Protective efficacy of priming with recombinant vaccine in chickens challenged with Egyptian highly pathogenic H5N1 avian influenza virus

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ABSTRACT

Highly pathogenic avian influenza (HPAI) H5N1 virusesare now endemic in Egypt inducing economic losses in poultry industry and subsequent risk of human infection. Vaccination is an effective method for controlling avian influenza virus infections.This study was conducted to evaluate the efficacy of the commercially applied AI vaccine regimens against challenge with three H5N1 HPAI viruses isolated from chickens, Egypt during 2009-2011. Both inactivated and recombinant fowlpox virus expressing H5 (rFP-AI-H5) vaccines were measured. Vaccine efficacy was evaluated by percentage of protection from mortality, morbidity and reduction in virus shedding from respiratory and/orintestinal tracts. Despite the H5 antibody responses in vaccinated chickens with program I (rFP-AI-H5" vaccine then Re-5) being significantly lower prior to challenge, it provided good protection (73.3%) against the lethal A/chicken/Egypt/SHAH-1403/2011 (H5N1) AIV challenge, with no evidence of virus shedding. Keeping in mind that each bird was potentially challenged with 10^5 EID_{50}, which may not be available to all birds under field condition. The foremost vaccine regimen can provide a safe, effective and considerable tool for the control of HPAIV in commercial chickens. Maximizing the beneficial role of recombinant and reassortant vaccines in single product could be utilized.

Key words: AI vaccines, rFP-AI-H5, HPAIV, H5N1, Egypt

Introduction

Avian influenza is an infectious disease of birds caused by type A Influenza viruses, f*amily Orthomyxoviridae*, resulting in huge economic losses in poultry industry and pose a great risk for human health (WEBBY R.J. and WEBSTER R.G., 2003; PEIRIS J.M. et al., 2007). Many outbreaks of highly pathogenic avian influenza (HPAI) virus H5N1 have been reported in various bird populations; their ongoing spread remains a major global concern (LI K.S. et al., 2004). Egyptian poultry industry includes both commercial enterprises and backyard rearing. In February 2006, H5N1 virus has emerged in Egypt and caused noteworthy economic losses (ALY M.M. et al., 2006; ABDELWHAB E.M. and HAFEZ H.M., 2011). The virus spread rapidly throughout the country within a short period either in commercial sector or backyard flocks (GRUND C. et al., 2011) and became endemic since 2008, despite intensive control efforts (ABDELWHAB E.M. and HAFEZ H.M., 2011).

Egypt adopted a strategy to control the disease based on mass vaccination, surveillance and culling of infected birds (ALY M.M. et al., 2008; HAFEZ M.H. et al., 2010). Nevertheless, the

HPAI virus H5N1 was reported in many poultry species and mammals (ABDELWHAB E.M. et al., 2010; EL-SAYED A. et al., 2010; ABDEL-MONEIM A.S. et al., 2010). Different types of vaccines are used in the field in Egypt, including inactivated (H5N1 and H5N2) and live recombinant vaccines based on the fowl pox and Newcastle disease viruses (FAO, 2006). The incompatibility of the vaccine seed virus strain and the circulated strains, along with the presence of antigenic drift (LEE C.W. et al., 2004) are among the probable causes and challenges confronting the use of inactivated vaccines. However, the vaccination is still a compulsory tool for reduction of viral load in the environment due to the predominance of backyard poultry, consequently decreasing the risk of virus transmission to humans. Previous studies strongly recommend the need for proper selection of vaccines, routine evaluation against avian influenza field viruses and challenge studies to assess efficacy of AIV vaccination campaigns (GRUND C. et al., 2011; HASSAN M.K. et al., 2012). Therefore, in this study, we evaluate the efficacy of the commercially applied AI vaccines regimens commonly used in Egypt against challenge of vaccinated broiler chickens with HPAI viruses isolated during 2009-2011.

MATERIALS & METHODS

***Viruses*:** The following Egyptian AIV isolates; A/chicken/Egypt/SHZA-0412/2009 (referred as Egypt/2009), A/chicken/Egypt/SHMK-1903/2010 (referred as Egypt/2010) and A/chicken/Egypt/SHAH-1403/2011 (referred as Egypt/2011) were isolated from backyard chickens in Sharkia, Egypt. They were typed as influenza A H5N1 viruses by RT-PCR. Each virus was propagated in the allantoic cavity of 9-day specific pathogen-free embryonated chicken eggs (ECEs). The intravenous pathogenicity index (IVPI) for each of the three isolates was determined in accordance with the instructions of (OIE, 2009).The IVPI values were 2.74, 2.90 and 2.69, indicating that they were HPAIV. The median embryo infectious dose (EID_{50}) of each isolate was determined by the method of (REED L. and MUENCH H., 1938). Virus stocks were diluted in phosphate-buffered saline and standardized to 10^5 EID_{50}/0.1 ml.

Vaccines:Three H5 available commercial vaccines used commonly in Egypt were used in this study; 1. Trovac AIV H5 "Avian influenza- fowl pox vaccine" (live fowl pox vector, H5 subtype). It contained haemagglutinin gene of AIV strain A/trk/Ireland/1378/1983 H5N8, Merial Company, List no: RFAID – 9475. 2. Reassortant avian influenza virus vaccine, inactivated (H5N1 subtype, Re-5 strain), from Chinese strain A/Duck/Anhui/1/2006, Merial Company, Batch no: 11051457. 3. Volvac AI K.V "Avian influenza, killed virus" (inactivated AI virus type A, subtype H5N2), from Mexican strain A/Chicken/Mexico/232/94/CPA, BoehlingerIngelheim Company, Lot no: 1106015 A.

Birds:A total of 135, day-old Cobb500 chicks obtained from EL-BanaCompany, Egypt were housed under suitable-temperature and relative-humidity conditions. The birds were reared in experimental unit, Faculty of Veterinary Medicine, Zagazig University. Strict hygienic conditions were applied.

Experimental design:The birds were divided equally into nine groups (15 birds/group). To evaluate the efficacy of the commercially applied AI

vaccines regimens commonly used in Egypt. Two vaccine programs were investigated; program I (Trovac "rFP-AI-H5" vaccine at one day old and booster dose with Re-5 "reassorted inactivated H5N1" at 10 days old) and program II (Volvac "inactivated H5N2" at one and 10 days old). Two weeks later, the vaccine efficacy was assessed by challenge of vaccinated broiler chickens with threeHPAIV field strains(10^5 EID_{50}/0.1ml) via intranasal route. Additionally, in two vaccinated groups (group in each program) remained non-inoculated and another group remained non-vaccinated non-infected and were housed separately from the infected ones (Table 1). Oropharyngeal and cloacal swabs were collected on days 2 and 5 days post-challenge (dpc) for virus isolation. Blood samples were collected weekly all over the experiment period for detecting antibody response post vaccination. Chickens were observed twice daily throughout the study for clinical signs and/or deaths. Necropsy was performed to examine gross lesions. Respiratory and intestinal tracts were collected separately and prepared for virus re-isolation.

Virus re-isolation: The collected swabs and tissue samples were prepared and inoculated into 9-day-old ECEs via allantoic cavity for virus isolation as recommended (OIE, 2009). Allantoic fluid harvested from eggs with dead embryos and from eggs incubated for five days post-inoculation was examined for hemagglutination activity. Tissue and swab samples received three blind egg passages when no hemagglutination was detected in the allantoic fluids.

Reverse transcription and Polymerase chain reaction: Total RNA was extracted from positive HA allantoic fluids using GeneJET RNA Purification Kit (Cat#K0731, Fermentas, EU) according to the manufacturer's instructions. RNA from each sample was reversed transcribed to produce cDNA using RevertAid^{TM} H Minus First Strand cDNA synthesis kit (Cat#K1611, Fermentas, EU) following the manufacturer's instructions. PCR was performed with specific H5 primers as described earlier (NJOUOM R. et al., 2008).

Hemagglutination inhibition (HI) test: Serum samples were collected weekly from all groups. Measurement of H5 antibody levels was carried out using micro-HI test using 4 HA units of the reference inactivated AIV H5N2 antigen (BoehlingerIngelheim) as recommended previously (THAYER S.G. and BEARD C.W., 1998).

Statistical analysis:ANOVA was used to analyze the results of the hemagglutination inhibition assay for the levels of antibodies.

RESULTS

Protective efficacy of the two vaccination regimens in broiler chickens: The efficacy of two commercially applied AI vaccines regimens commonly used in Egypt was investigated in commercial broiler chickens challenged with Egypt/2009 (groups 1 and 2), Egypt/2010 (groups 3

Table 1
Experimental design

Groups	1st vaccination	2nd vaccination	Challenge
	1 day old [a,b]	10 day old [c,d]	25 day old
1	Trovac	Re-5	Egypt/2009
2	Volvac	Volvac	
3	Trovac	Re-5	Egypt/2010
4	Volvac	Volvac	
5	Trovac	Re-5	Egypt/2011
6	Volvac	Volvac	
7	Trovac	Re-5	-
8	Volvac	Volvac	-
9	-	-	-

[a]Trovac at one day old (0.2 ml S/C)
[b]Volvac at one day old (0.3 ml S/C)
[c] Re-5 at ten day old (0.5 ml S/C)
[d]Volvac at ten day old (0.5 ml S/C)

and 4) or Egypt/2011 (groups 5 and 6) viruses.Two groups of vaccinated chickens were not challenged with virus (groups 7 and 8) as well as one group kept as non-vaccinated non-infected (group 9).

In program I, the vaccinated birds were protected with 73.3% against Egypt/2011 virus infection; while in contrast, 40% and 46.7% withstand Egypt/2009 and Egypt/2010 virus infections, respectively. Meanwhile the vaccinated ones with vaccine program II revealed protection rates 53.3%, 53.3% and 60% against the challenge viruses, Egypt/2009, 2010 and 2011, respectively (Figure 1). Control groups (non-vaccinated non-challenged and vaccinated non-challenged) chickens showed no clinical signs or mortalities along the period of observation.

In program I, the onset of mortalitystarted on day two post challenge in groupsinfectedwith Egypt/2009 (n = 2) and Egypt/2010 (n = 3). At that day, chickens infected with Egypt/2011 were only slightly depressed and died on days 4, 6, 10 and 15 (n = 4) post challenge. The unprotected birdssho-wed clinical signs commonly observed in HPAIV infections such as cyanosis of comb and wattles, hemorrhages on the shank, oculo-nasal discharges, greenish and whitish diarrhea. Add-itionally, nervous mani-festation was recorded only in one bird in each of groups 1, 5 and 6 within 9 dpcin form of paralysis in legs and torticollis. On necropsy, the foremost pathological findings observed in the dead birdswerecongestion and/or hemorrhage in brain, heart, pancreas, proventriculusandnecrosis in pancreas and spleen.

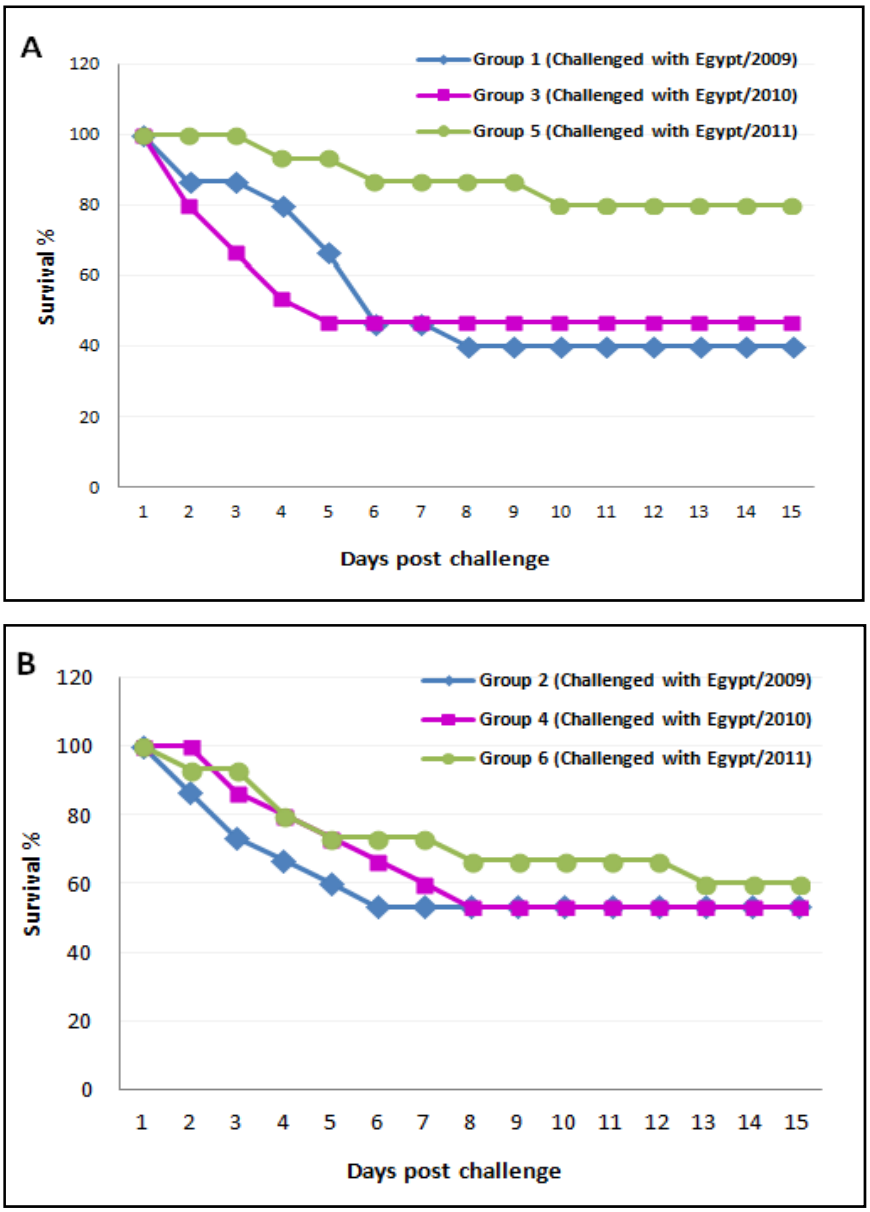

Figure 1: Survival of vaccinated chickens (n = 15 per group) after challenge with HPAIV isolated during 2009-2011, Sharkia, Egypt. A) Vaccinated chickens with vaccine program I "Trovac, rFP-AI-H5" vaccine at one day old and revaccinated with "Re-5, Reassortant inactivated H5N1" vaccine at 10 days old. B) Vaccinated chickens with vaccine program II "Volvac inactivated H5N2 at one and 10 days old"

Replication of HPAI viruses in vaccinated chickens: There was no evidence of the chickens being infected with avian influenza as oropharyngeal and cloacal swabs collected at the beginning of the experiments tested negative by virus isolation. No virus was isolated from birds in group 5 (vaccinated with vaccine program I and challenged with Egypt/2011). However, the same challenged virus was isolated at day 5 from program II vaccinated birds (Table 2) as tested by virus isolation on ECEs and identification by RT-PCR. Moreover, the virus was isolated from the respiratory and intestinal tissues of the vaccinated chickens (Data not shown).

Table 2
Virus isolation from oropharyngeal and cloacal swabs following challenge of vaccinated chickens

Challenge virus	Nil vaccine[a]		Vaccinated with program I		Vaccinated with program II	
	Day 2	Day 5	Day 2	Day 5	Day 2	Day 5
Egypt/2009	-/- [b]	-/-	-/+	-/+	+/-	-/-
Egypt/2010	+/-	-/-	+/-	-/+	-/-	-/+
Egypt/2011	-/+	-/-	-/-	-/-	-/-	-/+

[a] Previously published results (EL SISI M.A. et al., 2013)
[b] Virus isolation from pharynx/cloaca

Serologic Responses to Commercial Avian influenza Vaccines: The immunogenicity of the vaccines was examined by determining serum HI antibody titers in vaccinated chickens (Figure 2). There was no statistical difference between birds within the same group, but there were significant differences ($p < 0.05$)between groups vaccinated with program I and II. Before challenge at 21 days old, the vaccinated birds with program I induced relatively low geometric mean titer (GMT=8) compared to birds in vaccine program II (GMT=11.3).

DISCUSSION

Broiler production is the main economic pillar in the Egyptian poultry industry (ABDELWHAB E.M. and HAFEZ H.M., 2011). Protection of broilers is essential to combat HPAI in Egypt. Vaccination, a supportive tool in AI virus control strategies, was implemented to limit the spread of H5N1 and minimize their economic losses (LEE C.W. and SUAREZ D.L., 2005). In this study, the efficacy of two commercially applied AI vaccine regimens commonly used in Egypt was investigated in commercial broiler chickens challenged with HPAIV. Vaccine efficacy was compared in terms of reduction in mortality, clinical symptoms and virus replication in the trachea and cloaca.

The application of vaccine programs I and II induced protection rates up to 73.3% and 60%, respectively against HPAI H5N1 field viruses. TAHA M.M. et al. (2009) reported that the using of rFP-AI-H5 vaccine alone is not suitable to protect poultry flocks in Egypt against the circulating AIV. The vaccine program I achieved a significant high protection (73.3%) against Egypt/2011. This could be attributed to the priming with recombinant vaccine at one-day-old and boostering with inactivated vaccine "Re-5" at 10-day-old which is comparable to the findings of VEITS J. et al. (2006) who confirmed that live recombinant vaccines has ability to rapidly form adequate immunity. In Egypt, OMAR L.M. et al. (2011) reported that 88.2% and 94.1% from challenged chickens vaccinated with “rFP-AI-H5-Scotland then inactivated H5N2” or rFP-AI-H5-Ireland then inactivated H5N2, respectively were protected.

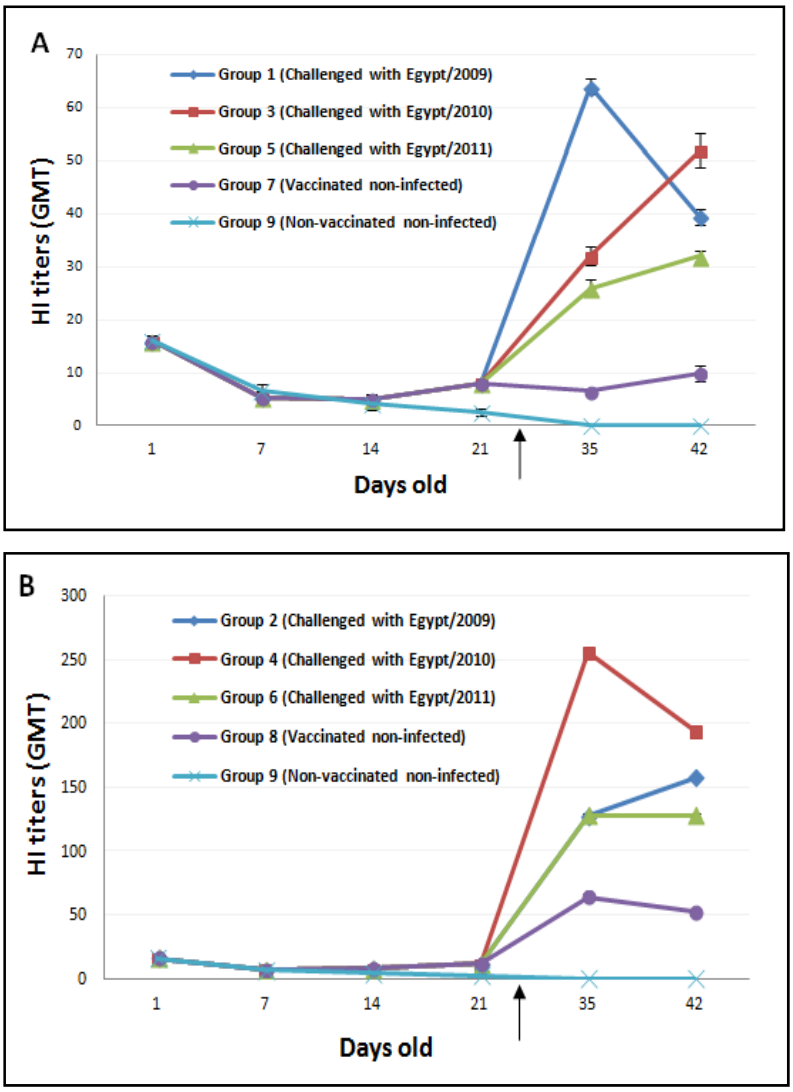

Figure 2: Cross-reactive antibody titers of chickens vaccinated with commercial avian influenza vaccines as measured byhemagglutination inhibition (HI) assay. A) Vaccinatedchickenswith vaccine program I "Trovac, rFP-AI-H5" vaccine at one day old and revaccinated with "Re-5, Reassortant inactivated H5N1" vaccine at 10 days old. B) Vaccinated chickens with vaccine program II "Volvacinactivated H5N2 at one and 10 days old". Bar indicates SD of five sera per group. Arrow indicates challenge day (25 day old).

On the other hand, the same vaccine program I produced less protection 40% and 46.7% against Egypt/2009 and 2010, respectively with shedding challenge viruses from trachea and cloaca. This could be attributed to the degree of sequence similarity between vaccine and challenge virus (SWAYNE D.E. et al., 1999 and 2000). Comparably in Egypt, TAHA M.M. et al.(2009) confirmed the low protection percentage of using rFP-AI-H5 vaccine; 40% against 2006 strain and 0% against 2007 strain was attributed to the lower identity percentage of HA sequencing (87% and 85%) between the two Egyptian field strains and vaccinal strain.

The second program "Volvac at one and 10 days old" succeeded to protect 53.3%- 60% against HPAI H5N1 challenge viruses which similar

toBOGOR G. (2009). However, this program did not prevent virus shedding via both respiratory and intestinal tracts. Proper vaccination scheme with killed vaccines seemed not sufficient (POETRI O. et al., 2011; SWAYNE D.E. and KAPCZYNSKI D, 2008; VEITS J. et al., 2008). In contrast, TIAN G. et al. (2005) have demonstrated that the vaccine, produced by reverse genetics to express an H5 antigen from Asian H5N1 virus, can protect field ducks and geese from mortality and morbidity, with reduced shedding of the challenge virus.

A key factor of successful vaccine usage for eradication of AIV is to reduce the spread of virus (VAN DER GOOT J.A. et al., 2005). In the current experiment, no virus was detected in oropharyngeal or cloacal swabs in protected birds. Similarly homologous vaccine program applied by KYDYRBAYEV Z.K. et al. (2010) succeeded to prevent virus shedding in tracheal and cloacal swabs in vaccinatedchickens.These results indi-cated that the vaccine could prevent the virus shedding of Egypt/2011, but not Egypt/2009 and Egypt/2010. Similar finding was reported by LEE C.W. et al. (2004) in Mexico with H5N2 AIV and PARK K.J. et al. (2011) in Korean with H9N2 AIV, whereby the vaccine was unable to prevent virus shedding when chickens were challenged with antigenic drift isolates. In the future, new vaccine strategies to induce cross protection against HPAIV may overcome these limitations and increase the value of vaccination (ABDELWHAB E.M. et al., 2011).

It is believed that induction of an antibody response is critical for protective immunity against many pathogens, such as viruses. Serological monitoring of H5 vaccinated flocks by the HI test using the homologous vaccine antigen is a routine laboratory procedure to evaluate vaccination efficacy of poultry (HAFEZ M.H. et al., 2010). However, in this study, both vaccine regimens did not induce substantial HI antibody response in the vaccinated chickens, although vaccine program I provided good protection against Egypt/2011. Previously it was reported that titers >4 log2 have been declared to be an indicator for clinical protection and titers >6 log2 for prevention of viral shedding (TIAN G. et al., 2005; KUMAR M. et al., 2007). Contrary to these findings, the relatively lower HI titers reported here in both vaccine programs is indicative to low correlation between HI titers and protection against challenge viruses as explained by (POETRI O. et al., 2011) who reported that vaccinated birds with low or undetectable antibody titres were protected against disease and mortality, but infection and transmission still occurred.

The mechanism of protection in the absence of measurable antibody remains an inquiry.In ferrets immunized with an inactivated whole-virus H5 vaccine (derived by implementing reverse genetics) containing the HA and NA genes of subtype H5N1 virus and the internal genes of subtype H1N1 virus, almost no detectable HI antibody response was detected, although the vaccine provided protection against a lethal A/Vietnam/1203/04 (H5N1) virus challenge (GOVORKOVA E.A. et al., 2006). An additional explanation is that the protection offered by the H5 influenza virus vaccines in absence of detectable antibodies may be induced by cell-mediated immune responses rather than by antibody-mediated protection (EPSTEIN S.L. et al., 1998; KIM J.K. et al., 2008). The antibody-mediated immune responses are mostly effective against homologous strains of influenza A viruses as they target external viral coat proteins. In contrast, T-cell-mediated immune responses can

be effective against both homologous and heterologous viral strains, since the responses can target more conserved internal proteins. This property gives vaccines that induce protective cellular immune responses the potential to protect against heterologous viral strains (THOMAS P.G. et al., 2006).

In conclusion, Priming with recombinant "rFP-AI-H5" vaccine at one day old and boostering with Re-5" Reassortant inactivated H5N1"at 10 days old succeeded in protection more than 70% of birds as well as prevention of virus shedding. Vaccination with inactivated vaccines alone seems to be not sufficient even with two successive doses. The follow up of HI results pre and post challenge evidenced irrelevance between the GMT level and protection against field virus challenge. Even so, enforcement of biosecurity measures plus elimination of infected birds remain a critical point in controlling avian influenza virus infections. Overall, these results could be utilized in recent AIV vaccine design with a prospective feature of dual benefits of reassortant and recombinant vaccine in one product.

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