Avian Pathology
Publication details, including instructions for authors and subscription information: http://www.tandfonline.com/loi/cavp20

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Accepted author version posted online: 23 Sep 2014.

To cite this article: Walid Kilany, Gwenaelle Dauphin, Abdullah Selim, Astrid Tripodi, Mohamed Samy, Heba Sobhy, Sophie VonDobschuetz, Marwa Safwat, Mona Saad, Ahmed Erfan, Mohamed Hassan, Juan Lubroth & Yilma Jobre (2014): Protection conferred by recombinant HVT avian influenza (rHVT-H5) vaccine in rearing period in two commercial layer chicken breeds in Egypt, Avian Pathology, DOI: 10.1080/03079457.2014.966302
To link to this article: http://dx.doi.org/10.1080/03079457.2014.966302

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Protection conferred by recombinant HVT avian influenza (rHVT-H5) vaccine in rearing period in two commercial layer chicken breeds in Egypt

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Received: 31 July 2014
Abstract

The effectiveness of recombinant turkey herpes virus with avian influenza (A/swan/Hungary/4999/2006(H5N1)) clade 2.2 virus (rHVT-H5) vaccine was evaluated in two layer chicken breeds (WB and BS). One dose of rHVT-H5 vaccine was administered at day 1 and birds serologically (HI test) and virologically monitored for 19 weeks. Maternally-derived antibody (MDA) and post-vaccination H5 antibody titers were measured using the Chinese (A/Goose/Guangdong/1/96(H5N1)) and the Egyptian (A/chicken/Egypt/128s/2012(H5N1)) HAs as antigens. The challenge was conducted at 19 weeks of age and on six experimental groups (I(WB) and II(BS), both vaccinated and challenged); (III(WB) and IV(BS), both vaccinated but not challenged); (V and VI, are unvaccinated SPF chickens, serving respectively as positive and negative controls). The challenge virus was the clade 2.2.1 HPAI H5N1 A/chicken/Egypt/128s/2012 at the dose of $10^6$ EID$_{50}$.

For both breeds, complete MDA waning occurred at the age of 4 weeks. The immune response to rHVT-H5 vaccination was detected from the 6th week. The seroconversion rates for both breeds reached 85.7%-100% on the 8th week of age. Protection levels of 73.3%, 60% and 0% were respectively recorded in Groups I, II and V. No mortalities occurred in the unchallenged groups. Group I showed superior results for all measured post-challenge parameters. As conclusion, a single rHVT-H5 hatchery vaccination conferred a high level of protection for a relatively extended period. This vaccine could be an important tool for future A/H5N1 prevention/control in endemic countries. Further studies on persistence of immunity beyond 19 weeks, need for booster with inactivated vaccines, breed susceptibility and vaccinal response, and transmissibility are recommended.
Introduction

In February 2006, a combination of control measures including AI vaccination, stamping-out, quarantine, and movement control were used to contain the first wave of HPAI outbreaks in poultry in Egypt. Due to several factors, these attempts were unsuccessful in terms of limiting the spread of the disease in the country. Highly pathogenic avian influenza (HPAI) H5N1 virus is now endemic in Egypt and continues to pose a significant economic and public health threat (Aly et al. 2006). Initially, emergency vaccination was used to protect breeders (grand-parents and parent flocks) but, as the disease spread to most Governorates, the decision was made in March 2006 to vaccinate all commercial flocks in the country (Abdelwhab & Hafez, 2011).

Subsequently, mass AI vaccination of household poultry started in May 2007 and was implemented by the public veterinary services. A study conducted by FAO, however, showed that AI vaccination in household poultry had only limited impact due to low coverage and low flock immunity levels (Peyre et al. 2009). In 2009, through a stakeholder consultative process, an AI vaccination plan with an exit strategy was developed for both the household and commercial poultry production sectors (Peyre et al. 2009). However, due mainly to a too weak reinforcement of the capacity of the public veterinary services, the strategy could not be fully implemented. At present, AI vaccination is still widely practiced in the commercial poultry sector but with little or no post-vaccination monitoring. The household AI vaccination was stopped since 2010.

However, without adequate explanation, the veterinary services have recently (2014) resumed AI vaccination in household poultry sector using inactivated vaccines in localized areas with confirmed HPAI outbreaks.

Turkey herpes virus (HVT) has been extensively used as a vaccine against Marek’s disease, and is usually administered at day old. Recently, CEVA Santé Animale® developed a
new live cell-associated rHVT-H5 (A/swan/Hungary/4999/2006 (H5N1)) vaccine, which in theory is capable of break through passive immunity from both the vector (HVT) and the insert (H5) and is consequently applicable at the hatchery (Rauw et al. 2012a). This guarantees vaccination of almost all hatched birds (Kapczynski et al. 2010; Rauw et al. 2011) and thus increases the vaccination coverage at flock level. In addition, the administration does not require vaccinators entering into poultry production premises which at times lead to serious biosecurity breaches. The rHVT-H5 vaccine confers both humoral and cellular immunity following single administration (Rauw et al. 2011). Besides, laboratory trials have shown protection against both HPAI and the immunosuppressive effect of Marek’s disease. Conversely, classical inactivated vaccines confer only partial protection after single administration (Abdel Hakim et al. 2013; Kilany et al. 2010) and therefore require booster injections (Abdelwhab et al. 2012b). This sub-optimal immune response may favour viral mutation and antigenic drift of field viruses from the vaccine strain (Hafez et al. 2010; Cattoli et al. 2011; Grund et al. 2011; Abdelwhab et al., 2012a).

More than 20 inactivated vaccines, derived from H5N1 or H5N2 strains, are currently licensed and marketed in Egypt. These vaccines are often used in the commercial poultry sector (Abdelwhab & Hafez, 2011). Several studies on currently used inactivated H5 vaccines under Egyptian field conditions indicated limited effectiveness (Kilany et al. 2011; Abdelwhab et al. 2012; Kilany et al. 2013). HPAI H5N1 outbreaks were recorded in many vaccinated broiler, layer breeder and turkey flocks (Hafez et al. 2010; Kilany et al. 2010; Abdelwhab et al. 2012).

Since 2007, different reference and national laboratories in Europe, Asia, USA and Egypt have evaluated rHVT-H5 under laboratory and field conditions using specific pathogen free (SPF) and commercial broilers chickens (De Vriese et al. 2009; Kapczynski et al. 2010; Rauw et
al. 2011, Kaspczynski et al. 2012; Kilany et al. 2012; Rauw et al. 2012a, b; and Retno et al. 2012). The results obtained from these studies indicated that the rHVT-H5 vaccine is able to confer good protection against different HPAI H5N1 viruses and clades, and that it is able to overcome the neutralizing effect of MDA against H5 (Rauw et al. 2012a).

In September 2012, a rHVT-H5 vaccine (Vectormune-AI, CEVA) was registered and commercialised in Egypt. In 2013, this rHVT-H5 vaccine was used in 53 million broilers and 7 million layer chickens in the country. In Egypt, hatchery vaccination against H5N1 is now considered as a potential tool, combined with other disease control measures, in effectively controlling enzootic HPAI H5N1. However, very limited evaluations have been made to measure its efficacy and effectiveness under Egyptian field conditions and in different poultry production and farming systems (Kilany et al. 2012). The present study was conducted to fill some of the prevailing knowledge gaps about rHVT-H5 and assess its effectiveness in local field conditions. The study was specifically designed to monitor the post-vaccination serological response of a single dose of rHVT-H5 vaccine in two layer chicken breed flocks before their egg-laying (rearing period) age and evaluate protection conferred against challenge with A/H5N1 clade 2.2.1 (A/chicken/Egypt/128s/2012), the dominant virus strain currently circulating in Egypt (NLQP-FAO, 2014 unpublished surveillance data).

Materials and methods

The present study consisted of an on-farm monitoring and a controlled challenge experiment.

On-farm monitoring. Study location and animals. The study was conducted in a commercial layer chicken farm located in Giza Governorate, Egypt, where flocks of two layer breeds (White
Bovans (n=43,104) and Brown Shaver (n=10,000) were raised under similar husbandry and health care conditions. Both breeds were obtained from parent flocks that had received AI vaccinations using the inactivated re1 H5N1 vaccine at 2, 8, 16, 22 and 40 weeks of age. Farm biosecurity measures are not rigorously and consistently applied. The farm has an annual production capacity of about 20 million table eggs. All birds in the study farm were vaccinated only once at day-old against H5 using rHVT-H5 vaccine (Serial No.: 395-012; Virus titer: 3.8x10^3PFU/dose, 0.2 ml/ chick S/C). The flocks were also vaccinated against other prevalent poultry diseases ((Newcastle disease (ND), infectious bronchitis (IB), infectious bursal disease (IBD), Infectious Coryza (IC), infectious laryngotracheitis (ILT), and fowl pox (FP)).

**Sampling procedures and serological monitoring.** Following the standard procedures for sero-monitoring (OIE, 2013), individual blood samples from twenty-eight randomly selected birds from each breed were, collected at week 1 (day 1), 2, 4, 6, 8, 16, 18, and 19, and sera were harvested, identified and stored at -20°C until testing. Serum samples were tested by: i) N1 ELISA (ID Screen® AI N1 antibody competition for neuraminidase NI antibody detection in order to assess possible A/H5N1 infection in the study flocks; ii) haemagglutination inhibition (HI) tests (OIE, 2012) in order to measure the A/H5N1 antibody profile during the study period. MDA and post-vaccination H5 antibody (against rHVT-H5 vaccine) titers were respectively determined, respectively using homologous Re1-H5N1 Chinese HA antigen (A/Goose/Guangdong/1/96 (H5N1)) (YP_308669) and A/H5N1 (2012) Egyptian HA antigen (A/chicken/Egypt/128s/2012 (H5N1)) (JQ858485) to rHVT-H5 vaccine. The similarity of the later virus with the H5 insert was confirmed by a prior antigenicity and cross HI test done at NLQP. Arithmetic means of HI titers were expressed as reciprocal log2 and inhibition of
haemagglutination at a dilution of $\geq 2^3$ were considered as specific antibody positive to AIV (Rauw et al. 2012a). The sero-conversion rates from both antigens were estimated as the proportion of birds with positive HI titers and were calculated using the following formula:

$$\text{Sero-conversion rate (\%)} = \frac{\text{Number of positive birds with HI titers} \geq 2^3}{\text{number of birds tested}} \times 100.$$

**The Challenge Experiment.** Prior to trial initiation, oropharyngeal and cloacal swabs were collected at 17th and 19th weeks of age from 25 randomly selected chickens from each of the two breeds. The samples were tested by rRT-PCR for common poultry viral infections in Egypt (VNDV, IBV, A/H5N1 and A/H9N2).

*The challenge virus.* The challenge virus used in the present study was A/Chicken/Egypt/128s/2012 (H5N1), which represents currently circulating HPAI H5N1 clade 2.2.1 viruses in Egypt. The challenge was administered by intra-nasal inoculation at a dose of $10^6$ EID$_{50}$ in 100 µl/chicken.

*Experimental animals and challenge protocol.* At the age of 17 weeks, 25 randomly selected and apparently healthy chickens from each of the two breeds in the study farm as well as 20 SFP chickens raised in a separate specialized farm were transferred to the animal facilities at NLQP. These birds were kept for 2 weeks in biosafety and biosecurity level 3 (BSL-3) chicken isolators in order to allow proper acclimatization prior to the initiation of the challenge experiment. Their health status was monitored and freedom from locally prevalent viral infections tested by rRT-PCR. The experiment was conducted according to the NLQP (2013) guidelines on research ethics in animals.
The challenge experiment was carried out on chickens aged 19 weeks and birds allotted into six groups:

Groups I and II comprised 15 White Bovans and 15 Brown Shaver chickens, respectively, both vaccinated with rHVT-H5 at day-old and experimentally challenged with A/Chicken/Egypt/128s/2012 (H5N1).

Groups III and IV consisted in 10 White Bovans and 10 Brown Shaver chickens, respectively, vaccinated with rHVT-AI vaccine as DOCs but were not experimentally challenged.

Groups V and VI, each consisted of 10 unvaccinated SPF chickens. Group V was challenged with A/Chicken/Egypt/128s/2012 (H5N1) and served as positive control, while Group VI was neither vaccinated nor challenged and hence served as negative control.

The challenge experiment was considered valid if the mortality rate within 5 days post challenge (dpc) in the positive control group (Group V) was ≥80%. Experimental birds were monitored daily for 14 consecutive dpc.

**Real Time RT-PCR Assay.** Tracheal swabs from individual chickens were separately collected and tested. Detection and quantification of influenza type A matrix gene were conducted from tracheal swabs by rRT-PCR test according to Spackman, et al. (2002). RNA extraction was performed according to the manufacturer’s recommendations using the QIAamp viral RNA Mini kit (Qiagen, Germany, GmbH). Moreover, gene amplifications for VNDV, IBV and A/H9N2 were conducted according to procedures, respectively, described by Wise et al. (2004), Meir et al. (2010), and Ben Shabat et al. (2010). Genomes detection and result analysis were done in a Stratagen MX3005P machine to amplify DNA.
Excreted virus, AIV matrix gene specific RNA, were quantified in a Taqman rRT-PCR. An absolute quantification was done relatively to a standard curve based on tenfold dilution of an in vitro transcribed RNA template of the challenge virus. A threshold (Ct) value of 40 was selected as the cut-off point between positive and negative results; therefore samples with higher than 40 Ct value were considered as negative for AIV according to the standard curve.

**Measured parameters.** The parameters measured in the challenge experiment include clinical onset and presentation, duration of the patent period, virus load at 3, 6, 10 and 14 dpc, number and proportion of shedder birds, and daily mortality. The virus load was expressed as the mean daily number of copies of M gene/ml in swab sample ($\log_{10}$ PCR copies). The mean virus load shed per group was calculated only for live positive shedders per group per day. The proportion of shedder birds was calculated as the percent of shedder birds from the total surviving birds in the group during the specified sampling occasion. The level of protection (survival rate) conferred by rHVT-H5 vaccine was computed as the proportion of surviving birds after challenge.

**Data management and statistical analysis.** Data collected from the farm and challenge experiment were collated, and statistical analysis was conducted using SPSS 21 (IBM, 2010). For the farm study, the variations within and between the White Bovans and Brown Shaver breeds in mortality rates, mean antibody titers, sero-conversion rates, as well as the HI titers obtained with the Chinese and Egyptian strain antigens were compared. In the challenge experiment, the overall group mortality was calculated based on daily mortality counts. The variation in mortality, mean daily virus shedding ($\log_{10}$) and percent of shedders between groups were
compared using paired T- and bivariate correlation tests, as appropriate, and \( p \leq 0.05 \) was considered statistically significant.

Results

**On Farm monitoring.** *Morbidity and mortality rates.* During the 19 weeks monitoring period, no overt clinical signs suggestive of H5N1 HPAI were observed in the study flocks. The overall flock mortality rate was 17.52% and no statistically discernible variation (\( p > 0.05 \)) was observed between the White Bovans (14.2%) and Brown Shaver (17.9%) breeds. The weekly mortality rate in both breeds remained below 1% during the entire study period with the exception of weeks 1, 2, 5, 9 and 13. Indeed, during the first two weeks of life and on week 13, the weekly mortality rate in Brown Shaver breed was significantly higher (\( p < 0.05 \)) than their White Bovans counterparts. Conversely, significant higher weekly mortality rates were recorded in White Bovans breed what could be partly linked to the IBD vaccination at 5-6 weeks of age, and partly to the confirmed occurrences of IBV and A/H9N2 infections on the 9th week of age (Table 1).

**Serological findings.**

a. **N1 ELISA**

All serum samples tested by N1 ELISA were negative and confirm that no A/H5N1 infection occurred in the flocks during the 19 weeks of the field study period.

b. **Maternally-derived antibody (MDA) titers against H5**

By using the homologous Chinese strain antigen (Ag) in the HI test, the mean MDA titers on the first day were \( 8.84 \pm 0.9 \) and \( 6.7 \pm 1.04 \) in White Bovans and Brown Shaver breeds, respectively. The MDA titers during the first two weeks of life were significantly higher (\( p < 0.05 \)) in the White
Bovans than in the Brown Shaver birds. The results obtained by using the Egyptian strain as Ag showed that MDA mean titers in the first week of age were 5.8±0.9 and 3.7±0.9 in White Bovans and Brown Shaver breeds, respectively. The difference between breeds in weekly mean HI titers was significant (p<0.05) at weeks 1 and 2. In both layer breeds, the HI results fusing the Chinese as well as the Egyptian strain antigens showed that the MDA decay was completed and titers reached zero level at 4 weeks of age (Table 2).

c. Post-vaccination H5 antibody titers
The post-vaccination immune response, measured using the Egyptian strain Ag, showed that, in both breeds, an antibody response to H5 was detected at the age of 6 weeks. The mean HI titer then consistently increased up to the age of 19 weeks to reach 7.8±1.1log2 and 7.3±1.3log2 in White Bovans and Brown Shaver breeds, respectively. Statistically significant breed differences (p<0.05) in weekly mean HI titers were depicted only in weeks 6 and 8 (Table 2). Similarly, the HI results assessed by using the Chinese strain Ag showed that the post-vaccination immune response to rHVT-H5 vaccine was detectable at 6 weeks of age in both breeds, but with relatively weaker mean titer values (0.7±1.2 and 1±2 for White Bovans and Brown Shaver breeds, respectively, as compared with the results from the tests performed using the Egyptian strain Ag. In addition, in both layer breeds, the Egyptian strain Ag, gave significantly higher HI mean titers at weeks 6, 8, 16, 18 and 19 as compared to the Chinese strain Ag (Table 2).

d. Sero-conversion rates
For the Chinese and Egyptian antigens, the sero-conversion rates at the 8th week of age were 82.1 and 75% (in White Bovans), and 85.7 and 100% in Brown Shaver breeds respectively. Statistically significant breed differences (p<0.05) in weekly seroconversion levels were observed at the age of 6 and 8 weeks for the Egyptian antigen. As for the Chinese strain Ag, a
statistically significance (p<0.05) breed variation in seroconversion rates occurred at weeks 2 and 16 (Table 3, Fig. 1). Within breed variation in the post-vaccination seroconversion levels was significant at 6, 8 and 16 weeks for the Brown Shaver breed, whereas no significant difference was depicted within the White Bovans breed (Table 3).

**The challenge experiment.** The results of pre-challenge virological monitoring conducted on weeks 17 and 19 confirmed that all experimental birds were free of field A/H5N1, A/H9N2, NDV and IBV infections.

*Morbidity and mortality.* In experimentally challenged groups, clinical signs suggestive of H5N1 HPAI infection occurred as early as the 1\(^{st}\)dpc (Group V), 3\(^{rd}\)dpc (Group II) and 6\(^{th}\)dpc (Group I). None of the unchallenged birds in Groups III, IV and VI did manifest clinical signs suggestive of HPAI. The overall specific mortality rates for Group I, II and V were 26.7%, 40% and 100%, respectively. Apparently healthy birds in Groups I and II survived and did not shed any virus during the course of the study. There was no mortality in unchallenged birds of Groups III, IV and VI. The protection rates for Group I, II and V were 73.3%, 60% and 0%, respectively. There was a statistically significant breed difference (p<0.05) in post-challenge protection levels, with Group I (White Bovans) showing superior results (Table 4).

**Virus shedding.** As shown in Table 5, shedding in Group I occurred between the 6\(^{th}\) and 9\(^{th}\)dpc. In Group II, shedding started earlier, on the 3\(^{rd}\)dpc, and extended up to the 10\(^{th}\)dpc. In Group V, shedding also started on the 3\(^{rd}\)dpc and continued until all birds died on the 5\(^{th}\)dpc. The mean virus load shed by Group I, II and V ranged between 2 to 5; 2.6 to 4.8 and 3.7 to 4.5,
respectively. The results clearly indicated that Group I birds shed relatively lower amount of virus and for a shorter duration as compared with Group II birds with earlier start and extended shedding (Fig. 2). The mean number of virus copies shed by Group II and Group V was significantly higher (p<0.05) than that for Group I. On the other hand, there was no statistically significant variation (p>0.05) in the mean number of virus copies between Groups II and V. Moreover, the amount of virus shed by both Groups I and II was lower (by 2 logs) than in Group V. A similar trend was also observed in the proportion of shedder birds. In Group I, 4/15 (26.7%) birds, in Group II 6/15 (40%) and in Group V, all birds (100%) shed virus. All birds shedding HPAI H5N1 virus died, indicating viremia due to the challenge (Table 5). A significant correlation (p<0.05) was depicted between the amount of excreted virus and the number of shedder birds in Group I.

Variations within the Challenged (I, II, V) groups. For Groups I, II and V (challenged birds), HI titers measured prior to challenge (19 weeks of age), and other post-challenge parameters measured are summarized for birds with and without clinical signs and is presented in Table 6.

There was no statistically significant (p>0.05) variation in the overall mean HI titer between Group I (7.7±0.9) and II (7.3±1.6). Similarly, there was no statistically significant (p>0.05) variation in the mean HI titer values between those birds in Group I with (7.3±1) and without (7.8±0.9) clinical signs. Conversely, the mean HI titers of birds in Group II with clinical signs (6.0±1.7) were significantly lower (p<0.05) than those without any clinical manifestation (8.2±0.8). Similarly, the birds with clinical manifestation in Group II have significantly lower (p<0.05) mean HI titers (6.0±1.7) than those with clinical signs in Group I (7.3±1) (Table 6).
In general, only 4/15 (26.7%) and 6/15 (40%) of the birds in Groups I and II, respectively, and 10/10 (100%) of those in Group V manifested clinical symptoms suggestive of H5 HPAI. All birds that manifested clinical illness invariably died from the disease. There was no statistically discernible difference (p>0.05) in mean of clinical signs between Groups I and II. But, the appearance of clinical pictures took significantly shorter (p<0.05) duration (2 dpc) for Group V as compared to the other two challenged Groups (I and II). The mean patent period, death time (MDT) and virus shed significantly varied (p<0.05) between the three challenged groups, with Group I showing superior results (longer patent period and MDT and lowered virus load), and Group V worst results in all these parameters (Table 6).

Discussion

Vaccination is an important tool in the protection of poultry against avian influenza (AI). For field use, the overwhelming majority of AI vaccines produced are inactivated whole virus formulated into an oil emulsion and to a lesser degree recombinant vectored vaccine (meaning viruses expressing AI genes) (Kapczynski, et al. 2010). In endemic H5 HPAI countries, such as Egypt, however, the use of classical inactivated vaccines is hampered by i) the interference with MDA in farms where breeding stocks are systematically vaccinated; ii) the frequent antigenic drift of the AIV that requires continuous updating of the vaccines to keep up the antigenic match and corresponding efficacy; iii) the poor quality of vaccine application at farm level and heterogeneous vaccine coverage of the poultry populations at risk; and iv) the limited duration of the induced immunity and, therefore, the necessity for booster vaccinations (FAO, 2013). Recent advances in vaccine manufacturing, using recombinant technology, has led to the availability of avian influenza vaccines for administration to day-old chicks (DOCs) at hatcheries. The avian
influenza recombinant vector vaccines commercialized in recent years include: three fowl pox vectored vaccines (r’FP-H5), two Newcastle disease virus vectored vaccines (r’NDV-AI/r’L-H5) and a herpes virus vectored vaccine (rHVT-H5) (FAO, 2013). Application of recombinant hatchery vaccines is labour saving and cost effective (Tripathy & Schnitzlien, 1991). Several studies demonstrated the potency of NDV-HA vaccines expressing either modified or native H5 or H7 AIV glycoproteins (Römer-Oberdörfer et al. 2008). Ramp et al., (2011) reported the development of NDV as a vaccine vector for avian influenza, including HPAI H5N1; clinical protection against both H5N1 and NDV after single dose immunization with r’NDV-AI in chickens was common (Römer-Oberdörfer et al. 2008). This system can protect against both NDV and AIV infection in poultry (Schroer et al. 2009; Geet al. 2010). However, genetic matching of the vaccine to currently circulating viruses was shown to be critical (Römer-Oberdörfer et al. 2008; Lardinois et al. 2012). Swayne et al. (2000), however reported the ineffectiveness of recombinant hatchery vaccines using r’FP–H5 due to prior exposure or vaccination of breeders with Fowl pox virus (FPV) alone and therefore interference with MDA against the vector virus leading to vaccination failure. The highest the homology between the H5 gene in the commercially used AI vaccine (included in the rHVT-H5 and the inact-H5N1) and the circulated viruses, the highest the protection afforded from those vaccines (Grund et al. 2011; Rauw et al. 2012; Abdelwhab et al. 2012b, Kilany et al. 2012; Kilany et al. 2013).

So, the theoretical potential interest of producing a tailor-made rHVT-H5 vaccine to possibly increase the level of protection is still worth investigating (Rauw et al. 2012b). However, this is difficult due to the genetic and antigenic continuous variations of the AIV strains in endemic countries such as Egypt (Cattoli et al. 2011; Abdelwhab et al. 2012a).

The same reason also explains the failures recorded with classical inactivated vaccines and
the persistence of the infections (Hafez et al. 2010; Kilany et al. 2010). The lack of prior studies on rHVT-H5 vaccine effectiveness in layer chickens makes it difficult to draw comparison with the results of the present study. Unfortunately, sentinel birds were not used in the present study although transmission of virus from non-protected birds could still happen, representing one of the limitations in this study. However, the results from the present 19 weeks farm monitoring study on layer chickens vaccinated once, as DOCs, with rHVT-H5 vaccine, showed that both the overall and weekly mortality rates were within the acceptable (≤1%) range in the context of the Egyptian poultry production system. Besides, the results of N1 ELISA for anti-neuraminidase antibody detection showed negative results for A/H5N1. Therefore, the results from both clinical and serological monitoring strongly suggest the absence of any H5 HPAI infection in the study farm during the entire 19 weeks monitoring period.

In principle, rHVT-vectored vaccines could reduce the negative impact of MDA interference during early stages, thus facilitating and accelerating the appearance of an effective and active immune response. In the present study, for both White Bovans and Brown Shaver breeds, the MDA titers against H5 obtained by using Chinese Stain Ag was higher as compared with the results obtained by using Egyptian Stain Ag. This could be explained by the fact that the birds used in the present study originated from parent stocks that had received over four doses of re1 H5N1 inactivated Chinese vaccine. Abdelwhab et al. (2012b) have also previously observed that high MDA seropositive titers in DOCs were derived from breeder flocks that receive repeated vaccinations with inactivated H5 vaccines. In general, irrespective of the antigen used, the MDA titers in the White Bovans breed were significantly higher than those in the Brown Shaver breed. This could possibly be related to the variation in the egg laying stages and
vaccination schedules applied in the respective breeder flocks of the two breeds, or to genetic differences with respect to the antibody response to this particular antigen.

The interference of MDA with the onset of immunity has been claimed to further jeopardize efforts to achieve protection of broiler chicks (De Vriese et al. 2010; Maas et al. 2011; Abdelwhab et al. 2012b). Our findings showed a high (8.84 log₂) to moderated (6.7 log₂) MDA titers in White Bovans and Brown Shaver breeds, respectively. These MDA levels seem to have delayed the post-vaccination humoral immune response by up to 6 weeks in Brown Shaver and 8 weeks in White Bovans breeds. As indicated in Fig. 1, this interference seems rather relevant and may have inhibited the replication of the vectored vaccine and subsequently delayed/suppressed the early post vaccination immune response up to 6-8 weeks of age. On the 6th week, significantly higher mean post-vaccination HI titer value (3.3 log₂) was obtained in Brown Shaver breed than for the White Bovans breed (1.8 log₂). Besides, the sero-conversion rate was significantly lower (17%) in the White Bovans breed as compared to their Brown Shaver (67.9%) counterparts. The breed variations in post-vaccination response and seroconversion rates disappeared in the following weeks. The results thus suggest that the higher the MDA titer at day old, the greater the relative delay in the post-vaccination antibody response. The studies of De Vriese et al. (2009); Retno et al. (2010) and Kilany et al. (2012), however indicated that rHVT-H5 has no interference with MDA. The study by Rauw et al. (2012b), indicated that MDA may have no or very low interferences that could influence the level of clinical protection. However, the level of MDA titers in DOC reported in these previous studies ranged between 4.2 to 5.8 log₂, which is lower than the levels (6.7 to 8.84 log₂) obtained in the present study. The MDA interference against the vector virus (rHVT) could not be measured in the present study mainly due to a lack of laboratory facilities. Further elaborated studies are therefore required to
determine the extent of MDA interference on both the vector vaccine and the insert as well as their impact on the effectiveness of rHVT-H5 vaccine, both in short- and long-cycle poultry farming systems. In the present study involving layer chickens, weeks 3, 4 and 5 could be considered as times with window for infection as the MDA level is declining to its lowest points and the humoral immune response just started ascending. This is an important improvement compared to the level of protection conferred by classical inactivated vaccines where the window of susceptibility is wider even after multiple vaccinations.

The post-vaccination titers steadily increased from weeks 6 to 19. Analysis of the seroconversion results indicated that there was a measurable breed difference in post-vaccination antibody response to rHVT-H5 up to the age of 8 weeks. Beyond this age, however, a 100% sero-conversion was attained in both breeds, which persisted at the same level until the end of the study period. Previous studies in Egypt in birds that received multiple doses of inactivated AI vaccines, showed HI mean titers of 6 log₂ (layer chicken) at 25 weeks of age (Hafez et al. 2010) and 7.2 log₂ (breeder chicken) at 52 weeks of age (Kilany et al. 2013). These results concur with the findings of the present study (7.8 and 7.3 log₂ in White Bovans and Brown Shaver breeds, respectively). The persistently high antibody titer observed is most likely due to the persistence of the HVT virus in the host. Rauw et al. (2012a) confirmed the long life latency nature of HVT virus in vaccinated birds. Okada et al. (1997) described that the latency is associated with CD4⁺ T cells, CD8⁺ T cells and B cells latently infected and activated.

The challenge trial showed that, at 19 weeks post-vaccination, rHVT-H5 vaccine conferred 73.3% protection in White Bovans and 60% in Brown Shaver against the high dose experimental challenge infection. This can be considered as a relatively high level of protection owing to the fact that this is a result obtained after 19 weeks of a single dose of vaccination.
Similarly, De Vriese et al. (2009); Kilany et al. (2012); Rauw et al. (2012b) reported 80 to 100% clinical protection after challenge in broilers and SPF chicks (with or without MDA) vaccinated with rHVT-H5. The extended protection capacity makes it economically feasible, particularly in long cycle poultry production systems. Indeed, repeated booster vaccinations are required for classical inactivated vaccines to be able to confer similar or comparable levels of protection. For instance, mortality rates of 27% and 43% were reported in naturally infected layer (20 weeks) and broiler breeder (32 weeks) flocks vaccinated with inactivated H5 vaccines with 3 to 4 booster doses (Kilany et al. 2010; Kilany et al. 2013). The rHVT-H5 vaccine has also been studied in short cycle (broiler) birds and confirmed its efficacy with a protection range of 90 to 100% (depending on the presence or absence of MDA) as compared with 40-70% protection using inactivated vaccine administered at 8 days of age (Rauw et al. 2012b). Retno et al. (2012) also showed 95% and 75% clinical protection in broiler birds vaccinated with rHVT-H5 at day old and H5 inactivated vaccine at 7 days of age, respectively. Walid et al. (2014) conducted two controlled studies using two different challenge viruses and compared the efficacy of 3 inactivated H5 and rHVT-H5 vaccines. In one of these trials where rg-H5N1 (Eg), H5N2 and rg-H5N3 inactivated vaccines were compared with rHVT-H5 vaccine against challenge with an Egyptian HPAIV H5N1 2011 variant virus (Clade 2.2.1.1), protection levels of 66%, 60%, 40% and 80% were respectively obtained. In the second trial where the same vaccines were compared against a challenge with an Egyptian HPAIV H5N1 2012 classic virus (Clade 2.2.1), protection levels of 13, 40 and 80% 93.3% were respectively obtained. The authors thus concluded that a high level of protection can be conferred by a single rHVT vaccination at the hatchery in the broiler production sector.
The absence of breed variation in mean HI titer indicates that the overall post-vaccination immune response to rHVT-H5 vaccine in layer chickens is good. Generally, birds with high MDA titer died in both breeds. On a closer look, however, there were significant differences between the three challenged groups (I, II and V) in post-challenge morbidity parameters (considering clinical signs, duration of patent period, onset of detectable shedding, number of shedder birds and amount of challenge virus shed). Of the two breeds, the White Bovans showed superior results than the Brown Shaver in all the above-indicated measurements. However, these parameters were not significantly influenced by the level of pre-challenge HI titres. This variation in breed response to AI vaccination is an important finding and requires further and in-depth investigations. Previous works on ducks indicate existence of species and breed variations in the pathogenicity of H5N1 highly pathogenic influenza (HPAI) (Pantin-Jackwood et al. 2013) and response to vaccination with commercial inactivated AI vaccines (Cagle et al. 2011). The breed variation observed in the present study corroborates our field observations, in particular to the preference of farmers for the White Bovans over the Brown Shaver breed mainly due to its relative ability to withstand disease challenges and better production performance. Our findings indicated that single hatchery vaccination with rHVT-H5 vaccine confers protection against A/H5N1 in commercial layer chickens, at least during the rearing period (19 weeks). Poultry farmers using rHVT-H5 vaccines often administer a booster of inactivated vaccine on the 20th week just before lay. This represents often a prudent and risk mitigation measure at an important phase of the production cycle. It would be of interest to study the extent of persistence of the high level of immunity beyond the 19 weeks of age and to assess on whether or not a boost with an inactivated vaccine is required during the production cycle in layer hens vaccinated at day-old
with rHVT-H5 vaccine. Besides, it is necessary to determine the transmissibility of infection from shedder birds in vaccinated flocks.

To sum up, hatchery vaccination using rHVT-H5 vaccine is proved to be one of the viable tools for the prevention and control of endemic HPAI H5N1 in long cycle poultry production sectors. However, it is important to understand the possible limitations with respect to breed variations in both susceptibility and responses to AI vaccination, particularly in circumstances where H5N1 HPAI is endemic and natural infection challenge is often high.
Acknowledgements

This work was supported by the United States Agency for International Development (USAID) under the grant (AID-263-IO-11-00001, Mod.#3) and in the framework of OSRO/EGY/101/USA which is project jointly implemented by FAO, GOVS and NLQP. The study was also supported by the USAID-funded global project OSRO/GLO/301/USA and implemented by FAO HQ. We express our sincere gratitude to USAID for its continued support in addressing the endemic HPAI situation in Egypt. The authors would like to extend their sincere gratitude to the owner of the layer farm and CEVA-Egypt for their willingness to collaborate in the study and various support provided including access to the farm, sample collection, and provision of production and related data. The authors are also grateful to a number of colleagues from partner organizations that provided a wide range of direct and indirect supports.
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influenza in chickens pre-immunized with a fowl pox vaccine. Avian Diseases, 44, 132-137.


Figure 1. Weekly sero-conversion ratio obtained on using Chinese rH5N1 antigen (A/Goose/Guangdong/1/96(H5N1) and Egyptian H5N1 (A/chicken/Egypt/128s/2012(H5N1) strain antigens in White Bovans and Brown Shaver breeds of layer chicken vaccinated with rHVT-H5 vaccine at day-old.
Figure 2. Mean H5N1 virus titers (PCR log_{10} copies/ml) shed by birds in challenged groups (I, II and V) based on matrix gene quantification.

Groups I= White Bovans breed, vaccinated and challenged
Groups II= Brown Shaver breed, vaccinated and challenged
Groups V= SPF chickens, unvaccinated but challenged
The numbers in the middle of the bars show the number of shedder birds at that specific sampling time.
Table 1. *Weekly mortality rates* during the 19 weeks study period in layer chicken inoculated with **rHVT-H5** vaccine at day old

<table>
<thead>
<tr>
<th>Breed</th>
<th>White Bovans</th>
<th>Brown Shaver</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=43,104)</td>
<td>(n=10,000)</td>
<td>(n=53,104)</td>
</tr>
<tr>
<td>Age (week)</td>
<td>Count</td>
<td>Rate (%)</td>
<td>Count</td>
</tr>
<tr>
<td>1</td>
<td>1070(^a)</td>
<td>2.4</td>
<td>610(^b)</td>
</tr>
<tr>
<td>2</td>
<td>798(^a)</td>
<td>1.9</td>
<td>360(^b)</td>
</tr>
<tr>
<td>3</td>
<td>135</td>
<td>0.3</td>
<td>61</td>
</tr>
<tr>
<td>4</td>
<td>77</td>
<td>0.2</td>
<td>55</td>
</tr>
<tr>
<td>5</td>
<td>1890(^a)</td>
<td>4.5</td>
<td>160(^b)</td>
</tr>
<tr>
<td>6</td>
<td>350(^a)</td>
<td>0.9</td>
<td>50(^b)</td>
</tr>
<tr>
<td>7</td>
<td>135</td>
<td>0.3</td>
<td>38</td>
</tr>
<tr>
<td>8</td>
<td>107</td>
<td>0.3</td>
<td>30</td>
</tr>
<tr>
<td>9</td>
<td>275(^a)</td>
<td>0.7</td>
<td>35(^b)</td>
</tr>
<tr>
<td>10</td>
<td>176</td>
<td>0.4</td>
<td>39</td>
</tr>
<tr>
<td>11</td>
<td>198</td>
<td>0.5</td>
<td>36</td>
</tr>
<tr>
<td>12</td>
<td>173</td>
<td>0.4</td>
<td>45</td>
</tr>
<tr>
<td>13</td>
<td>250(^a)</td>
<td>0.6</td>
<td>80(^b)</td>
</tr>
<tr>
<td>14</td>
<td>139</td>
<td>0.4</td>
<td>34</td>
</tr>
<tr>
<td>15</td>
<td>93</td>
<td>0.2</td>
<td>39</td>
</tr>
<tr>
<td>16</td>
<td>70</td>
<td>0.2</td>
<td>30</td>
</tr>
<tr>
<td>17</td>
<td>60</td>
<td>0.2</td>
<td>35</td>
</tr>
<tr>
<td>18</td>
<td>50</td>
<td>0.1</td>
<td>30</td>
</tr>
<tr>
<td>19</td>
<td>70</td>
<td>0.2</td>
<td>32</td>
</tr>
<tr>
<td>Total</td>
<td>6116</td>
<td>14.2</td>
<td>1799</td>
</tr>
</tbody>
</table>

Different lower case letters in a raw denote the presence of significant variation between breeds (p<0.05); n= Number of layer chickens per flock
Table 2. Weekly mean HI (log2± SD) titers* obtained using Chinese and Egyptian strain antigens in White Bovans and Brown Shaver breeds of layer chicken vaccinated with rHVT-H5 vaccine at day old

<table>
<thead>
<tr>
<th>Mean HI titers (log2± SD)</th>
<th>Breeding</th>
<th>White Bovans (n=28)</th>
<th>Brown Shaver (n=28)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (week)</td>
<td></td>
<td>Antigens Used for HI</td>
<td>Chinese Ag*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>b6.7±1.04c</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chinese Ag*</td>
<td>b6.7±1.04c</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Egyptian Ag**</td>
<td>0</td>
</tr>
<tr>
<td>H5 MDA</td>
<td>1</td>
<td>a8.84±0.9c</td>
<td>a5.8±0.9d</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>a4.25±1.4c</td>
<td>a3.2±1.8d</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.2±0.5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.7±1.2c</td>
<td>a1.8±1.2d</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>3.8±1.8c</td>
<td>4.7±1.6d</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>a5.1±1.6</td>
<td>6.1±1</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>6.4±1.6</td>
<td>7.8±0.8</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>a7.1±1</td>
<td>7.8±1.1</td>
</tr>
</tbody>
</table>

*Chinese re1H5N1 antigen (A/Goose/Guangdong/1/96 (H5N1)  
**Egyptian HPAI H5N1 (A/chicken/Egypt/128s/2012)

Different letters (a, b) in the left-hand side in a raw denote the presence of statistically significant differences (p<0.05) between breeds in HI titers obtained using same antigen.  
Different letters (c, d) in the right-hand side in a raw denote the presence of statistically significant differences (p<0.05) between the results performed with the two antigens within the same breed.  
Different letters in column didn’t reflect the presence of any statistical differences.
Table 3. Weekly sero-conversion rates obtained using Chinese (A/Goose/Guangdong/1/96 (H5N1) and Egyptian (A/chicken/Egypt/128s/2012(H5N1) strain antigens in White Bovans and Brown Shaver breeds of layer chicken vaccinated with rHVT-H5 vaccine at day old

<table>
<thead>
<tr>
<th>Sero-conversion rate</th>
<th>Age (Weeks)</th>
<th>Breed</th>
<th>White Bovans (n=28)</th>
<th>Brown Shaver (n=28)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Chinese Ag</td>
<td>Egyptian Ag</td>
<td>Chinese Ag</td>
</tr>
<tr>
<td>H5 MDA</td>
<td>1</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>92.8</td>
<td>78.5</td>
<td>46.4</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>rHVT-H5 Post vaccination H5 antibody response</td>
<td>6</td>
<td>17</td>
<td>17</td>
<td>17.9</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>82.1</td>
<td>85.7</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>100</td>
<td>100</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>
Table 4. Daily mortality and protection levels in birds used in the challenge experiment

<table>
<thead>
<tr>
<th>Group</th>
<th>Days post challenge</th>
<th>Total mortality count</th>
<th>Total survived animals</th>
<th>Mortality rate</th>
<th>Protection level (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D0  D1  D2  D3  D4  D5  D6  D7  D8  D9  D10  D11  D12  D13  D14</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group I (n=15)</td>
<td>0  0  0  0  0  0  0  1  0  2  1  0  0  0  0</td>
<td>4</td>
<td>11</td>
<td>26.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>73.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group II (n=15)</td>
<td>0  0  0  0  0  1  1  2  0  0  1  1  0  0  0</td>
<td>6</td>
<td>9</td>
<td>40&lt;sup&gt;b&lt;/sup&gt;</td>
<td>60&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group III (n=10)</td>
<td>0  0  0  0  0  0  0  0  0  0  0  0  0  0  0</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Group IV (n=10)</td>
<td>0  0  0  0  0  0  0  0  0  0  0  0  0  0  0</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>100&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group V (n=10)</td>
<td>0  0  0  6  3  1  0  0  0  0  0  0  0  0  0</td>
<td>10</td>
<td>0</td>
<td>100&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group VI (n=10)</td>
<td>0  0  0  0  0  0  0  0  0  0  0  0  0  0  0</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

Different letters in a column denote the presence of significant differences (p<0.05) between different experimental groups:

- Groups I= White Bovans breed, vaccinated and challenged
- Groups II= Brown Shaver chickens vaccinated challenged
- Groups III= White Bovans breed, vaccination but not challenged
- Groups IV= Brown Shaver breed, vaccination but not challenged
- Groups V= SPF chickens, unvaccinated but challenge
- Groups VI= SPF chickens, unvaccinated but not challenged
Table 5. Proportion of shedder birds and shedding titers* in experimental groups that were challenged with the (A/Chicken/Egypt/128s/2012 (H5N1)) HPAI clade 2.2.1 virus

<table>
<thead>
<tr>
<th>Days post challenge (dpc)</th>
<th>Group I**</th>
<th>Group II**</th>
<th>Group V**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of shedder birds</td>
<td>Proportion of shedder birds (%)</td>
<td>*No. of PCR copies (log_{10})</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>3.2</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>2.7</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>6.7</td>
<td>3.5</td>
</tr>
<tr>
<td>8</td>
<td>2</td>
<td>2</td>
<td>x</td>
</tr>
<tr>
<td>9</td>
<td>1</td>
<td>2.5</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>14</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Shedding was measured on 3, 6, 10 and 14 dpc. Other information provided in the Table are results obtained from examination of dead birds. Mean virus shedding is computed based only on positive shedders.

**Groups I= White Bovans breed, vaccinated and challenged; Groups II= Brown Shaver breed, vaccinated and challenged; Groups V= SPF chickens, unvaccinated but challenged.

‘x’ indicates that there is no surviving bird to be tested.
Table 6. Summary results of post-challenge parameters measured in experimentally infected groups (I, II & V)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group I*</th>
<th>Group II*</th>
<th>Group III*</th>
<th>Group IV*</th>
<th>Group V*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(range)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2HI log₂ titer</td>
<td>a 7.3 ± 1 (6-8)</td>
<td>a 7.8 ± 0.9 (6-9)</td>
<td>b 6.0 ± 1.7 (4-8)</td>
<td>c 8.2 ± 0.8 (7-9)</td>
<td>d 0 ± 0</td>
</tr>
<tr>
<td>Onset of signs (Days)</td>
<td>a 5.5 ± 1.3 (4-7)</td>
<td>0</td>
<td>a 5.2 ± 1.9 (3-8)</td>
<td>0</td>
<td>b 2 ± 0</td>
</tr>
<tr>
<td>3Patent period (Days)</td>
<td>a 3.5 ± 0.6 (3-4)</td>
<td>0</td>
<td>b 2.5 ± 0.8 (2-4)</td>
<td>0</td>
<td>c 1.6 ± 0.7 (1-3)</td>
</tr>
<tr>
<td>4MDT (Days)</td>
<td>a 8 ± 1.4 (6-9)</td>
<td>0</td>
<td>b 6.7 ± 2.3 (4-10)</td>
<td>0</td>
<td>c 3.6 ± 0.7 (3-5)</td>
</tr>
<tr>
<td>5Virus shedding (log₁₀)</td>
<td>a 2.6 ± 0.7 (2-3.5)</td>
<td>0</td>
<td>b 3.2 ± 0.9 (2.6-4.8)</td>
<td>0</td>
<td>c 4.2 ± 0.4 (3.7-4.9)</td>
</tr>
</tbody>
</table>

1 Mean ± SD = Mean values ± standard deviation between individuals birds; 2HI log₂ titer= the mean HI titer for the collected sera samples before challenge at 19 weeks of age using the Egyptian HPAI (A/chicken/Egypt/128s/2012(H5N1) Clade 2.2.1 antigen; 3Patent period (days)= the mean number of days from the onset of clinical signs and death; 4MDT= mean death time; 5Virus shedding (log₁₀) = Mean virus shedding is computed based only on positive shedder. (Log₁₀/PCR copies/Ml ); *Groups I= White Bovans breed, vaccinated and challenged; Groups II= Brown Shaver breed, vaccinated and challenged; Groups V= SPF chickens, unvaccinated but challenged. Different letters (a, b, c, d) in the left-hand side in a raw denote the presence of statistically significant differences (p<0.05); Different litters in column didn’t reflect any statistical differences