Efficacy of Inactivated H9N2 Avian Influenza Vaccine against Non-highly Pathogenic A/Chicken/Iran/ZMT-173/1999 Infection

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Summary

An experimental avian influenza (AI) oil-emulsion vaccine was formulated with a ratio of 4 parts oil adjuvant ISA-70 and 1 part formalin inactivated A/Chicken/Iran/ZMT-101(101)/98(H9N2) antigen. Thirty 2-week-old Aryan broilers and thirty 2-week-old white Hy-line pullets were vaccinated subcutaneously. The latter was delivered a booster 10 weeks after primary vaccination. All vaccinated and control birds were bled for hemagglutination-inhibition (HI) test at least one week intervals. Half of the birds were challenged via intranasal and intravenous routes with a H9N2 strain at 8 and 27 weeks of age in broiler and layer birds, respectively. A high HI titers were observed in both vaccinated and unvaccinated birds, when examined at 2 weeks postchallenge (PC). Viral isolation or shedding from tracheal and cloacal swaps of both vaccinated broiler and layer was decreased at 2 weeks PC, as compared with unvaccinated control birds. All control birds became morbid, and egg production decreased on day 3 PC. The results suggested that the inactivated oil-emulsion H9N2 AI vaccine may be protects both chickens against viral shedding and egg drop in field conditions.

Key words: avian influenza, oil-emulsion vaccine, vaccination, broiler, layer

Introduction

The avian influenza viruses (AIVs) have been associated with numerous disease outbreaks in domestic poultry throughout the world (Brugh & Perdue 1991, Crawford *et al* 1998, Garcia *et al* 1998, Zanella *et al* 2001). Variable morbidity and mortality have characterized these outbreaks (Alexander 2000). AIVs belong to type A and all 15 hemagglutinin and 9 neuraminidase subtypes have been reported from domestic birds (Alexander 2000). Such AIVs have been experimentally pathotyped as either highly pathogenic AI (HPAI) or non-highly pathogenic AI (nHPAI) viruses, including mildly pathogenic (MP), low pathogenic (LP) and non-pathogenic (NP) AI viruses (Alexander 2000, Swayne *et al* 1997). High mortality and severe egg production drops typically characterized in H5 and H7, HPAI outbreaks. In contrast, the disease associated with nHPAI outbreaks in chickens has been less dramatic, with most affected birds demonstrating no or minimal signs of diseases (Ziegler *et al* 1999).

During 1994-1999 years, infections of poultry with influenza viruses of H9 subtype have been noticeably common. Outbreaks due to H9N2 subtype occurred in domestic ducks, chickens and turkeys in Germany during 1995-1998, chickens in Italy (1994 and 1996), pheasants in Ireland (1997), ostriches in South Africa (1995), turkeys in the USA (1995 and 1996), chickens in Korea (1996), in China (1994), and more recently (1999) in chickens in Pakistan (Alexander 2000, Naeem *et al* 1999). An AI outbreak was occurred in chickens in Iran in 1998 and a nHPAI Virus of H9N2 subtype was isolated (Vasfi Marandi & Bozorgmehri Fard 2001). The disease caused severs economic losses in poultry industry (Ziggers 1999).

Several experimental studies have demonstrated that inactivated monovalent and polyvalent subtypes of AI vaccine, are capable of inducing antibody and providing protection against mortality, morbidity and low egg production (Butterfield & Campbell 1979, Stone 1987, Swayne *et al* 2001, Wood *et al* 1985, Xie & Stone 1990). Until recently vaccination has not been employed against AI, in chickens in the USA, primarily due to concerns of vaccine serologic responses hampering surveillance program of natural avian influenza virus infections (Crawford *et al*

1998). However, in the case of nHPAI, inactivated vaccines are used in turkey industry (Karunakaran *et al* 1987). There are pros and cons to the use of vaccination to control avian influenza in chickens (Beard 1992). In principle, any method that will reduce the level of virus replication in infected chickens will slow down spread of the virus and, if used systematically, could eradicate both the HPAI and nHPAI viruses (Webster *et al* 1996). Vaccination of chickens with inactivated oil emulsion vaccines was employed during the recent H5N2 and H7N3, avian influenza outbreaks in Mexico and Pakistan respectively (Garcia *et al* 1998, Naeem 1998).

The present study describes the preparation and evaluation of a H9N2 subtype inactivated oil-emulsion vaccine in broiler and layer chickens.

Materials and Methods

Antigen. A/Chicken/Iran/ZMT-101(101)/98(H9N2) was isolated from layer chickens in 1998 in Iran (Vasfi Marandi & Bozorgmehri Fard 2001). This strain was propagated in embryonated chicken eggs (ECEs) and harvested as described by Swayne *et al* (1998). The allantoic fluid virus harvest possessing approximately $10^{9.2}$ ELD_{50}/ml, inactivated with 0.1% formalin as described by Vasfi Marandi & Bozorgmehri Fard (2001). Virus inactivation was confirmed by ECEs inoculation.

Vaccine preparation. Water-in-oil-emulsion vaccine was prepared with one part allantoic fluid antigen in a 4 parts oil adjuvant ISA-70 (SEPPIC, Cosmetics-Pharmacy Division, France) as described by Stone (1987).

Virus challenge. A/Chicken/Tehran/ZMT-173/99(H9N2), a nHPAI or LPAI virus isolated from a broiler flock in Tehran province, was used as challenge strain. Each chicken was challenged intranasally with 0.5ml and intravenously with 0.1ml of undiluted infectious allantoic fluid containing approximately $10^{9.2}ELD_{50}$/ml. Following challenge, the chickens were observed daily for clinical signs and then slaughtered and given a complete postmortem examination.

Experimental designs. Sixty Aryan broiler chickens and sixty Hy-line pullets has been brooded in separate rooms. The birds were kept on cages and fed with complete

feeds. Vaccination, antibody titration and challenge were carried out according to two different experimental protocols as described below.

Expt. 1). Thirty broiler chickens were sub-cutaneously vaccinated at 2-week-old. Besides another thirty chickens were not vaccinated. Half of vaccinated and unvaccinated chickens were used respectively as vaccinated and non-challenged control group as well as unvaccinated and non-challenged control group. Blood samples were taken at one week interval postvaccination (PV) and all sera were tested individually for the presence of hemagglutination inhibition (HI) antibodies as described by Beard & Wilkes (1973). Two groups of vaccinated and unvaccinated chickens were challenged with $10^{9.2}$ ELD$_{50}$/ml of H9N2 strain via intranasal and intravenous routes at 8 weeks of age. For virus isolation from tracheal and fecal materials all of challenged chickens were slaughtered at 2 weeks postchallenge (PC). The viral recoveries were done in 9-10-day-old ECEs by standard procedure (Swayne *et al* 1998).

Expt. 2). Thirty Hy-line pullets were sub-cutaneously vaccinated at 2 and 12-week-old. Besides another thirty chickens were not vaccinated. Half of vaccinated and unvaccinated chickens were used respectively as vaccinated and non-challenged control group as well as unvaccinated and non-challenged control group. Blood samples were taken at least 1 to 6 weeks PV and all sera were tested for HI titers. Two groups of vaccinated and unvaccinated layer chickens were challenged with $10^{9.2}$ELD$_{50}$/ml of H9N2 strain via intranasal and intravenous routes at 27-week-old. The viral shedding test from tracheal and cloacal swaps was carried out at two weeks PC in 9-10-day-old ECEs.

Results

Expt. 1). HI antibody titers in fifty of 2-week-old broiler chickens immunized with inactivated H9N2 vaccine at 1 to 8 weeks of age were shown in figure 1. HI antibody titers were not increased until 8 weeks of age in unvaccinated chickens. A rapid increase in HI titer to 6.6 at 2 weeks PC was observed in

unvaccinated chickens. Whereas, those in vaccinated group was slowly increased to 6.8 in the same period. Virus recoveries from nasal and cloacal swaps of slaughtered vaccinated chickens were highly decreased as compared with those in unvaccinated chickens at 2 weeks PC. Virus recovery in vaccinated chickens challenged only via intranasal rout with field strain was not detecting (data not shown).
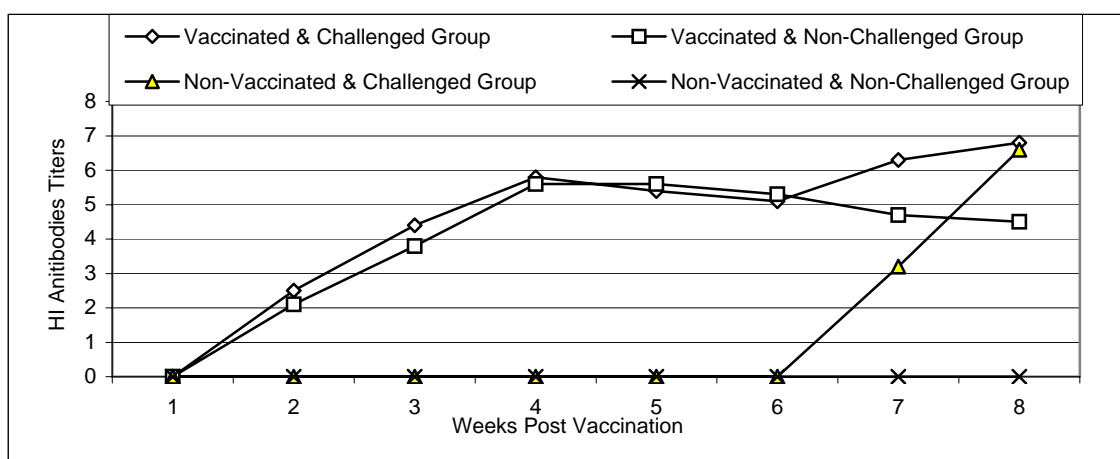

Figure 1. *The hemagglutination inhibition antibody titers (log2) of four groups of broiler chickens were immunized at 2 wks old*

Expt. 2). HI antibody titers in fifty of 2-week-old pullets immunized with inactivated H9N2 vaccine at 2 and 12 weeks of age and at various intervals of age were shown in figure 2.
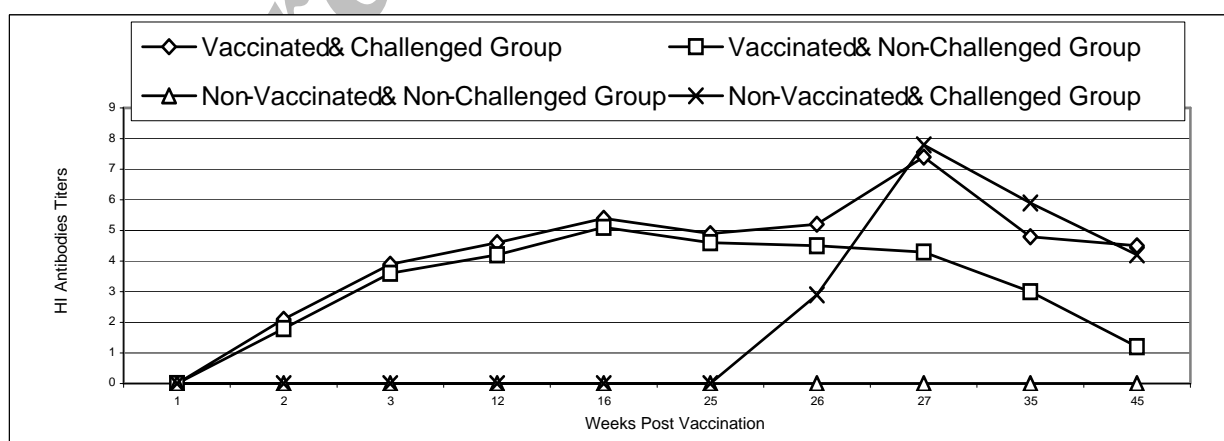
Figure 2. *The hemagglutination inhibition antibody titers of four groups of pullets were immunized at 2 and 12 wks old*

HI titers were not increased in unvaccinated pullets until 28-week-old. A rapid increase in HI titer to 7.3 at 2 weeks PC was observed in unvaccinated chickens. Whereas, those in vaccinated group was increased to 7.8 in the same period. Virus shedding from nasal and cloacal swaps of vaccinated pullets was highly decreased as compared with those in unvaccinated chickens at 2 weeks PC.

The percentage of egg production in vaccinated layer chickens were not effected when challenged at 189 days of age (27-week-old), whereas, those in unvaccinated layer chickens were decreased from 86.66 % to 66.66 % at 190-day-old (Figure 3).
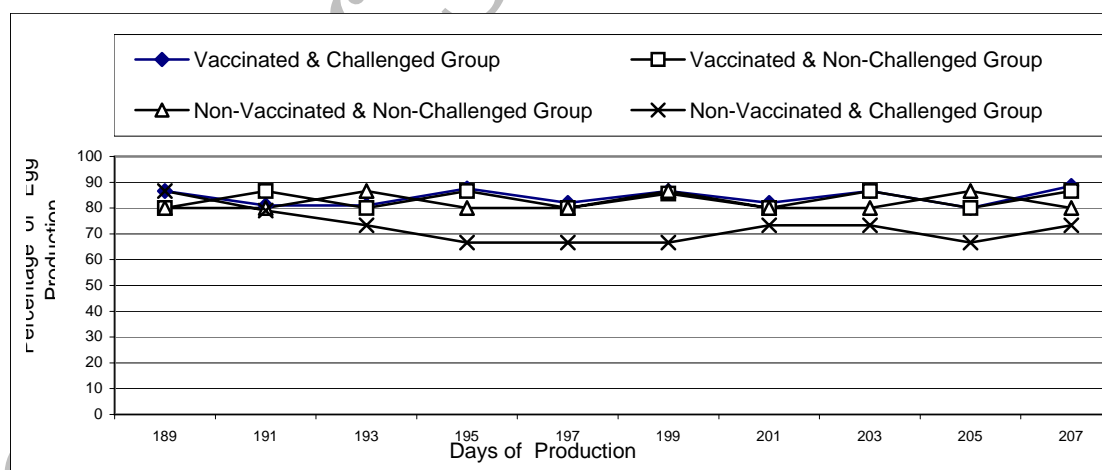
Figure 3. *The percentage of egg production in four groups of layer vaccinated and non-vaccinated with an inactivated vaccine, when challenged with a H9N2 field strain at 189 days (27 wks) old*

Discussion

In July 1998, decreased egg production (up to 75%) and increased mortality (up to 80%) in layer, breeder and broiler flocks, were associated with serological and virological evidence for H9N2 avian influenza outbreaks in Iranian poultry industry. Three H9N2 viruses, isolated from these flocks, caused no disease signs when experimentally inoculated into specific pathogen free (SPF) chickens. Therefore, the representative Iranian H9N2 strains were pathotyped as nHPAI

or NPAI virsues (Vasfi Marandi & Bozorgmehri Fard 2002). Because eradication of the AI disease in possible future outbreaks in Iran potentially could be too costly and unsuccessful, vaccination is an alternative or supplemental control procedure that may need to be considered to control H9N2 subtype.

Since avian influenza due to H9N2 subtype in Iran in chickens, was seen concurrently with a prolonged environmental heat stress at the time of first outbreak in July 1998 and with other viral and/or bacterial agents in next outbreaks (Vasfi Marandi & Bozorgmehri Fard 2000), it is impossible to evaluate vaccine efficacy against AI clinical signs. For this reasons there has been no good challenge method available to determine the efficacy of oil-emulsion H9N2 vaccine against AI disease by using nHPAI or NPAI virus strains. Therefore, in this study the vaccinated birds were challenged by both intranasal and intravenous routes. The challenge virus designated as A/Chicken/Tehran/ZMT-173/99(H9N2), produced coughing, depression, anorexia and diarrhea signs and airsacculitis at two wks PC in broiler chickens. Besides, a nHPAI or NPAI H9N2 strain, designated as A/Chicken/Iran/ZMT-101(101)/98(H9N2) isolated from a commercial layer flock during the primary outbreaks in Tehran province was previously characterized (Vasfi Marandi & Bozorgmehri Fard 2001). This strain had a high ability to replicate in ECEs and was proposed as vaccinal strain (Vasfi Marandi & Bozorgmehri Fard 2002). We used this strain for antigen and vaccine preparation.

The results of virus isolation and viral shedding in both vaccinated and unvaccinated broiler and layer birds showed that the immunized chickens were protected against viral shedding in broiler and layer chickens and egg drop in experimental infection. Garcia et al (1998) and Naeem (1998) reported that an inactivated oil-emulsion vaccine was protected the chicken against clinical disease both in experimental and field conditions. However, the reliability of these results should be evaluated in filed conditions before large-scale vaccination.

Two reports about AI control by vaccination, presented by Rivera and Naeem at the 4th international symposium on avian influenza in may 1997 indicated that H5N2 and H7N3 AI in Mexico and Pakistan is largely controlled against AI disease but not

eradicated (Naeem 1998, Garcia *et al* 1998). This may be due to circulating AIV in flocks of vaccinated birds (Webster *et al* 1996). Following large scale utilization of inactivated vaccines in Mexico and Pakistan and successful control of AI in their poultry industries, US industry is urged to work with the government to have vaccines available when eradication is not successful. Webster et al (1996) emphasized this.

A slow decreasing of HI antibodies in commercial layer birds at 28 weeks of age in laboratory settings indicate that one or two booster vaccinations of broiler breeder pullets before 20-week-old may be needed in eventual field application of AI inactivated vaccines in broiler breeder chickens. Since HI antibodies following inactivated vaccine utilization in pure line and grand parent flocks interfere with serological monitoring of H5 and H7, HPAI subtypes in AGP or ELISA tests, therefore this inactivated vaccine should not be used in these flocks. The relatively slow time to peak protective immunity by one dose vaccination of broiler chickens at 2-week-old, propose several obstacles in field application, as AI was observed in broiler flocks having four weeks in Iran.

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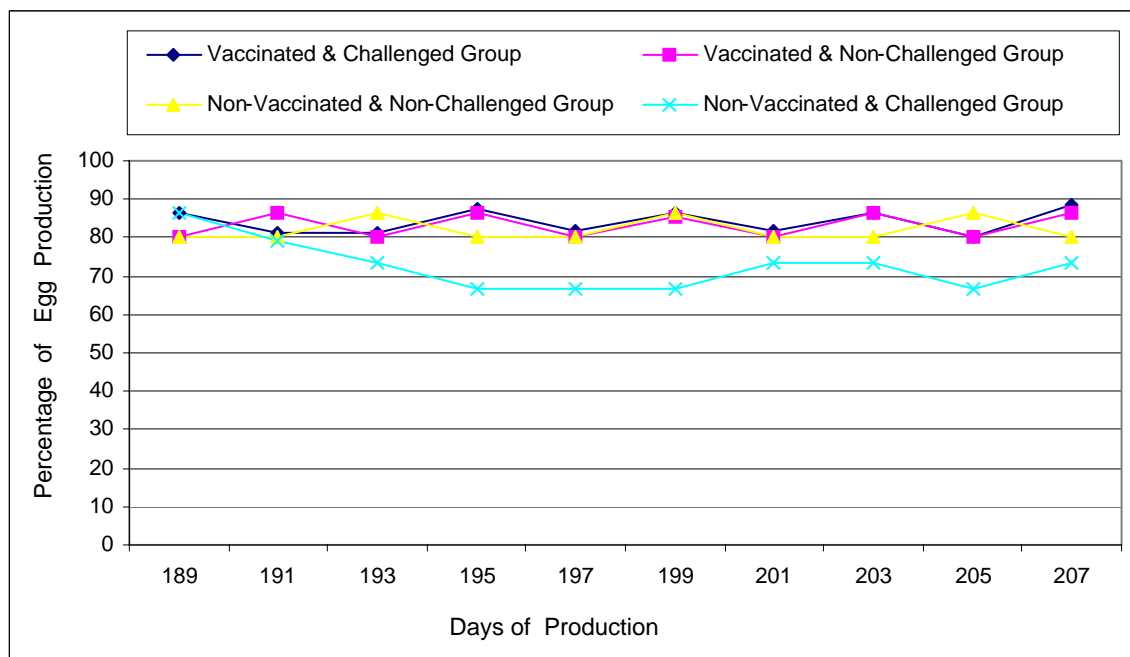
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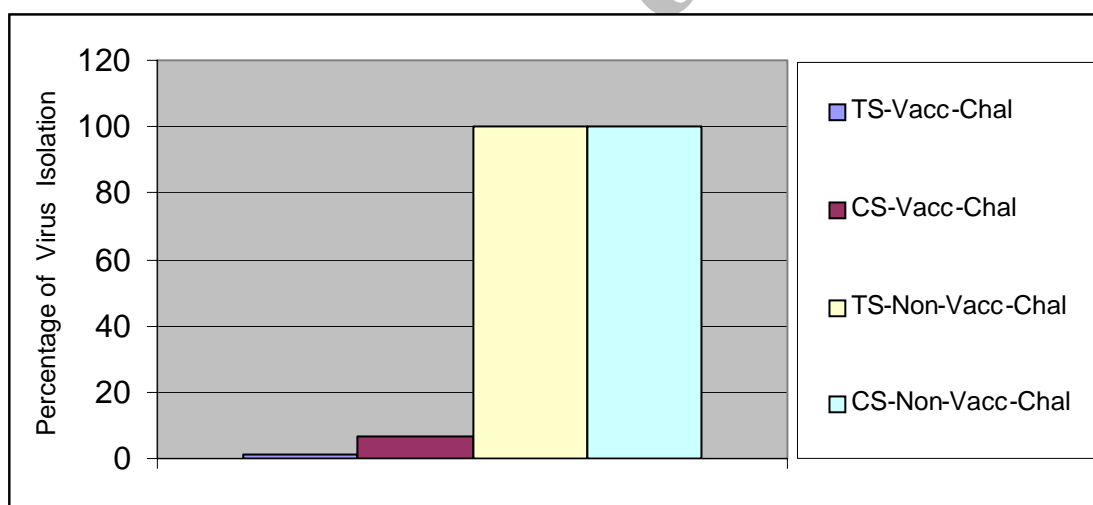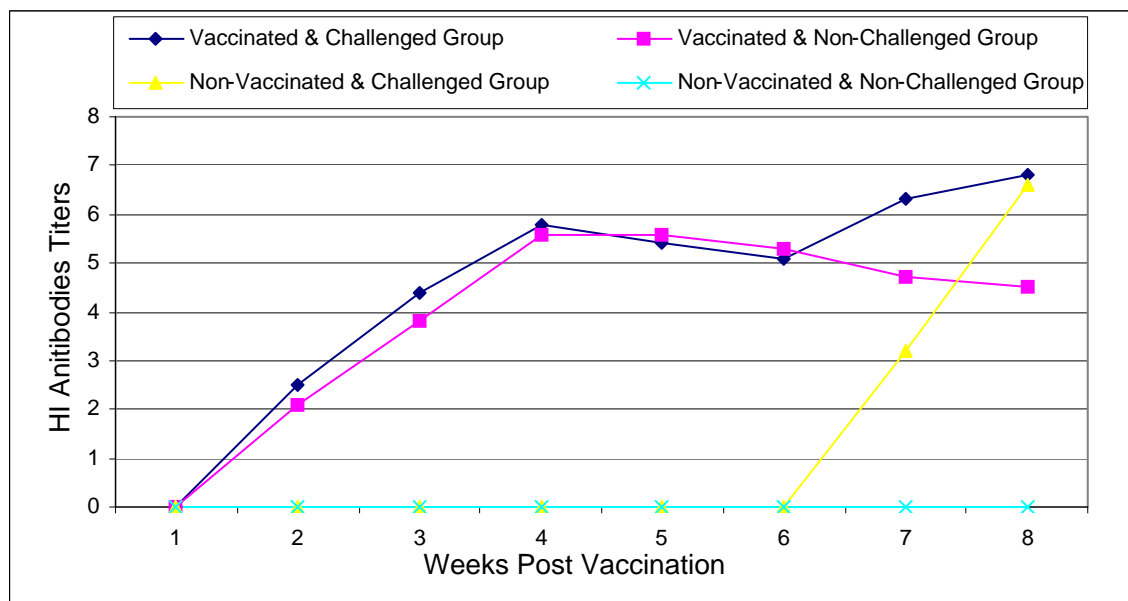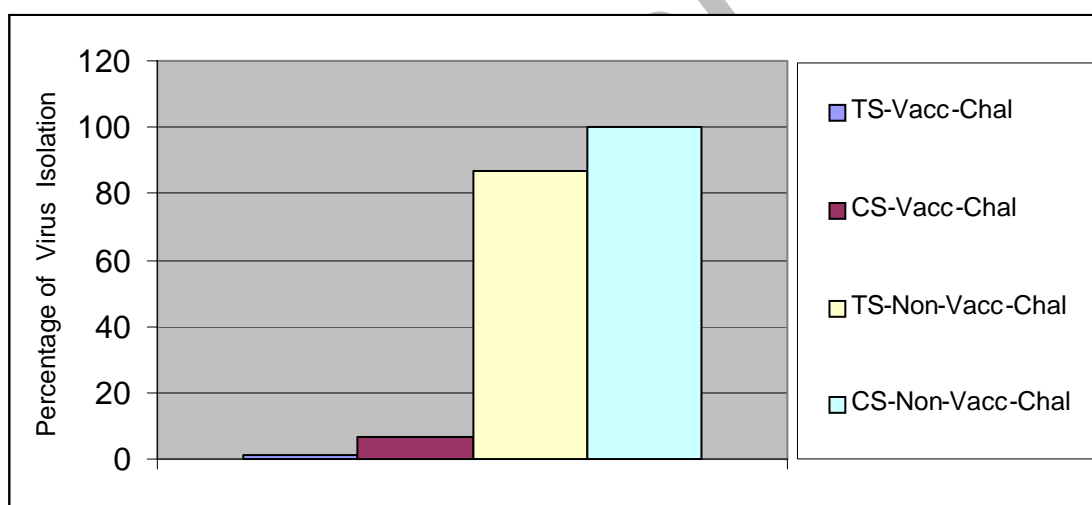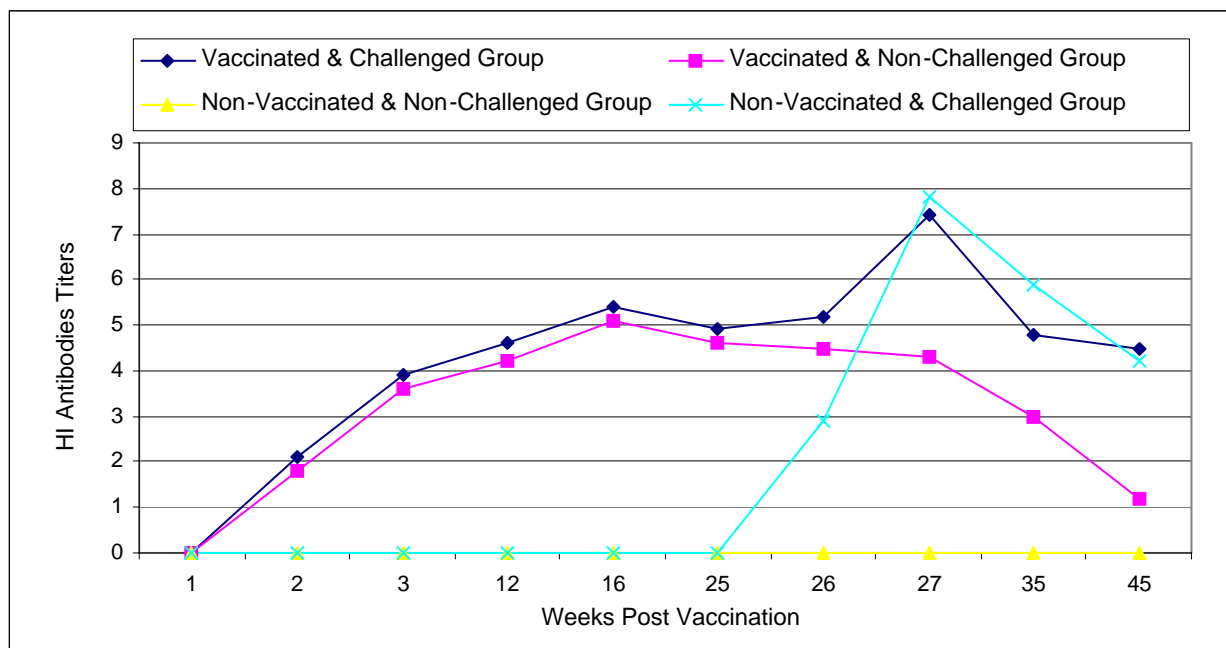
The hemagglutination inhibition antibodies titers (log 2) of four groups of broiler chickens were measured by HI test. Two groups of chickens were immunized at 2 wks old with an inactivated H9N2 vaccine and two groups were challenged with field strain at 8 wks old.
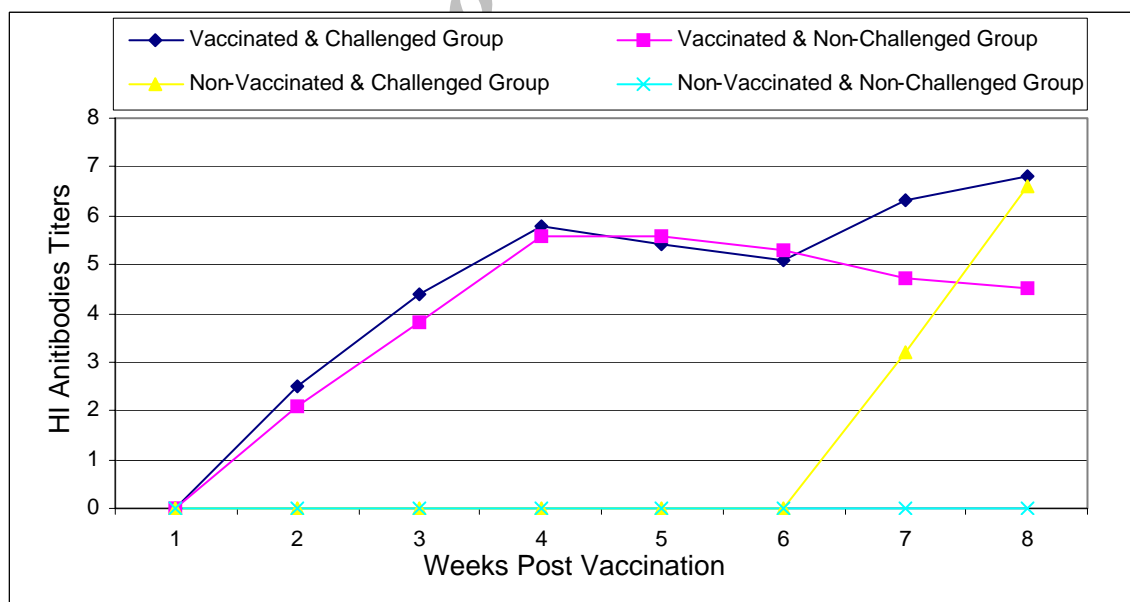
Figure 2 :

H9N2 avian influenza virus (AIV) isolation in tracheal (TS) and cloacal swaps (CS) at two wks post challenge, prepared from broiler chickens vaccinated and challenged (Vacc-Chal) and from non-vaccinated and challenged (Non-Vacc-Chal) groups in embryonated chicken eggs (EGEs).
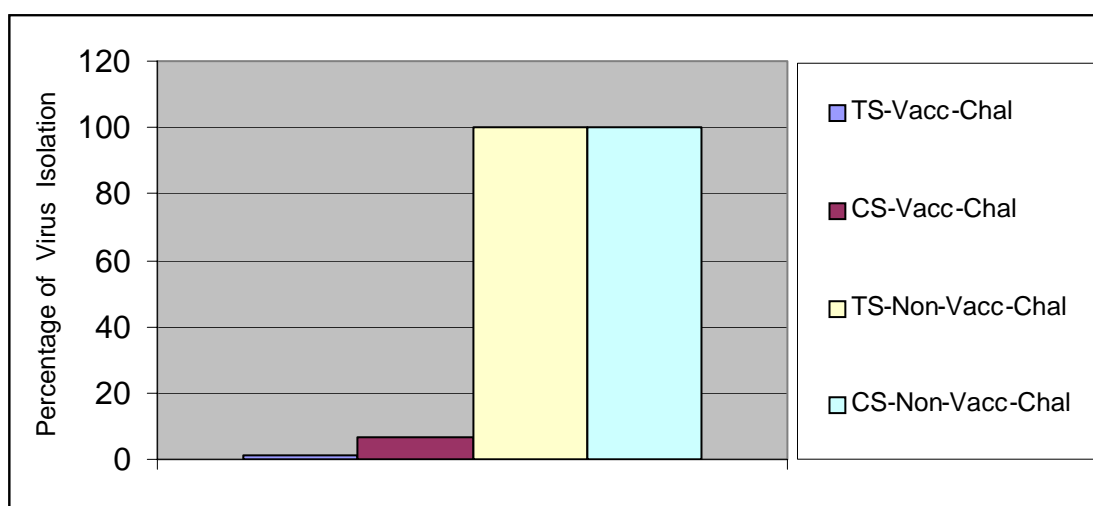
Figure 3 :

The hemagglutination inhibition antibodies titers of four groups of pullets were measured by HI test. Two groups of chickens were immunized at 2 and 12 wks old with an inactivated H9N2 vaccine and two vaccinated and non-vaccinated groups were challenged with field strain at 27 wks old or 25 wks post vaccination.
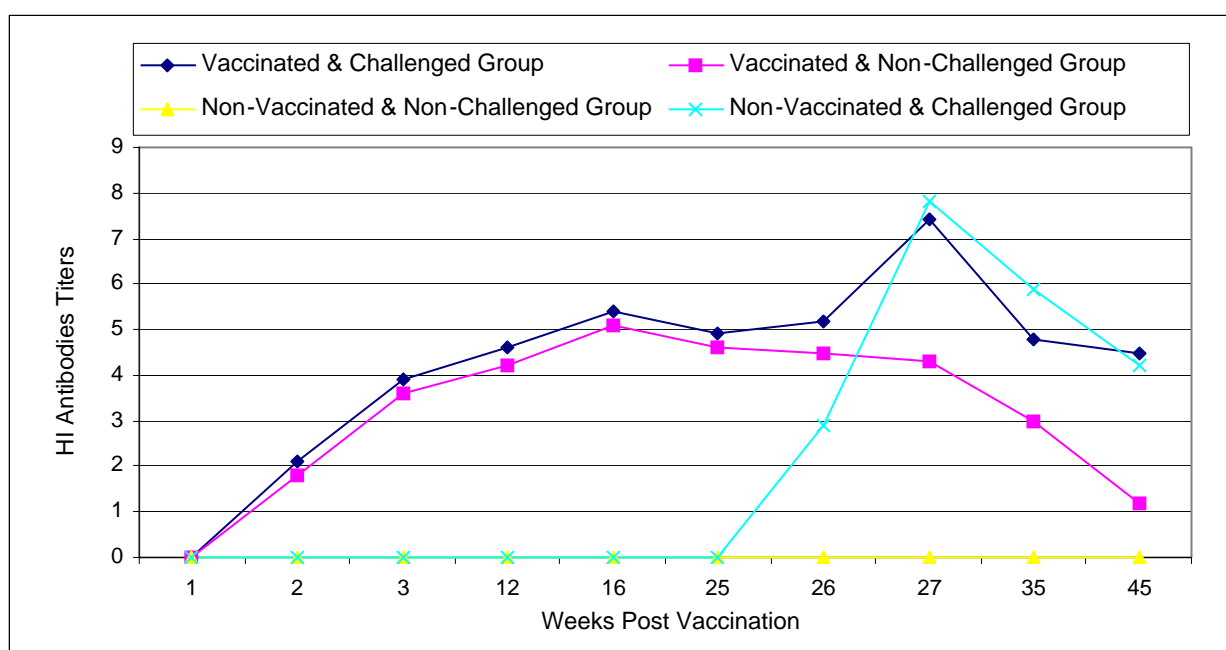
Figure 4 :

       H9N2 avian influenza virus (AIV) shedding in tracheal (TS) and cloacal swaps (CS) prepared at two wks post challenge, from commercial layer chickens vaccinated and challenged (Vacc-Chal) and from non-vaccinated and challenged (Non-Vacc-Chal) groups in embryonated chicken eggs (EGEs).
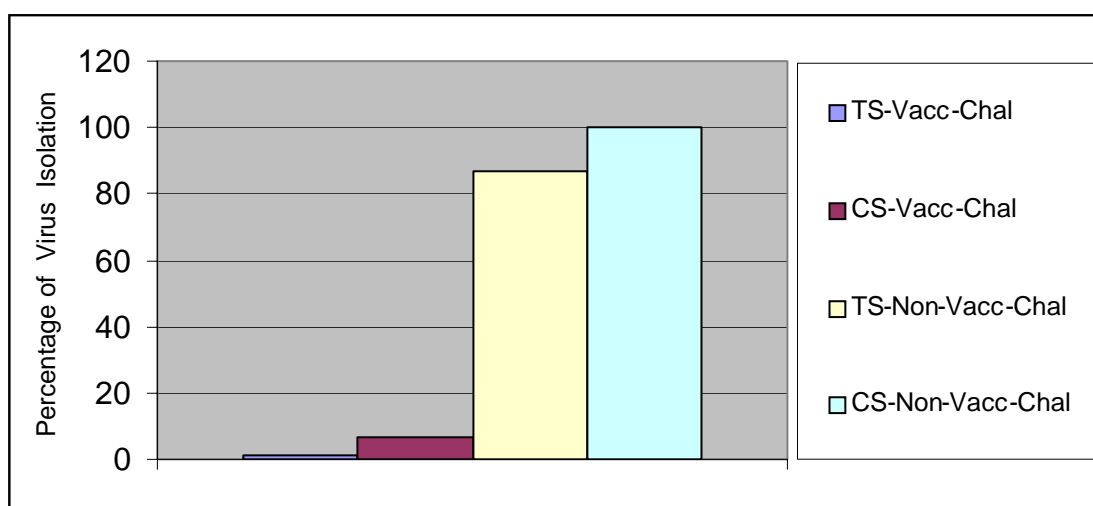
Figure 5 :

     This figure is showing the percentage of egg production in four groups of layer vaccinated and non-vaccinated with an inactivated vaccine, when challenged with a H9N2 field strain at 189 days (27 wks) old.