



The Combined Use of rHVT-H5 and rHVT-F Vector Vaccines in the Hatchery Enhances Immunity against Highly Pathogenic Avian Influenza H5N1 and Velogenic Newcastle Disease Viral Infections in Commercial Chickens

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Abstract

Highly pathogenic avian influenza H5N1 and Newcastle disease viral infections cause severe illness in chickens and vaccination is a strategic tool of controlling these diseases. Hence, this study was conducted to evaluate the efficacy of using both recombinant herpesvirus of turkey (rHVT-H5 and rHVT-F) vector vaccines at day-old, in the hatchery, under field conditions. Vaccinated chickens were challenged at 33 days of age with 100 µL containing 10⁶ embryonated infective dose 50 of either highly pathogenic avian influenza H5N1 or very virulent (velogenic) Newcastle disease viral strains through the intranasal route and monitored for 7 days for clinico-pathological changes. Tracheal and cloacal swabs and blood samples were also collected for determination of viral shedding using RT-PCR and immune responses using hemagglutination inhibition test. Absolute (100%) protection was recorded in vaccinated group against challenge with H5N1. In all time points, the challenge virus shedding was either not detected or greatly reduced in the trachea and the cloaca of vaccinated chickens compared to non-vaccinated, challenged chickens. Excretion of H5N1 challenge virus was not detected in the trachea of vaccinated birds at 3 and 5 days post-challenge and results of hemagglutination inhibition test revealed an average of 3.2 log₂ titres at 5 days post-challenge. Protection achieved against challenge with very virulent Newcastle disease virus was 79%. There was 80-100% reduction in tracheal shedding at 3, 5, and 7 days post-challenge, and an average of 6.2, 6.0 and 6.4 log₂ hemagglutination inhibition titres were recorded, respectively. Cloacal shedding of challenge Newcastle virus was greatly reduced in vaccinated groups compared to non-vaccinated, challenged chickens. These data support the efficacy of the combined use of rHVT-H5 and rHVT-F vector vaccines against highly pathogenic avian influenza and Newcastle disease viral infections under field conditions.

Introduction

Both highly pathogenic Avian Influenza (HPAI) and Newcastle disease (ND) are well-known avian contagious diseases affecting many species

of poultry, causing significant economic burden. In Egypt, the diseases are endemic and, thus, continue to hinder the profitability of poultry

industry (ELbayoumi *et al.*, 2013). Recent reports have confirmed H5N1 infections in many Egyptian governorates (<http://www.fao.org/AVIANFLU/EN/index.html>). Moreover, recent outbreaks from ND infection were detected in Egyptian poultry across the country (Abdel-Glil *et al.*, 2014, Al-Habeed *et al.*, 2013, Radwan *et al.*, 2013, Osman *et al.*, 2014).

As widely reported, ND infections induce a wide range of symptoms which differs according to several factors, including the infecting pathotype, age of infection and host immunity and health. The world-wide impact of ND is huge since infections represent a major drain to the global economy and hinder the international poultry trade (Alexander, 2003; OIE, 2012). Despite being recognized for almost 90 years, being caused by one single serotype of Paramyxovirus and having commercially available very efficient vaccines, ND still remains as a challenging disease for veterinarians and farmers all around the world. More recently, the advances in the technology have allowed manufacturers to develop products based on the concept of vectored vaccines which are safer and more efficacious than conventional vaccines against ND.

In Egypt, HPAIV H5N1 is endemic and therefore it poses a constant threat for the profitability of the poultry industry (Osman *et al.*, 2015). Moreover, the presence of Avian Influenza (H5N1 and H9N2) circulating with velogenic ND and Infectious Bronchitis (IB) complicates further any prevention program. Previous literature has shown that the recombinant herpesvirus of turkey expressing the F protein gene from NDV (rHVT-F) provided a good protection against velogenic NDV in chickens following administration subcutaneously at one day of age (Rauw *et al.*, 2010). Moreover, the rHVT expressing the HA (H5) gene from H5N1 HPAIV clade 2.2 (rHVT-H5) also revealed a high protection against challenge with two antigenically different strains of Egyptian H5N1 clade 2.2.1 in subcutaneously-inoculated day-old SPF chickens (Rauw *et al.*, 2011).

Control of AI, as well as ND, infections in poultry in endemic countries, as in Egypt, requires an implementation of strategic means, including vaccination, zoning and restriction of bird movement, enhancing biosecurity measures and adopting surveillance and monitoring programs. With regards to the vaccination strategy, both H5 and ND vector vaccines

(rHVT-H5 and rHVT-F) have been developed to 1) provide early and long lasting immunity, 2) increase the vaccination coverage by hatchery administration, 3) express maternally-derived antibodies (MDA) immune evasion, 4) minimize field viral transmission by better shedding control and 5) allow differentiating infected from vaccinated animals (DIVA) (Rauw *et al.*, 2010; Palya *et al.*, 2012; Gardin *et al.*, 2016). As the need for better control of both diseases is increasing, the need for hatchery application of both vectors is, therefore, demanded. Hence, this field trial was designed to demonstrate, under field conditions, the benefits of a combined use of rHVT-F and rHVT-H5 in controlling infections with vvNDV and H5N1 HPAIV, respectively. The compatibility of these two vaccines has already been demonstrated in laboratory conditions (Rauw *et al.*, 2012).

Materials and Methods

Experimental design

A total of seventy (one-day-old) commercial broiler chickens were reared in sterilized litter and were given access to antibiotic-free feed and water *ad libitum*. Birds were divided into five groups, each of 14 birds. The first two groups of chickens were vaccinated with one dose of rHVT-H5 and rHVT-F at day old while the third and fourth group of birds were kept as non-vaccinated controls. Challenge was done intra nasal at 33 days of age with either HPAIV or vvNDV. The fifth group of chickens was kept as a non-vaccinated non challenged control. All examined groups, either vaccinated or non-vaccinated, were observed daily for clinical protection and appearance of clinical signs of illness.

Challenge viruses

The first four groups of chickens were challenged at 33 days of age with either HPAIV (H5N1) or vvNDV. Reference HPAI virus (A/Chickens/Egypt/11VIR4453-266/2010) was used and the vvNDV was supplied from Faculty of Veterinary Medicine, Mansoura University. Each bird was inoculated intra nasal with 100 μ l containing *ca* 1×10^6 embryonated infective dose₅₀ (EID₅₀). The EID₅₀ was calculated as described previously (Reed and Muench, 1938). Chickens were monitored for 7 days post-challenge (dpc). Clinical protection, antibody response and tracheal and cloacal viral excretion were determined.

Reverse transcription (RT)-PCR

Determination of tracheal and cloacal shedding of the challenge virus was done using RT-PCR at 3, 5 and 7 dpc. Samples were collected and RNA extraction was done using Thermo Scientific GeneJET Genomic RNA Purification Kit according to manufacturer's instructions and purified RNA was kept at -80°C until used. Determination of viral shedding post-challenge was performed using Qiagen One Step RT-PCR as previously described (Seal *et al.*, 1995, Slomka *et al.*, 2007, Mazumder *et al.*, 2012). Amplification and production of specific PCR products for AI were performed using the following primer sequence H5-kha-1:(5'-CCT CCA GAR TAT GCM TAY AAA ATT GTC-3') and H5-kha-3: (5'-TAC CAA CCG TCT ACC ATK CCY TG-3') with the following cycle profile: one cycle at 50°C for 30 min, one cycle at 95°C for 15 min, 40 cycles at 94°C for 30 s, 58°C for 60 s and 68°C for 2 min and one cycle at 68°C for 7 min. Amplification and production of specific PCR products for ND were performed using the following primer pair (5'-TGGAGCCAAACCGCGCACCTGCGG-3') and (5'-GAGGATGTTGGCAGCAT-3') with the following cycle profile: one cycle at 50°C for 30 min, one cycle at 95°C for 15 min, 30 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 1 min and one cycle at 72°C for 10 min. The amplified RT-PCR products were subjected to agarose gel electrophoresis and were visualized in an image documentation system.

Hemagglutination inhibition (HI) test

Measuring antibody responses was carried out using the standard hemagglutination inhibition (HI) test. Newcastle disease virus (NDV) antigen, laSota strain, and Avian Influenza virus (AIV) antigen, H5N2, were used to test blood samples collected (up to 10 samples per each group) for specific antibody titers against NDV and AIV. Standard HI test was performed as previously described (Allan *et al.*, 1978, Alexander, 2009) at the day of challenge and at 3, 5, and 7 days after challenge. Briefly, two-fold serial dilution of each 50 µL of sample serum was done in V-shaped micro titer plates using sterile normal saline 0.9%. Four hemeagglutinating units (HAU) of NDV were used as well as positive and negative controls. Following 30 min of incubation at room temperature, 50 µL of 0.5% washed chicken RBCs were added in each well and plates were left for 20 min. The reciprocal of the highest

dilution that causes inhibition of agglutination was referred as HI titer and geometric mean titer (GMT) was calculated.

Statistical analysis

Data were analyzed using Student t test using GraphPad software. Differences between vaccinated-challenged and non-vaccinated challenged groups were considered significant if $P < 0.05$.

Results and Discussion

Clinical protection following HPAIV or vvNDV challenge

The protection efficacy following vaccination with rHVT-H5 or rHVT-F has been previously described (Rauw *et al.*, 2010; 2011), but no single study, as yet, has studied the protection as a result of the combined use of both vector vaccines in a challenge experiment performed in commercial chickens under field conditions. In this study, no mortality was seen in the vaccinated chickens challenged with HPAIV, with 100% protection observed (Fig 1). However, mild depression was observed at 2 to 5 days post challenge with HPAIV. These protection results are comparable with the previous investigation which has shown better clinical protection in chickens' vaccinated rHVT-H5 compared to inactivated vaccines following challenge of commercial chickens with HPAI H5N1 clade 2.2.1 at 28 and 35 days of age (Kilany *et al.*, 2015). Results obtained have shown that simultaneous vaccination with rHVT-H5 and rHVT-F at day-old could provide 79% protection against vvNDV challenge when infection occurred at 33 days of age. Commercial turkeys vaccinated with rHVT-F have demonstrated full protection against challenge with vvNDV genotype IV as early as 21 days of age (El Khantour *et al.*, 2017). Moreover, Palya *et al.*, (2014) have shown that single application of rHVT-F in one-day-old commercial laying chickens provided long lasting protection measured till 72 weeks of age with 95-100% protection at 4 weeks of age against challenge with velogenic NDV genotype VII. In the present study, the combined vaccination with both vectors seems to greatly enhance the clinical protection against AI and ND, although ND protection seems to be a bit delayed. This could be also influenced by many factors, including challenge age, infecting dose and poultry breed examined. In this study, all non-vaccinated, challenged control chickens

showed 100% mortality (Fig 2), depending on the challenge virus type. In contrast, there was neither mortality nor clinical signs observed in

the non-vaccinated non challenged negative control chickens.

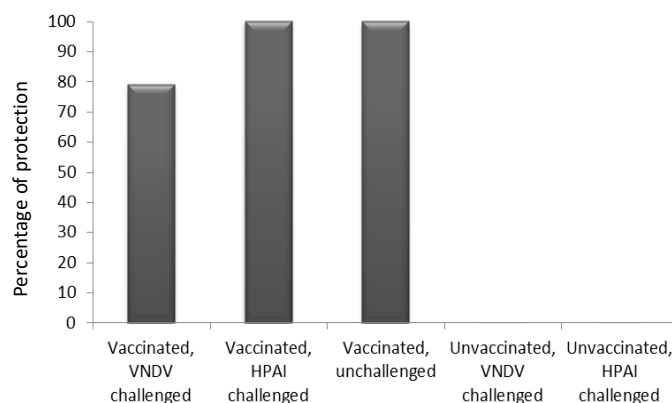


Figure 1. Clinical protection post-challenge with HPAIV or vvNDV.

Percentage of protection was calculated from the number of survivors post-infection compared to the total number of chickens per group. Chickens were monitored for 7 days post-challenge. rHVT-H5 and rHVT-F-vaccinated chickens were challenged with either vvNDV (group 1) or HPAIV (group 2) or kept as non-vaccinated challenged positive controls (group 3 (ND) and 4 (AI)) and non-vaccinated unchallenged negative control (group 5).

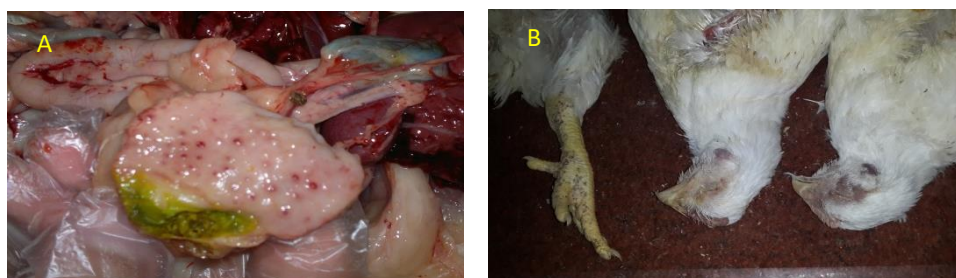


Figure 2. Post mortem picture of positive control chickens following challenge with vNDV (A) and HPAIV (B).

A; petechial hemorrhages on tips of proventricular glands, B; subcutaneous hemorrhages of shanks and comb cyanosis.

Viral shedding post-challenge with HPAIV or vvNDV

Challenge viral excretion was determined in the trachea and cloaca of experimentally infected chickens using RT-PCR at 3, 5, and 7 days post challenge (Fig. 3). Data presented here have shown a significant reduction ($P < 0.05$) in HPAIV and vvNDV viral load in the respiratory and digestive tracts of vaccinated chickens at 3 to 7 days post challenge. Reduction of viral excretion following challenge of SPF chickens with AIV and NDV at 4 and 8 weeks has been seen under laboratory conditions (Rauw *et al.*, 2012). Indeed, the data presented in this study have shown that the combined vaccination with rHVT-H5 and rHVT-F at day old resulted in 80-

100% and 60-100% reduction in the viral shedding of HPAIV and vvNDV, respectively, when challenge performed at 33 days of age. These results are in line with previous literature which has demonstrated enhanced tracheal and cloacal shedding following vaccination with either rHVT-H5 or rHVT-F (Kilany *et al.*, 2014a,b; Palya *et al.*, 2014). It seems like the simultaneous administration of both vector vaccines does not negatively impact their capability to reduce the challenge viral shedding as it is clearly shown that the combined vaccination with rHVT-H5 and rHVT-F at day-old induced a significant reduction of tracheal and cloacal viral shedding.

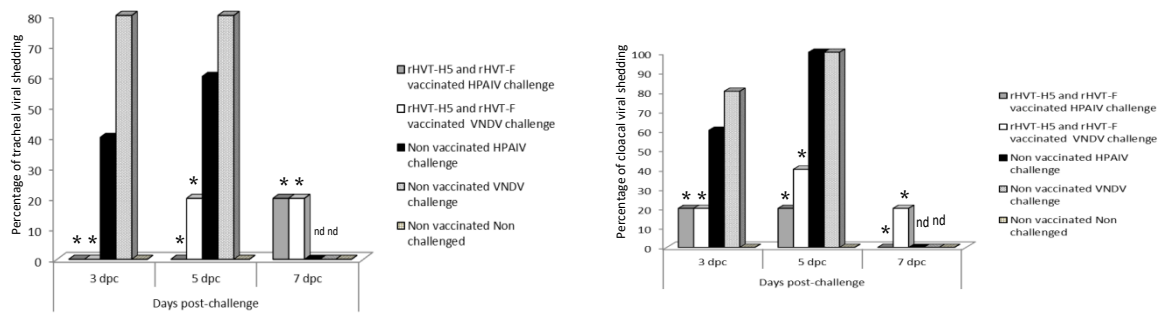


Figure 3. Tracheal and cloacal viral shedding following HPAIV or vvNDV challenge.

Data presented as the percentage of positive swabs compared to the total number of swabs examined. Five tracheal and cloacal swabs were taken from each group and tested by RT-PCR for either AI or ND viral shedding. * Asterisks indicate significant difference between vaccinated and non-vaccinated challenged groups ($P < 0.05$).

Antibody responses following HPAIV or vvNDV challenge

Humoral immune responses to H5AIV or NDV were measured in all experimental groups using HI test (Table 1). In this study, the obtained data have shown a significant increase ($P < 0.05$) in the magnitude of HI titers of group 3 and 4 in response to infection with either HPAIV or vvNDV at 3 and 5 days post challenge. This increase in HI titers is highly influenced by humoral immune responses developed as a result of infection. Moreover, chickens vaccinated with rHVT-H5 and rHVT-F vector vaccines seem to seroconvert to infection with

the virulent viruses, with a significant elevation ($P < 0.05$) in the immune responses developed to HPAIV infection at 7 day post challenge. Nonetheless, the overall mortality data, shedding and serology data clearly confirm the good protection achieved by using both vaccines at day-old. These serological results are in line with Kilany *et al.* (2015) who reported comparable HI titers at 4 and 5 weeks of age following vaccination of day-old commercial broilers with rHVT-H5. Palya *et al.* (2014) has also reported protective HI and ELISA titers to single dose vaccination with rHVT-F in commercial layers.

Table 1. Antibody response post-challenge

Group	Challenge virus	Measured antibody titre	Days			
			0 day	3 dpc	5 dpc	7 dpc
1	vNDV	ND	5.5 ± 1.1*	6.2 ± 1.2*	6.0 ± 1.4*	6.4 ± 1.3*
2	HPAIV	H5 AIV	1.7 ± 1.3*	2.3 ± 0.8*	3.2 ± 1.6*	4.3 ± 2.5*
3	vNDV	ND	0.7 ± 0.7	2.7 ± 1.7	5.2 ± 2.2	nd
4	HPAIV	H5 AIV	0.6 ± 0.7	2.1 ± 1.4	4.0 ± 2.3	nd
5	Non challenged	ND	0.3 ± 0.4	0.1 ± 0.3	0.1 ± 0.3	0.1 ± 0.3
		H5 AIV	0.1 ± 0.3	0.0 ± 0.0	0.2 ± 0.6	0.2 ± 0.4

Specific humoral immunity to ND or H5 AIV presented as Log_2 titres ± standard deviation of the mean at the day of challenge (0 day) and 3, 5, and 7 days post-challenge. Group 1 and 2; rHVT-H5 and rHVT-F vaccinated chickens, group 3 and 4; non-vaccinated challenged positive controls, group 5; non vaccinated non challenged negative control. * Asterisks indicate significant difference between vaccinated and non-vaccinated non challenged groups ($P < 0.05$). nd = not done.

In this study, it is clearly evidenced that the combined vaccination with both vectors has positively impacted the humoral immune responses. Future studies should be conducted to determine pattern of the rHVT-H5 and rHVT-F vaccine-take in the vaccinated chickens and to emphasize the cellular immune responses.

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