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Effectiveness of two H9N2 low pathogenic avian influenza conventional inactivated oil emulsion vaccines on H9N2 viral replication and shedding in broiler chickens

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Summary

The objective of this study was to compare the effect of two conventional H9N2 avian influenza (AI) vaccines on replication and shedding of the H9N2 AI virus in broiler chickens. These inactivated oil emulsion vaccines contain either a UAE or an Iranian H9N2 AI isolate. One hundred and fifty one-day-old commercial broiler chickens were randomly divided into six groups. The birds, except for the control group (group 4), were challenged with a low pathogenic A/Chicken/Iran/SH-110/99(H9N2) virus isolate. Birds in groups 1 and 5 were vaccinated with an Iranian AI vaccine and groups 2 and 6 with an UAE vaccine type. Birds in groups 5 and 6 were also vaccinated with an H120 strain of infectious bronchitis live vaccine. On days 3, 7, 11, and 15 post inoculations (PI) the trachea, lungs, kidneys and faeces were collected for molecular detection and quantitation of the H9N2 AI virus using TaqMan real time PCR assay. The results showed that frequency of virus recovery and viral titration was generally higher for unvaccinated challenged birds (group 3) on all days PI. No virus was detected in the chicks of group 1. The virus was detected in some cases in the tracheas and lungs of chicks in groups 2, 5 and 6. However, there was no statistically significant difference in viral replication in the trachea and lungs between chicks vaccinated with the UAE and Iranian type vaccines. The most frequent detection of the virus was in the kidneys in comparison with the other samples. The viral titer in the kidneys of unvaccinated challenged birds (group 3) on day 3, 7, 11 and 15 PI was higher than those of the same organs in the vaccinated challenged birds (groups 1, 2). The highest titer of the virus was observed in the faeces of unvaccinated challenged and the chicks vaccinated with the IB and UAE type vaccine (group 6) on day 7 PI. There was a statistically significant difference in viral shedding between groups (1 and 3), (2 and 3) and (5 and 6) (P=0.008). Infectious bronchitis live vaccine could increase the AI virus propagation and shedding in co-infected groups (groups 5 and 6). Altogether, both AI H9N2 vaccines could effectively reduce viral replication and shedding in broiler chicken, however, in order to achieve efficient control of the disease, vaccination should be accompanied with other preventive measurements including biosecurity practices.

Key words: H9N2 avian influenza, Vaccination, TaqMan real time-PCR

Introduction

Avian influenza (AI) is a contagious viral disease of global concern. Influenza viruses are unique among respiratory viruses with their segmented genome and great antigenic diversity. Influenza viruses are classified in the family *Orthomyxoviridae*. These viruses are further categorized into three distinct genus “type” A, B, and C based on serologic reactions to the internal proteins, principally NP and M1 proteins. All avian influenza viruses are type A. Type A influenza viruses are divided into subtypes based on the antigenic relationship in the surface glycoprotein haemagglutinin (H) and neuraminidase (N). The virus is additionally subjected into two distinct pathogenic groups; highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI) (Swayne and Halvorson, 2008).

The most prevalent situation and endemic form of H9N2 AI viruses in high populated commercial chicken farms in Asian countries, in which concurrent infection with H1N1 and H5N1 AI viruses may occur, should have an alarm notice to scientists and government officials for achievement of more effective measures to control the virus spread before any potentially dangerous reassortment viruses appear (Alexander, 2007; Naeem *et al.*, 2007).

In addition to biosecurity measures, the vaccination practice is an important tool for prevention and control of avian influenza viruses in chickens.

Several experimental studies have demonstrated that inactivated AI vaccines are capable of inducing antibody response, which aids in the protection of the infected birds from mortality, and egg production decline (Bublot *et al.*, 2007; Capua and Alexander, 2008). There are several different types of influenza vaccines licensed for humans, animals and birds: inactivated, attenuated and recombinant vaccines.

In July 1998, H9N2 subtype influenza A virus of low pathogenicity was reported in the industrial poultry populations of Iran (Vasfi Marandi and Bozorgmehrifard, 1999). Due to the widespread occurrence of the H9N2 LPAI viruses, and the zoonotic potential of the virus, vaccination of chicken with water – in – oil-emulsion vaccine was employed to control the disease in the country (Nili and Asasi, 2003). Despite extensive application of inactivated vaccine against H9N2 subtype, the virus is circulating in Iranian chicken flocks causing notable mortality, particularly in broiler chickens. However, limited information is available on the role and the efficacy of H9N2 LPAI conventional inactivated oil emulsion vaccines use in the country. The quantitation of viral replication and shedding in infected bird is a valuable tool for evaluation of AI vaccine effectiveness, because an effective vaccine not only prevents clinical disease, but also reduces the viral shedding, thereby decreasing the likelihood of transmission of the virus to new susceptible hosts (Swayne and Halvorson, 2008).

In recent years, real-time PCR has been increasingly used for detecting avian pathogens based on its rapidity, simplicity, sensitivity, specificity and ability to quantify infection levels (Edwards *et al.*, 2004; Ellis *et al.*, 2007). The present study was conducted to study the effects of two conventional inactivated oil emulsion vaccines on viral replication and shedding in broiler chickens challenged with an H9N2 low pathogenic avian influenza virus isolate using TaqMan real time PCR.

Materials and Methods

Virus and vaccines

The virus isolate used in this study was A/Chicken/Iran/SH-110/99(H9N2). Two different inactivated oil emulsion commercial vaccines used in this experiment were: vaccine number 1, containing an Iranian H9N2 isolate and vaccine number 2, containing a UAE H9N2 isolate. An infectious bronchitis (IB) vaccine (H120 strain, Razi Vaccine and Serum Research Institute, Iran) was used in this study as well.

Birds

One hundred and fifty one-day-old commercial broiler chicks (AA plus) were randomly divided into six groups including five experimental and one control group (25 chicks in each group) (Table 1). Each group was reared separately in an isolated room in the Animal Research Unit of the Veterinary School of Shiraz University. Feed and water were available *ad libitum*.

Experimental design

At 10 days of age, birds in group 1 were vaccinated with H9N2 AI vaccine number 1 (0.5 ml/bird) by the sub-cutaneous route in the back of the neck. The birds in group 2 were vaccinated with H9N2 AI vaccine number 2 by the same route. In frequent field observation IB vaccination was incriminated in enhancing the severity of H9N2 AI clinical symptoms, so the IB vaccination was included in this study. So birds in group 5 and 6, at 30 days of age, were vaccinated with the IB vaccine (1 dose/bird) via drinking water. Finally, the

Table 1: Experimental design for evaluation of effectiveness of two kinds of H9N2 avian influenza inactivated oil emulsion vaccines in commercial broiler chicken

Groups	Vaccination with AI vaccine No. 1 (day 10)	Vaccination with AI vaccine No. 2 (day 10)	Challenge with H9N2 virus (day30)	Inoculation of normal saline 0.5 ml (day 10)	Inoculation of normal saline 100 µl (day 30)	Vaccination with infectious bronchitis vaccine (day 30)
1	+		+			
2		+	+			
3			+	+		
4*				+	+	
5	+		+			+
6		+	+			+

* Negative control group, Vaccine number 1 and number 2 contained an Iranian H9N2 isolate and a UAE H9N2 isolate, respectively. Number of birds in each group was 25

birds in the negative control group (group 4) received an equal volume of normal saline.

Prior to challenge with AI virus (day 0), 5 birds from each group were euthanized and their organs and faeces were tested by real-time reverse transcriptase polymerase chain reaction (RRT-PCR) to confirm the birds to be negative to AI virus. Challenge of vaccinated and unvaccinated birds (groups 1, 2, 3, 5 and 6) was carried out with the LPAI A/Chicken/Iran/SH-110/99(H9N2) at 30 days of age. The birds were inoculated by the intra-nasal (100 µl) of allantoic fluid containing 10^6 EID50 of the virus. The EID50 was calculated according to the Reed and Muench formula (Reed and Muench, 1938). The birds of the control group were inoculated by an equal volume of normal saline.

On days 3, 7, 11 and 15 post inoculation (PI) five chicks from the experimental and the control groups were randomly selected. The trachea, lungs, kidneys and faeces were collected separately for virus detection and titration. All samples were immediately stored at -70°C until used.

RNA isolation and detection

Briefly, 50-100 mg of tissue samples was homogenized and total RNA was isolated according to the RNX™ (-Plus) kit (CinnaGen Inc.) protocol. After the isolation step, the pellet was dissolved in 50 µl of sterile distilled water containing 1 mM EDTA. RNA was extracted from 140 µl of the supernatants of 10% (w/v) faecal suspensions using the QIAamp®Viral RNA kit (Qiagen, Germany) according to the manufacturer's protocol. Viral RNA was dissolved in 60 µl elution buffer. The extracted RNA was treated with RNase inhibitor and DNase before storing at -70°C.

Real time-PCR was performed as a screening test to detect the presence of influenza virus genome in the samples.

The cDNA was synthesized using AccuPower® RT PreMix kit (BioNeer Corporation, South Korea) according to the manufacturer's protocol. Reaction was performed with a mixture of 20 pmol random hexamer and 20 pmol of primer that was specific to a highly conserved region of matrix protein gene of influenza A virus (Table 2) and previously described (Ward *et al*., 2004). The cDNA synthesis was performed at 42°C for 60 min, heated to 95°C for 5 min, cooled to 4°C then stored at -70°C until used.

Real time-PCR

The real-time PCR primers and TaqMan probe used in this study were previously described (Ward *et al*., 2004) (Table 2). The primers amplified a 104 bp fragment in the M1 gene of influenza A. The assays were performed on a 48-well microtitre plate (BIO-RAD MiniOpticon™ System) with a 20 µl reaction mixture. The reaction mixture contained 5 µl of target cDNA, 1 µl of each primer at a concentration of 10 pmol/ µl, 0.6 µl of the TaqMan probe at a concentration of 10 pmol/µl, 2.5 µl of dUTP mix, 2.4 µl of $MgCl_2$ at a concentration of 50 mM, 0.2 µl of each Uracil DNA Glycosylase (UDG) and Taq polymerase enzyme (5 U/µl), and 2 µl of ×10 buffer in a final volume of 20 µl. The RRT-PCR program consists of 2 min at 50°C and 10 min at 95°C. The cDNA was amplified by 40 two step cycles (15 sec at 95°C, 1 min at 60°C).

Positive samples for influenza by RRT-PCR were subsequently tested by quantitative real time-PCR (qRT-PCR) to quantify the titer of H9N2 subtype avian

Table 2: RT-PCR and real time-PCR primer and probe sequences used to detect the H9N2 avian influenza genome in tissue samples

Name	Sequences
cDNA synthesis forward primer	5´TCT AAC CGA GGT CGA AAC GTA 3´
Real time PCR forward primer	5´AAG ACC AAT CCT GTC ACC TCT GA 3´
Real time PCR reverse primer	5´CAA AGC GTC TAC GCT GCA GTC C 3´
Real time PCR probe	[a]5´FAM TTT GTG TTC ACG CTC ACC GT TAMRA 3´

[a] FAM, 6-carboxy fluorescein; TAMRA, 6-carboxy tetramethylrhodamine

influenza virus genome. The cDNA synthesis and the real time-PCR assay were carried out as previously mentioned. Viral copy numbers (expressed as copies per 1 µg total RNA) were quantified by comparison with a 10-fold serially diluted plasmid standard of known concentration. The standard recombinant plasmid containing the real time qPCR amplicon was prepared previously in the Laboratory of Virology, School of Veterinary Medicine; Shiraz University (Mosleh *et al.*, 2009b). The data and standard curves were obtained during target cDNA and recombinant plasmid amplification.

Statistical analysis

Statistical analysis was performed using SPSS version 13. The non-parametric Kruskal-Wallis test was applied for comparison of vaccinated and unvaccinated groups. Differences were considered significant at $P<0.01$. The Mann-Whitney U-test was also used for comparison between groups. A p-value of <0.05 was considered as statistically significant.

Results

Viral detection

The presence of the virus in different organs of the birds was determined by RRT-PCR and is shown in Table 3. The virus was detected in the trachea, lungs, kidneys and faeces of the infected chickens, in a variable manner, on days 3, 7, 11 and 15 post inoculation (PI). The virus was detected in the trachea of the vaccinated challenged birds in groups 2, 3 and 6 on day 3 PI (20%, 20% and 20%) and on day 7 PI (20%, 40% and 20%), respectively. The viral RNA was not detected in the trachea of chicks in groups 1 and 5. The virus was detected in the lung of birds in groups 3 and 6 on day 3 PI (20% and 0%), on day 7 PI (40% and 20%), and on day 11 PI (20% and 20%), respectively. In the kidneys the virus was detected in the chicks of groups 3 and 6 on days 3 and 15 PI (20%), in groups 2, 3, 5 and 6 on day 7 PI (60%, 80%, 60% and 80%) and on day 11 PI (20%, 20%, 0% and 20%), respectively. In faecal samples the virus was detected in groups 2, 3, 5 and 6 on day 3 PI (0%, 0%, 20% and 0%), on day 7 PI (60%, 100%, 60% and 100%), and on day 11 PI (20%, 40%, 0% and 0%), respectively. The virus was not detected in the chicks of group 1 on days 3, 7, 11 and 15 PI. No virus was detected prior to the challenge of the H9N2 AI virus, nor was any virus detected in any tissues of the chicks of the uninfected control group.

Viral titration

The qRT-PCR results are shown in Table 3. The mean copies of the virus per 1 µg of total RNA in the trachea of chicks in groups 2, 3 and 6 on day 3 PI were 247, 671 and 1099, respectively. The viral load continued to increase in the trachea on day 7 PI (1314 in group 2, 3550 in group 3 and 1750 in group 6). The mean of AIV RNA levels in the lungs of chicks in groups 3 and 6 were (169344, 10813 copies) on day 7 PI, (18960, 28586 copies) on day 11 PI, and (2467, 0 copies) on day 3 PI. The viral genome was not detected in the lungs of chicks in groups 1 and 2 (groups vaccinated by AI vaccines and unvaccinated by IB vaccine) on the above mentioned days PI. The mean of the viral copies in the kidneys of chicks in groups 3 and 6 were (21, 155 copies) on day 3 PI. It was 10853 copies in group 2, 5912 copies in group 3, 15934 copies in group 5 and 95727 copies in group 6 on day 7 PI, respectively. The viral load continued to decrease on day 15 PI (1284 in group 3 and 51 in group 6).

The mean of the AIV RNA level in faecal samples on day 7 PI was 73784

copies in the chicks of group 2, 3353919 in the chicks of group 3, 209660 in the chicks of group 5 and 2477847 in the chicks of group 6. The AIV RNA copies were also detected in faecal samples of chicks in groups 2 and 3 (9471, 29441) on day 11 PI. The viral genome was not detected in the faecal samples of chicks in group 1 on all days PI studied. There was a significant difference in viral copies between the faecal samples of groups 1 and 3, groups 2 and 3 and groups 5 and 6 (P=0.008), but there was no significant difference between groups 1, and 2.

The frequency of avian influenza virus detection and the No. of RNA copies in the samples of chicks in groups 5 and 6, which were additionally vaccinated by IB vaccine, were higher than those in the chicks of groups 1 and 2.

Discussion

In the present study, we compared the efficacy of two conventional H9N2 inactivated oil emulsion vaccines on viral replication and shedding in broiler chickens challenged with H9N2 LPAIV.

In the trachea and lung tissues, the highest titer of the H9N2 AIV was observed in the chicks of group 3 that was challenged with H9N2 virus on day 7 PI. However, there was no statistically significant difference between the efficacies of the two AI vaccines.

Detection of the virus from the trachea and lung indicates that H9N2 AI virus is pneumotropic. Shamseddini *et al.* (2002) detected H9N2 virus antigen in the trachea, lungs, and kidneys of experimentally infected 6-week-old broiler chickens using immunoperoxidase assay. It was reported that A/Chicken/HS/K5/01(H9N2) viral antigens were detected in the kidneys, spleen, trachea, lungs, thymus, bursa and cecal tonsils of 3-week-old SPF chickens on day 5 post-inoculation (Kwon *et al.*, 2008).

In our study the most frequent detection of the virus was in the kidneys and the virus was recovered in this organ for a longer time

Table 3: Virus detection and molecular quantitation of H9N2 AIV in the organs of experimentally infected broiler chicks on different days post inoculation

Days post inoculation		Group 1	2	3	4	5	6
0[a]	T	0/5[b]	0/5	0/5	0/5	0/5	0/5
	L	0/5	0/5	0/5	0/5	0/5	0/5
	K	0/5	0/5	0/5	0/5	0/5	0/5
	F	0/5	0/5	0/5	0/5	0/5	0/5
3	T	0/5	1/5 (247[c])	1/5 (671)	0/5	0/5	1/5 (1099)
	L	0/5	0/5	1/5 (2467)	0/5	0/5	0/5
	K	0/5	0/5	1/5 (21)	0/5	0/5	1/5 (155)
	F	0/5	0/5	0/5	0/5	1/5 (29306)	0/5
7	T	0/5	1/5 (1314)	2/5 (3550)	0/5	0/5	1/5 (1750)
	L	0/5	0/5	2/5 (169344)	0/5	0/5	1/5 (108313)
	K	0/5	3/5 (10853)	4/5 (59120)	0/5	3/5 (15934)	4/5 (95727)
	F	0/5	3/5 (73784)	5/5 (3353919)	0/5	3/5 (209660)	5/5 (2477847)
11	T	0/5	0/5	0/5	0/5	0/5	0/5
	L	0/5	0/5	1/5 (18960)	0/5	0/5	1/5 (28586)
	K	0/5	1/5 (216)	1/5 (496)	0/5	0/5	1/5 (697)
	F	0/5	1/5 (947)	2/5 (29441)	0/5	0/5	0/5
15	T	0/5	0/5	0/5	0/5	0/5	0/5
	L	0/5	0/5	0/5	0/5	1/5 (2765)	0/5
	K	0/5	0/5	1/5	0/5	0/5	1/5 (1287)
	F	0/5	0/5	0/5	0/5	0/5	0/5

[a] Prior to inoculation of the virus, [b] No. of samples positive for virus recovery/total samples, and [c] Mean titer of AI virus. T = Trachea, L = Lungs, K = Kidneys, and F = Faeces. For groups description see Table 1

(15 days PI) in comparison with the other organs. The majority of infection in the kidneys was detected in groups 3 and 6 on day 7 PI (80%). Although the viral genome was not identified in the kidney of chickens vaccinated with the Iranian type vaccine on all days PI, there was no statistically significant difference in viral replication between chicks vaccinated with the UAE and the Iranian type vaccines. The presence of H9N2 AI viral antigens in the kidneys may have resulted from transient viremia or virus transfer from a localized infection in the lungs through abdominal or caudal thoracic air sacs which are adjacent to kidney lobes. Mosleh *et al*. (2009a) showed the presence of the virus genome in spleen of the chickens following intranasal challenge of the virus that might be an index for occurrence of viremia. On the other hand, according to Shalaby *et al*. (1994), the respiratory tract can allow communication and transportation of infectious agents from outside of the body into the coelomic cavities. Presence of the virus in the kidneys indicates that H9N2 AI virus is also nephrotropic following IN inoculation. It has also been noted that H9N2 LPAI virus was detected and recovered from kidneys and spleen of experimentally infected 35-week-old commercial layers (Lee *et al*., 2007).

The viral genome was identified in the faecal samples, except in group 1, on day 7 and 11 PI. The copy number of the viral genome in the faeces was higher than those in the internal organs of the birds. One study showed H9N2 antigen in cloacal swabs following IN inoculation on days 5 and 7 PI (Kwon *et al*., 2008). Presence of the virus in faeces may have resulted from replication of the virus in the GI tract. As shown in Table 3, the mean of the viral titer was highest in the unvaccinated group (group 3) as compared with the vaccinated group (groups 1, 2) on days 7 and 11 PI. Furthermore, there was also significant difference in viral copies between the faecal samples of groups 5 and 6. This data indicates that two AI vaccines can decrease H9N2 viral shedding in broiler chicks. In addition, if the vaccine is produced with the Iranian subtype of the AI virus, it can be more effective. Vasfi Marandi *et al*. (2002) vaccinated broilers and layer pullets, subcutaneously, by using A/Chicken/Iran/ZMT-101(101)/98(H9N2) as experimental oil-emulsion formalin inactivated vaccine. They concluded that immunized chickens were protected against viral shedding in broilers and layer pullets and egg drop in 190-day-old layers in experimental infection (Vasfi Marandi *et al*., 2002).

It was shown that conventional H5N9 vaccine reduces shedding in specific-pathogen-free birds challenged with HPAI H5N1 A/chicken/Yamaguchi/7/2004 (Terregino *et al*., 2007). Bublot *et al*. (2007) compared the efficacy of two avian influenza (AI) H5-inactivated vaccines against challenge with an H5N1 highly pathogenic avian influenza isolated from a chicken in Thailand. Their studies showed that vaccinated challenged birds were protected against morbidity and mortality and both vaccines prevented cloacal shedding and significantly reduced oral shedding of the challenge HPAI virus (Bublot *et al*., 2007). Similarly, Beato *et al*. (2007) evaluated the efficacy of a conventional inactivated oil emulsion vaccine on tracheal and cloacal shedding challenged with HPAI H5N1 using RRT PCR. Their experiment showed that this vaccine suppressed viral shedding in commercial (Peking) ducks challenged with HPAI H5N1 virus (Beato *et al*., 2007). In our study no virus was detected in the faeces of birds vaccinated with the Iranian type vaccine. The UAE type vaccine significantly reduced shedding of the challenge H9N2 virus.

This study showed that both conventional H9N2 vaccines used in Iranian poultry flocks are efficient to decrease AI replication and shedding in broiler chicks; meanwhile there is a high morbidity and mortality in the vaccinated infected birds in Iran. Therefore, some other factors such as co-infections with other viruses or bacteria and poor management in poultry farms might be the cause of those economic losses. It was shown that avian influenza and infectious bronchitis co-infection is one of the most important reasons for the high mortality resulted from avian influenza in Iranian broiler chickens flocks (Haghighat-Jahromi *et al*., 2008; Seifi *et al*., 2009). In the present study, avian influenza virus

detection frequencies and the number of RNA copies in the samples of groups 5 and 6, which were additionally vaccinated by IB vaccine, were higher than those in groups 1 and 2 as shown in Table 3. The time of IB vaccination was chosen at 30 days of age to reproduce a co-infection, IBV and H9N2 AIV, as the natural situation frequently occurs in Iranian broiler chicken farms (Seifi *et al*., 2009).

In conclusion vaccination with H9N2 AI inactivated oil emulsion vaccines is a useful tool to control AI disease. Furthermore, in order to achieve efficient control of H9N2 AI disease, vaccination should be concurrent with other components including education, surveillance, biosecurity, movement restrictions and monitoring of infection in vaccinated flocks.

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