteins, hemagglutinin (HA) and neuraminidase (NA) can induce humoral antibody secretion, but HA plays a more important role than NA in protection against AI (Suarez and Schultz-Cherry, 2000; Capua and Alexander, 2008; Medina and García-Sastre, 2011). Therefore, the level of protection mainly depends on the degree of antigenic similarity between HA of vaccine strain and the circulating virus (Swayne and Kapczynski, 2008). The continuous antigenic drift that occurred in circulating AI viruses (AIVs) is the main cause of vaccination failure (Bashashati *et al*., 2013). The effect of immunological pressure by the vaccine and co-circulation of H9N2 with other subtypes of HPAI such as H5 and H7 in wild birds, can increase the probability of development of novel variants and reassortment of virus in Iran (Bashashati *et al*., 2013).

The recent Iranian H9N2 viruses (2010) show about 91.7% similarity in the HA gene compared to 1998 isolates (Bashashati *et al*., 2013). Numerous international and local vaccine producers offer inactivated whole virus H9N2 vaccines as monovalent or polyvalent mixed with Newcastle disease (ND) in the Iranian market. Based on the information provided by companies, nearly all AI inactivated vaccines in the market use isolates from the earliest Iranian H9N2 outbreaks as vaccine seed. This may lead to a significant antigenic difference between recent Iranian circulating H9N2 viruses and used vaccines. Consequently, although a high antibody response may arise due to vaccination, proper protection against the infection may not occur. Concerning these facts, we decided to evaluate the effects of antigenic changes in Iranian H9N2 viruses on the ability of commercial vaccines for inhibiting viral replication in target organs as a proper criterion of protection against H9N2 challenge and compared the results with an autogenous vaccine made from recent isolates. If the autogenous vaccine shows better performance in reducing H9N2 viral replication and antibody response, commercial vaccines producers need to replace old vaccine seed with recent circulating isolates in order to properly protect poultry against H9N2 infection.

Materials and Methods

Experimental design

A total of 100 one-day-old Ross 308 hybrid broiler chickens were obtained from a local hatchery. Necessary tests were done on the chickens to be sure that they are free of *Salmonella* spp. and *Mycoplasma* spp. Chickens were randomly divided into five equal groups, 20 birds in each group. Three groups were vaccinated with different vaccines; one group was vaccinated with commercial vaccine A, another was vaccinated with commercial vaccine B, and the last vaccinated group, was vaccinated with an autogenous vaccine (Autogenous H9N2 vaccine). Two remaining groups received no vaccination treatment, one of them was challenged concurrent with vaccinated groups (no vaccine + challenge), and another was not challenged (no vaccine+ no challenge). All groups were reared on the litter with the same conditions in separate isolated rooms. All groups were fed with a commercial pellet feed that was produced in three formulations as a starter, grower, and finisher based on broilers' requirements. The daily amount of feed provided to the broilers was restricted based on official Ross 308 broiler performance objectives (2014) provided by the Aviagen Company.

Autogenous vaccine

Based on molecular epidemiology studies on different H9N2 isolates in Iran during 2016-2017, an H9N2 strain (A/Chicken/Iran/UT-Barin/2017(H9N2)) was selected as a dominant strain for preparing the autogenous vaccine and for the challenge strain. The vaccine seed propagated in 10 day old specific pathogen-free (SPF) eggs to obtain the required antigen. Propagated H9N2 antigen was inactivated by formaldehyde and each dosage of the vaccine (0.2 ml) contained 4 HAU of H9N2 antigen. Montanide ISA 70 was added to the solution as an immunologic adjuvant.

Commercial vaccines

Commercial vaccines were selected from two different manufacturers. Both of them were well-known bivalent inactivated oil emulsified H9N2 plus ND vaccines in the Iranian market and are used by many broiler and layer farms in most regions of the country. Vaccine A and B use AI strain that belongs to earlier Iranian outbreaks. The recommended dosage for broiler chickens is 0.2 ml, and the recommended age for broiler vaccination of both vaccines is during the first week of age.

Vaccination program

Commercial vaccines were stored and administered based on the manufacturer's instructions. All birds in triple vaccinated groups received 0.2 vaccines subcutaneously (SC) on seven days of age (DOA).

Experimental infection

At the 35 DOA, all groups except one (no vaccine + no challenge) were challenged with (100 µL) 10^6 EID_{50} of the H9N2 virus (the same virus that was used for the autogenous vaccine) oculonasal. The groups were carefully monitored for clinical signs daily. Clinical manifestations such as depression, reluctance to move, sneezing, and oculonasal discharge was observed 48 h

post-challenge in over 50% of the chicks in all challenged groups.

Antibody response

Four day-old chickens from each group bleed randomly to determine the maternal antibody titer. The maternal antibody titer was used to determine the proper age for experimental infection. The blood samples (20) from each group were also collected on 28 DOA and the serum antibody titer of all birds determined. Blood samples were collected from the wing vein, and haemagglutination inhibition (HI) test was performed (Capua and Alexander, 2009).

Animal ethics

All chickens used in the studywere treated and euthanized based on instructions provided by the Animal Ethics Committee of Veterinary Faculty, University of Tehran.

Sampling

To study the ability of each vaccine to reduce viral replication in primary target organs of H9N2 viruses, samples from the trachea and faecal swab were collected on 5 and 15 DPC. A total of 10 tracheal samples and 10 faecal samples were collected from each group on each mentioned sampling date. Collected samples were frozen at -20°C before preparing for quantitative real time-polymerase chain reaction (qRT-PCR).

qRT-PCR

To evaluate H9N2 viral replication in target organs, qRT-PCR was performed on collected samples. RNA was extracted from tissues by using Sina Pure Extraction Kit (Sinaclon, Iran). After extracting RNA, cDNA was made according to AccuPower CycleScript RT Premix (Bioneer, South Korea) protocols. SYBR green-based quantitative PCR was performed using the real Q plus master mix (real Q plus green without ROX, AMPLIQON, Denmark). Primers were designed to target the Matrix gene and were selected based on previous studies (Shayeganmehr *et al*., 2018).

Statistical analysis

The one-way ANOVA with significance level of 0.05 was used for comparing HI titers and viral load in tracheal and faecal samples using Graphpad 6 software.

Results

Antibody response

Hemagglutination inhibition test shows that the unvaccinated group has the lowest humoral antibody compared to the vaccinated groups, but the difference is just significant between the autogenous vaccinated group and unvaccinated groups ($P \leq 0.05$). The group that was vaccinated with the autogenous vaccine showed the highest HI titer with 3.52 ± 2.2 ($P \leq 0.05$) compared to commercial vaccine A (2.15 ± 0.36) and commercial vaccine B group (1.94 ± 0.74) (Table 1; Fig. 1). There was no significant difference in antibody response between the two commercial vaccines or within them and the unvaccinated groups.

qRT-PCR results

Trachea

The viral load on the first sampling date (5 DPC) showed that there is no significant difference within triple vaccinated groups. The same results were obtained when comparing the unvaccinated group with commercial vaccine A and B groups, while the difference in cycle threshold (C_t) of the autogenous vaccine and the no vaccine group was significant ($P \leq 0.05$) (Table 1). Similar results were obtained on the second sampling date (15 DPC), and no significant difference was observed between commercial vaccine groups (Table 1). The no vaccine-challenged group showed significantly lower C_t value compared to the autogenous vaccine group ($P \leq 0.05$) while the obtained C_t of the no vaccine group was not significantly different from groups that received commercial vaccines (Commercial A and B vaccines)(Fig. 2). Unlike the first sampling date that there was no significant difference in viral load within vaccinated groups (autogenous and commercial vaccinated groups), the autogenous group showed significantly higher C_t compared to commercial vaccine groups on 15 DPC ($P \leq 0.05$). In both sampling dates, the no challenged-no vaccinated group showed higher C_t value compared to all other groups ($P \leq 0.05$) (Fig. 3).

Faecal swab

At the first sampling day (5 DPC), the commercial vaccine B showed significantly ($P \leq 0.05$) (Table 1) lower C_t value compared to the autogenous and commercial vaccine A groups. The difference in viral load of this

Table 1: Mean±SD of HI titer (on 28 DOA) and C_t value of different groups on 5 (1st sampling) and 15 (2nd sampling) DPC

Groups/Subjects	HI titer (log_2)	Trachea viral load (C_t)		Faecal viral load (C_t)	
	28 days of age	1st sampling	2nd sampling	1st sampling	2nd sampling
Autogenous H9N2 vaccine	3.52 ± 2.2^{a}	26.27 ± 0.42^{b}	33.28 ± 1.04^{b}	28.11 ± 0.35^{b}	34.72 ± 1.22^{b}
Commercial H9N2 vaccine A	2.15 ± 0.36^{b}	26.33 ± 1.3^{bc}	30.28 ± 2.03^{c}	27.56 ± 1.55^{bc}	33.96 ± 2.04^{bc}
Commercial H9N2 vaccine B	$1.94 \pm .74^{bc}$	25.94 ± 0.83^{bcd}	29.32 ± 1.68^{cd}	25.94 ± 0.33^{d}	32.67 ± 1.13^{cd}
No vaccine + challenge	1.53 ± 0.53^{bcd}	24.86 ± 1.4^{cde}	30.99 ± 0.92^{cde}	26.18 ± 0.26^{de}	28.59 ± 1.33^{de}
No vaccine	1.53 ± 0.53^{bcd}	45.6 ± 1.07^{a}	45.8 ± 1.03^{a}	45.2 ± 0.29^{a}	45.6 ± 1.36^{a}

[a, b, c, d, e] Superscript with different letters in a column means significant difference ($P \leq 0.05$). HI: Haemagglutination inhibition, DOA: Days of age, C_t: Cycle threshold, and DPC: Days post challenge

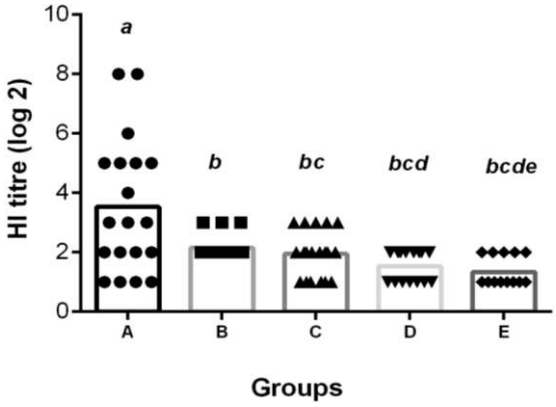

Fig. 1: HI mean titer of different groups on 28 days of age. HI: Hemagglutination inhibition, A: Autogenous H9N2 vaccine, B: Commercial H9N2 vaccine A, C: Commercial H9N2 vaccine B, and D: No vaccine. [a, b, c, d, e] superscript with different letters means significant difference (P≤0.05)

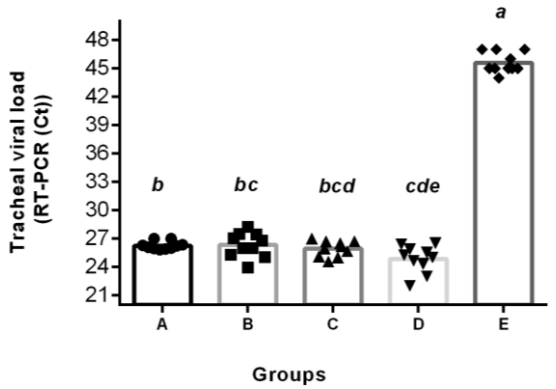

Fig. 2: Means of C_t values in trachea of different groups on day 5 post challenge. C_t: Cycle threshold, RT-PCR: Real time-polymerase chain reaction, A: Autogenous H9N2 vaccine, B: Commercial H9N2 vaccine A, C: Commercial H9N2 vaccine B, D: No vaccine + challenge, and E: No vaccine [a, b, c, d, e] superscript with different letters means significant difference (P≤0.05)

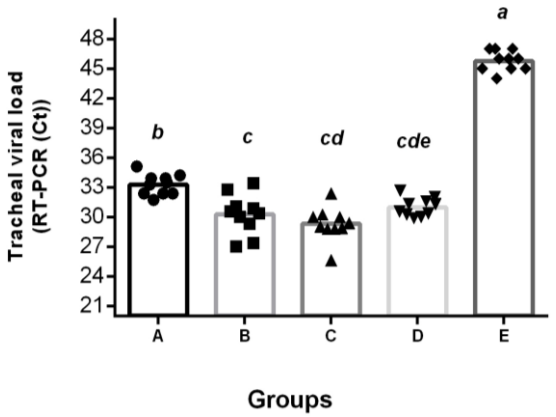

Fig. 3: Means of C_t values in trachea of different groups on day 15 post challenge. C_t: Cycle threshold, RT-PCR: Real time-polymerase chain reaction, A: Autogenous H9N2 vaccine, B: Commercial H9N2 vaccine A, C: Commercial H9N2 vaccine B, D: No vaccine + challenge, and E: No vaccine. [a, b, c, d, e] superscript with different letters means significant difference (P≤0.05)

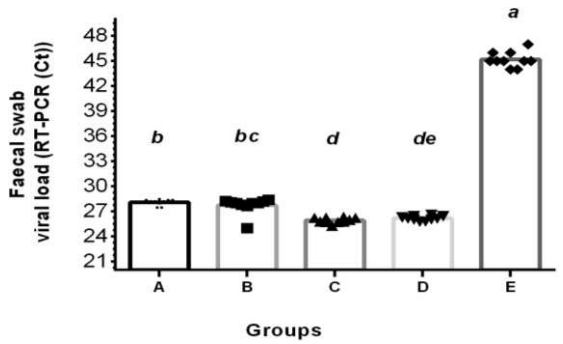

Fig. 4: Means of C_t values in a faecal swab of different groups on day 5 post challenge. C_t: Cycle threshold, RT-PCR: Real time-polymerase chain reaction, A: Autogenous H9N2 vaccine, B: Commercial H9N2 vaccine A, C: Commercial H9N2 vaccine B, D: No vaccine + challenge, and E: No vaccine. [a, b, c, d, e] superscript with different letters means significant difference (P≤0.05)

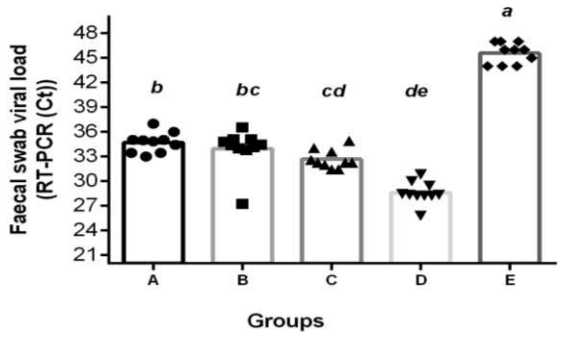

Fig. 5: Means of C_t values in faeces of different groups on day 15 post challenge. C_t: Cycle threshold, RT-PCR: Real time-polymerase chain reaction, A: Autogenous H9N2 vaccine, B: Commercial H9N2 vaccine A, C: Commercial H9N2 vaccine B, D: No vaccine + challenge, and E: No vaccine. [a, b, c, d, e] superscript with different letters means significant difference (P≤0.05)

group and no vaccinated group was not significant while autogenous and commercial vaccines A show significantly lower C_t compared to the unvaccinated group (P≤0.05) (Fig. 4). Also, in the second sampling (Fig. 5) date (15 DPC) (Table 1), the commercial vaccine B showed significantly lower C_t value compared to the two other vaccinated groups (P≤0.05). The no vaccine challenged group show significantly lower C_t compared to the autogenous and commercial vaccine A groups (P≤0.05) while the difference between this group and commercial vaccine B was not significant. Similar to tracheal viral load results, the no challenged-no vaccine group show significantly lower C_t value compared to any other groups in the first and second sampling date (P≤0.05).

Discussion

H9N2 subtype is the most prevalent subtype of AI in Eurasian terrestrial birds (Malekan *et al*., 2016). A similar condition exists in Iran, and even though some other subtypes including H5N1 and H5N8 have been reported in recent years from some parts of the country,

H9N2 is the only endemic subtype reported since 1998 (Ghafouri *et al*., 2017; Hosseini *et al*., 2017). Albeit Iranian H9N2 isolates are a non-HPAI virus, they can cause enormous economic losses through decreased weight gain and mortality (up to 65%) in unvaccinated broiler flocks (Nili and Asasi, 2002). Vaccination emerged as a proper solution in AI control in poultry, during the past two decades. Antibodies that mainly elicit against HA protein can increase resistance to infection and prevent clinical symptoms via inhibiting viral replication in respiratory and alimentary tracts (Swayne, 2006). Despite the development of novel vaccine technology including recombinant, subunit and inactivated vaccines are still the most used vaccines to prevent AI in most countries. Inactivated vaccines can improve poultry's resistance to AI infection and reduce AIV replication in target organs including respiratory, gastrointestinal, and kidneys (Capua and Alexander, 2008). An inactivated vaccine can efficiently reduce viral shedding through faeces and respiratory droplets (Moghaddam Pour *et al*., 2006; Choi *et al*., 2008), that means the lower infection is spreading within birds. Humoral immunity is the main protective immunity against AI in poultry, immunoglobulin M (IgM) and immunoglobulin G (IgG) response to both surface proteins, HA and NA are neutralizing and protective, with HA playing a more important role than NA in protection (Suarez and Schultz-Cherry, 2000). Despite widespread vaccination in commercial farms of Iran, H9N2 outbreaks occur routinely all over the country and impose huge economic losses annually (Shayeganmehr *et al*., 2018). Many factors may cause vaccination failure, continuous antigenic drifts that occur in circulating H9N2 viruses are the most important reason why vaccination is not entirely effective in protecting broiler farms against H9N2 infection (Bashashati *et al*., 2013). The level of protection obtained from vaccination depends mainly on the degree of antigenic similarity between HA of vaccine and challenged virus (Swayne *et al*., 1999). Using viruses from field outbreaks and producing an autogenous vaccine from them result in close relatedness between vaccine and field vaccine (Swayne, 2006). Studies that compared the effectiveness of commercial and autogenous vaccines show that autogenous vaccines can reduce viral replication and shed more efficiently (Gharaibeh and Amareen, 2015). In addition, autogenous vaccines elicit more HI titer compared to commercial vaccines (Gharaibeh and Amareen, 2015). So that, we can expect that the vaccine, which shows the most antigenic similarity with the dominant circulating isolate, offers the best protection. H9N2 is a candidate for a possible future influenza pandemic in the human population (Sun and Liu, 2015) so using more effective vaccines can lead to lower H9N2 shedding from infected birds to humans.

Many genetic studies have been done on Iranian H9N2 isolates, and all of them show that circulating viruses belong to the G1-sublineage (Ghalyanchi Langeroudi *et al*., 2013). Also, studies carried out on isolates obtained over the past 20 years revealed continuous changes in genetic sequencing of Iranian isolates (Bashashati *et al*., 2013; Ghalyanchi Langeroudi *et al*., 2013). Numerous inactivated vaccines from local and international companies are used in Iran; they mostly contain vaccinal viruses that are isolated from the first H9N2 outbreaks that occurred in the country about 20 years ago. The current study tries to evaluate effects of antigenic changes that occurred in recent Iranian H9N2 isolate on the ability of commercial vaccines to inhibit H9N2 viral replication in target organs of challenged broiler chickens and compare results with an autogenous vaccine.

Moreover, produced humoral antibodies arising from each vaccine were determined and compared. Real time-polymerase chain reaction was done on target organs to determine the viral load in the trachea and a faecal swab of infected broilers. Besides, the HI test was conducted on obtained serums to determine antibody response due to the vaccines. Commercial vaccines were selected based on their high consumption, vaccine A and B, used isolates from the first H9N2 outbreaks. Polymerase chain reaction results revealed that difference in tracheal viral load is not significant between unvaccinated group + challenge and two commercial vaccinated groups on 5 DPC, so that, commercial vaccines cannot inhibit AIV replication in tissues during first 5 DPC, while autogenous vaccine succeeded in inhibiting viral replication in the trachea in 1st and 2nd sampling date compared to unvaccinated and commercial vaccine groups ($P \leq 0.05$). Commercial vaccine A succeeded in reducing viral load in the trachea after 15 DPC, while commercial vaccine B was unsuccessful. In checking C_t values in faecal swabs, commercial vaccine A showed a similar performance on viral load compared to autogenous vaccine on both sampling dates, it could be because commercial vaccine A contains more antigenicity, similar to vaccinal strain to challenge strain compared to commercial vaccine B. These two groups (autogenous and commercial A) reduced viral load in faecal swabs at both sampling dates compared to commercial vaccine B and unvaccinated group ($P \leq 0.05$). Results obtained from commercial vaccines especially vaccine B are contrary to previous studies that showed inactivated vaccines from earlier outbreaks can inhibit viral replication significantly in broilers challenged with dominant circulating viruses (Vasfi Marandi and Bozorgmehri Fard, 2002; Moghaddam Pour *et al*., 2006).

This may be due to significant changes that occurred in Iranian H9N2 viruses during recent years. In trachea and faecal swab, the best results in inhibiting viral replication were observed in the autogenous vaccine group, it may be because surface antigens of recent Iranian isolate that was used in challenged birds have more similarity with the autogenous vaccine, which is in agreement with other studies on H9N2 autogenous vaccines (Gharaibeh and Amareen, 2015). Two commercial vaccines induced humoral immunity response at the same level, while the autogenous vaccine showed the highest HI titer compared to other groups ($P \leq 0.05$). It is in agreement with previous studies that

reported autogenous vaccines show the highest HI titer compared to conventional vaccines (Swayne *et al*., 1999; Suarez and Schultz-Cherry, 2000; Moghaddam Pour *et al*., 2006; Naeem and Siddique, 2006; Capua and Alexander, 2008; Choi *et al*., 2008; Gharaibeh and Amareen, 2015).

Autogenous vaccines can significantly inhibit AIV replication in the intestine and trachea of infected broilers. Lower viral replication leads to milder clinical signs and mortality, so the infected flock's performance is less affected by the AI. Furthermore, lower AIV load in target organs means less shedding of AIV in respiratory droplets and faeces. In addition, using autogenous vaccines leads to higher antibody titer compared to commercial vaccines that mean more resistance to infection. Using autogenous inactivated vaccines against H9N2 infection may be a proper solution to control AI in broilers, and they should replace commercial vaccines which contain vaccinal strains that have less antigenic similarity with current circulating viruses in a country or region.

The results of this study showed commercial vaccines that use vaccine seeds that belonged to two decades ago, cannot protect broilers against circulating H9N2 viruses anymore, and it is necessary to alter their seeds with isolates from more recent outbreaks. Also, because antigenic changes in circulating H9N2 viruses are permanent and continuous, it is essential to check the ability of available commercial vaccines routinely in providing proper immunity against circulating viruses.

Acknowledgements

The authors gratefully acknowledge the Ghalyanchilab experts for their help.

Conflict of interest

The authors declare that they have no conflicts of interest.

References

Abdel-Moneim, AS; Afifi, MA and El-Kady, MF (2012). Isolation and mutation trend analysis of influenza A virus subtype H9N2 in Egypt. Virol. J., 9: 173.

Bashashati, M; Marandi, MV and Sabouri, F (2013). Genetic diversity of early (1998) and recent (2010) avian influenza H9N2 virus strains isolated from poultry in Iran. Arch. Virol., 158: 2089-2100.

Capua, I and Alexander, DJ (2008). Avian influenza vaccines and vaccination in birds. Vaccine. 26: 70-73.

Capua, I and Alexander, DJ (2009). *Avian influenza and Newcastle disease: a field and laboratory manual*. 1st Edn., Milan, Italy, Springer. PP: 85-89.

Choi, JG; Lee, YJ; Kim, YJ; Lee, EK; Jeong, OM; Sung, HW; Kim, JH and Kwon, JH (2008). An inactivated vaccine to control the current H9N2 low pathogenic avian influenza in Korea. J. Vet. Sci., 9: 67-74.

Ghafouri, SA; Langeroudi, AG; Maghsoudloo, H; Tehrani, F; Khaltabadifarahani, R; Abdollahi, H and Fallah, MH (2017). Phylogenetic study-based hemagglutinin (HA) gene of highly pathogenic avian influenza virus (H5N1) detected from backyard chickens in Iran, 2015. Virus Genes. 53: 117-120.

Ghalyanchi Langeroudi, A; Karimi, V; Kheiri, MT and Barin, A (2013). Full-length characterization and phylogenetic analysis of hemagglutinin gene of H9N2 virus isolated from broilers in Iran during 1998-2007. Comp. Clin. Pathol., 22: 321-330.

Gharaibeh, S and Amareen, S (2015). Vaccine efficacy against a new avian influenza (H9N2) field isolate from the Middle East (serology and challenge studies). Avian Dis., 59: 508-511.

Hosseini, H; Ghalyanchilangeroudi, A; Mehrabadi, MHF; Sediqian, MS; Shayeganmehr, A; Ghafouri, SA; Maghsoudloo, H; Abdollahi, H and Farahani, RK (2017). Phylogenetic analysis of H9N2 avian influenza viruses in Afghanistan (2016-2017). Arch. Virol., 162: 3161-3165.

Malekan, M; VasfiMarandi, M; Ranjbar, MM and Bashashati, M (2016). Molecular evaluation of M2 protein of Iranian avian influenza viruses of H9N2 subtype in order to find mutations of adamantane drug resistance. Iranian J. Vet. Med., 10: 253-262.

Medina, RA and García-Sastre, A (2011). Influenza A viruses: new research developments. Nat. Rev. Microbiol., 9: 590-603.

Moghaddam Pour, M; Momayez, R and Akhavizadegan, M (2006). The efficacy of inactivated oil-emulsion H9N2 avian influenza vaccine. Iran. J. Vet. Res., 7: 85-88.

Naeem, K and Siddique, N (2006). Use of strategic vaccination for the control of avian influenza in Pakistan. Dev. Biol. (Basel), 124: 145-150.

Nili, H and Asasi, K (2002). Natural cases and an experimental study of H9N2 avian influenza in commercial broiler chickens of Iran. Avian Pathol., 31: 247-252.

Pazani, J; Marandi, MV; Ashrafihelan, MJ; Marjanmehr, SH and Ghods, F (2008). Pathological studies of A/Chicken/Tehran/ZMT-173/99 (H9N2) influenza virus in commercial broiler chickens of Iran. Int. J. Poult. Sci., 7: 502-510.

Shayeganmehr, A; Vasfi Marandi, M; Karimi, V; Barin, A and Ghalyanchilangeroudi, A (2018). Zataria multiflora essential oil reduces replication rate of avian influenza virus (H9N2 subtype) in challenged broiler chicks. Br. Poult. Sci., 59: 389-395.

Suarez, D and Schultz-Cherry, S (2000). Immunology of avian influenza virus: a review. Dev. Comp. Immunol., 24: 269-283.

Sun, Y and Liu, J (2015). H9N2 influenza virus in China: a cause of concern. Protein Cell. 6: 18-25.

Swayne, DE (2006). Principles for vaccine protection in chickens and domestic waterfowl against avian influenza: emphasis on Asian H5N1 high pathogenicity avian influenza. Annals of the New York Academy of Sciences. 1081: 174-181.

Swayne, D; Beck, J; Garcia, M and Stone, H (1999). Influence of virus strain and antigen mass on efficacy of H5 avian influenza inactivated vaccines. Avian Pathol., 28: 245-255.

Swayne, DE and Kapczynski, D (2008). Strategies and challenges for eliciting immunity against avian influenza virus in birds. Immunol. Rev., 225: 314-331.

Vasfi Marandi, M and Bozorgmehri Fard, MH (2002). Isolation of H9N2 subtype of avian influenza viruses during an outbreak in chickens in Iran. Iran Biomed. J., 6: 13-17.