Hussein *et al., J. of Virol. Sci.,* Vol. 4: 33-43, 2018
ISSN: 1685-1687

Journal of Virological Sciences

RESEARCH

Antigenic content of 500 HA units in H5N1 reassortant AI vaccine enhances protection and reduces shedding of HPAI H5N1 clade 2.2.1.2 in broiler chickens

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ABSTRACT

Background*:* Egyptian poultry industry has suffered from high economic losses as a result of the wide spread of highly pathogenic H5N1. Determination of the optimal antigen content of avian influenza virus vaccines is urgent to reach protective antibody titers and reduce virus shedding.

Methods: Groups of one-day old commercial broiler chicks were divided in to 8 groups 1, 2 and 3 were vaccinated with a prepared vaccine contain 500HAU of H5N1 reassortant antigen; while group 4, 5 and 6 were vaccinated with an imported reassortant vaccine with 500HAU antigen content of H5N1 at 1, 5 and 10 days of age; respectively. A group 7 was positive challenged control and group 8 negative challenged groups. All chicken groups were maintained at isolators along the experiment study. Blood samples were collected for weekly for 4 weeks and antibody titers were determined by HI test. All vaccinated groups were challenged 4 weeks post vaccination and tracheal and cloacal swabs were taken at 3, 5, 7, and 10 days post challenge and tested by real time RT-PCR (rRT-PCR) and virus isolation and titration in SPF ECE.

Results: Results of HI demonstrated significant difference between groups in relation to age of vaccination, where the groups vaccinated at 10 days of age were significantly higher compared to others with maximum titers at 4 weeks post vaccination. The protection % post challenge revealed 0, 20, 86 % and 0, 20 and 86 % in groups 1, 2, 3 and groups 4, 5, and 6; respectively. Results of rRT-PCR and virus isolation revealed that all chicken groups vaccinated at 1 and 5 days of age revealed 100% shedding at 3rd, 5th, 7th and 10th days post challenge. However, groups 3 and 6 which were vaccinated at 10 days of age demonstrated different shedding pattern where group 3 (vaccinated with local prepared 500HAU vaccine) showed at the 3rd and 5th days shedding by rRT-PCR and 80% and 20 % of the chickens in tracheal swabs and 80% and 40% in cloacal swabs when tested by virus isolation in eggs at 3 and 5 days post challenge; respectively. Whereas swabs of 7 and 10 days post challenge of group 3 were negative by rRT-PCR and virus isolation. On the other hand, group 6 (vaccinated with imported 500HAU vaccine) demonstrated shedding % at 3 and 5 days post challenge by rRT-PCR and for virus isolation were positive in 60% for tracheal swabs 3 day post challenge and no shedding at 5th post challenge and 60% and 20% for cloacal swabs; respectively. At 7th and 10th days post challenge shedding of all chickens in group 6 were negative by both rRT-PCR and virus isolation.

Conclusion*:* Vaccination against H5N1 AIV is greatly affected by both antigen content of vaccine and level of maternal immunity in vaccinated chicks.

Keywords: Avian influenza; antigen content; maternal immunity; challenge; rRT-PCR.

BACKGROUND

Avian Influenza (AI) virus is an Orthomyxoviruses type A and produces disease syndromes in various poultry species. AI virus subtypes are hemagglutinin (H1-H18) and neuraminidase (N1-N11) subtypes (Tong *et al.*, 2013). Infection with AI virus can cause great economic losses in the poultry industry worldwide and represents a serious threat to public health (Capua *et al.*, 2004). Conventional control strategies are potentially based on surveillance, stamping out of infected flocks and biosecurity measures enforcement (Swayne, 2009). There were magnificent losses due to spread of the infection estimated by several billions of culled birds, and

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the disease became endemic in many infected countries. The estimated loss of the Egyptian poultry industry after the first emergence of highly pathogenic AI H5N1 in February 2006 was 1 billion US$ and affected the income of 1.5 million people who are mainly rely on poultry (Meleigy *et al.*, 2007). About 30 million birds were culled in Egypt in the first wave of 2006. Beside the biosecurity and monitoring infection particularly in the populated poultry areas, vaccination also represents an option as a supportive tool in AI virus control strategies to limit the spread of H5N1 and to reduce the losses (EFSA, 2008). Different types of vaccines which are already in use decrease virus shedding, morbidity, mortality, and increase resistance to infection; and reduce field virus replication (van den Berg *et al.,* 2008). Antibodies to the circulating virus strain had been detected in day-old chicks in Egypt (van Eck *et al.,* 1991). To date, broiler production sector in Egypt apply different AI vaccination programs involved vaccination at 7 to 10 days of age (Kilany *et al.,* 2016). Vaccination may hide the clinical signs of disease but can't prevent the infection of vaccinated birds as well as shedding, "silent" circulation of the virus in vaccinated birds is considered as one of important risk of virus spread among poultry flocks and humans (Savill *et al.,* 2006). An effective vaccine must provide a high and long-lasting immune response at a low antigen dose. Therefore, formulation of vaccines using adjuvants to stimulate immune response is an important approach because it helps reduce the antigen dose. Indirect methods, such as the HA titer and EID50, are more frequently used (Swayne *et al.,* 1999). The day-old chicks derived from vaccinated dams should not be vaccinated immediately (Kim *et al.,* 2010). On the other hand, the broiler chicks are bad antibody forming birds than layers and breeders, so it is not necessary to vaccinate broilers obtained from immune breeder flocks (Nasr, 2008). In birds, maternal antibodies are transferred into the yolk, and the chick depend on this source of passively acquired immunity during the 1st weeks of its life (Abdelwhab *et al.,* 2012). Although these antibodies can protect the chicks against viral disease (Maas *et al.,* 2011), they also can hinder and retard the immune response to vaccination as seen with infectious bursal disease (Naqi *et al.,* 1983), Newcastle disease virus (NDV) (van Eck *et al.,* 1991), and AI vaccines (Abdelwhab *et al.,* 2012).

Most of the work of vaccine evaluation has been done in chickens based on EID50 before virus inactivation rather than hemagglutinin content after inactivation. This is despite HA being the major influenza protein that elicits a protective immune response that is readily detected and estimated serologically (Swayne and Kapczynski, 2008). In this study, the increase in antigenic content in the inactivated H5N1 AI vaccine and their impact on serological response, protection and reduction of virus shedding of vaccinated commercial broiler chickens were carried out in a challenge trial using the official challenge strain of H5N1 isolated in 2015 (genetic clade 2.2.1.2). Five hundred Hemagglutination units (HAU) as optimal antigen content were chosen based on the evaluation data in the central laboratory for evaluation of veterinary biologics. We expect it will provides antibody response in commercial broiler chickens which protect against challenge and reduce the virus shedding.

MATERIALS AND METHODS

Viruses and Vaccine preparation:

Reassortant Avian Influenza Virus A/Chicken/Egypt/Q1995D/2010(H5N1) and A/Duck/Egypt/M2583D/2010 (H5N1) were developed by national research center and used for preparation of local influenza vaccines in Veterinary Serum and Vaccine Research Institute, Newcastle disease unit, Abbassia, Cairo-Egypt. The viruses were propagated in 9-11 days old specific pathogen free-embryonated chicken eggs (SPF – ECE) (Beard *et al,* 1989) via their inoculation of 500 EID50 into allantoic cavity and incubation at 36°C for 36-38 hours. The virus

harvests were inactivated with formalin solution 0.1%. Samples from the inactivated virus before addition of adjuvant were tested by at least two passages in 9-11-day old SPF embryonated eggs (0.1 ml/egg) via the allantoic cavity to confirm complete inactivation. All embryos that died or remained alive after 24 hours and up to 6 days were examined for the presence of virus in the collected allantoic fluid by the rapid HA. The vaccine was prepared by mixing oil adjuvant Montanide ISA-70 (Seppic, France) at a ratio of (70/30) yielding stable white emulsion. In addition, the vaccine was supplemented with gentamicin (200 mg/ml) and thiomerosal (0.102 mg/ml). The entire process of preparing laboratory specimens of the inactivated vaccine was carried out under conditions of the Biological Safety Laboratory 3. Virus stock was amplified in SPF ECE and virus titer was determined by 50% Egg Infectious Dose (EID50). The titer used in the prepared vaccine was 10^{11} and 10^{12} for chicken and duck seed viruses in a percentage of 50/50; respectively.

Challenge trial:

Two hundred one – day-old broiler chickens (Cobb breed) were obtained from Commercial Hatcheries-Egypt, and housed in separate isolators all over the challenge trial. The chicks were reared under proper Hygienic conditions ventilated under positive pressure with HEPA- filtered air and maintained under continuous lightening, feed and water supplied ad libitum. Birds were randomly divided into groups (n = 25/group). Birds in Groups 1–6 were vaccinated subcutaneously (SQ) with 0.5 mL of the H5N1 vaccines. Groups 1, 2 and 3 were vaccinated with the prepared vaccine; groups 4, 5 and 6 were vaccinated with one of the imported reassortant H5N1 containing 500 HAU at 1, 5 and 10 days of age; respectively. Groups 7 and 8 were positive and negative groups for the challenge trial. Four weeks post vaccination, each bird was challenged intra-nasally (I.N.) with 10^6 EID50 of HPAIV/bird (A/Duck/Egypt/CLEVB-24-N00238/2015); Accession no: EPI579780 on GISAID obtained from viral strain bank of CLEVB and used for challenge tests. All chickens were daily observed and monitored for 10 days post challenge (DPC) in order to report the clinical sings as well as record mortalities and detection of virus shedding for each group. Swabs (oropharyngeal and cloacal) were taken from live birds at day 3,5,7 and 10 post challenge in all groups for quantification of virus shedding using real-time RT-PCR (OIE, 2015) and for virus re-isolation in ECE. RNA was extracted from the oropharyngeal and cloacal swabs using QI Amp Viral RNA Mini Kit supplied from (QI Amp Viral RNA Mini Kit (QIAGEN) catalogue No. 52904. Virus isolation and detection in embryonated chicken egg were carried out as previously described (OIE, 2015). Both oropharyngeal and cloacal swabs have been placed in isotonic phosphate-buffered saline (PBS), pH 7.0–7.4 with antibiotics. Penicillin (2000 units/ml), streptomycin (2 mg/ml), gentamycin (50 µg/ml) and mycostatin (1000 units/ml) for oropharyngeal swabs, but cloacal swabs received five-fold higher concentrations (OIE, 2015). For virus inoculation in ECE, these suspensions were filtered through 0.22µm Millipore filter. Five 9-days-old SPF EGE were inoculated and candled daily for embryo viability for 7 days (Beard *et al.,* 1989). Allantoic fluid from embryos dead 24 h post inoculation was collected aseptically and tested for the presence of AI H5 virus by rapid slide Hemagglutination test (Anon *et al.,* 1971).

Serological monitoring of antibodies:

Blood samples were collected from jugular vein and kept in a slope position at 37^{o}C for one hour then at 4 ^{o}C overnight. Sera were then separated by centrifugation at 3000 rpm for 10 minutes and stored at -20 ^{o}C. Sera were inactivated at 56 ^{o}C for 30 minutes before testing. Ten serum samples were collected from each group (1-8) at 7th, 14th, 21th and 28th days PV for post vaccination monitoring in the first experiment, while it was collected at 1st, 5th, 7th, 14th, 21thand 28th day old and from non-vaccinated non challenged group (gp 7) for follow-up of the

maternally derived antibodies. Serum samples were subjected for (HI) **(OIE, 2015)** using the (A/Chicken/Egypt/Q1995D/2010(H5N1) homologous antigen for the local prepared vaccine, (H5N1) license no.: veterinary Bio-drug (2014) 080018076 antigen for the imported vaccine by using standard 4 HAU of the antigen.

Statistics:

The results represent the mean with standard error of at least triplicate determinations (n=3). Statistical significance was determined by two- way analysis of variance (ANOVA) with LSD post hoc test using statistical software program SPSS (version 21.0).

RESULTS:

HI titers of MDA

The results of HI test to determine the maternal immunity are represented in Figure (1). There were high to moderate levels of maternal antibodies against AI (H5N1) on the 1st and 5th day of age vaccinated chicks and gradually decreased starting from 7 days and disappeared at 28 days of age.

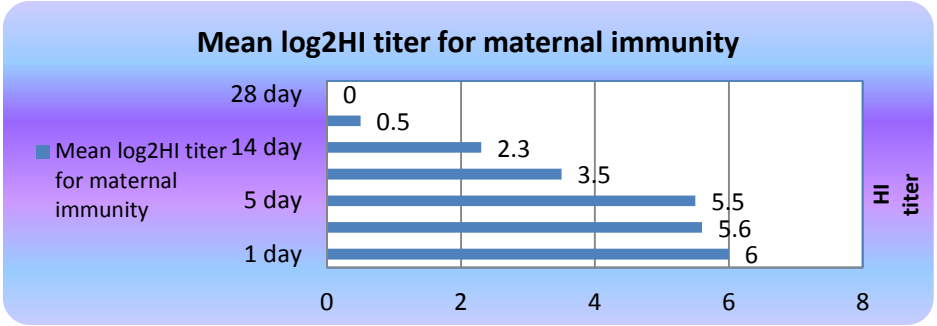

Figure (1): The mean HI titer of the maternal antibodies to H5N1 in non-vaccinated chicks

HI antibodies to the H5N1 vaccines

Results of HI test in the first week post vaccination there were statistically differences with in titers which were high in groups 1 and 4 compared to groups 2 and 5. HI titers were low in groups 3 and 6. However, at the 2nd week significant higher titers were recorded in groups 1, 3, 4 and 6 in comparison with groups 2 and 5. By the 3rd week significant higher antibody titers were in groups 3 and 6. Significant higher titers were in groups 3 and 6 compared to others in groups 2 and 5 whereas groups 1 and 4 demonstrated zero titers at 4 weeks post vaccination.

Table (1). Serum antibody response following vaccination with local and imported inactivated AIV (H5N1) vaccines containing 500 HAU in chicken groups at different ages.

Groups	Days post vaccination			
	Virus titer (Log2)			
	1st week	**2nd week**	**3rd week**	**4th week**
Group 1	3.3 ± 0.5^{bc}	3.0 ± 1.0^{abc}	0.33 ± 0.5^{a}	0^{a}
Group 2	2.8 ± 0.4^{b}	2.0 ± 0.7^{a}	1.8 ± 0.8^{a}	2.4 ± 0.5^{b}
Group 3	1.4 ± 0.5^{a}	4.0 ± 0.7^{bc}	5.4 ± 0.5^{b}	7.4 ± 0.8^{c}
Group 4	4.0 ± 1.0^{c}	3.3 ± 1.15^{b}	1.0 ± 1.0^{ac}	0^{a}
Group 5	2.8 ± 0.8^{b}	2.4 ± 0.5^{a}	2.2 ± 0.8^{c}	2.8 ± 0.4^{b}
Group 6	1.2 ± 0.4^{a}	3.6 ± 0.8^{c}	5.6 ± 0.5^{b}	7.2 ± 0.8^{c}

Means with different superscript letters (a, b, c, d) within the same column are significantly different at P value < 0.05 between chicken groups. Group1: vaccinated with local vaccine at 1day, Group2: vaccinated with local vaccine at 5days, Group 3: vaccinated with local vaccine at 10 days, Group4: vaccinated with imported vaccine at 1day, Group5: vaccinated with imported vaccine at 5days, Group 6: vaccinated with imported vaccine at 10 days.

Protection % against challenge with HPAIV

The characteristic clinical signs for HPAI observed 3 days post challenge with mortalities occurred in different challenged groups. Sick birds displayed cyanosis of comb and wattle, ecchymosis on the shanks and feet, facial edema, greenish diarrhea and nervous signs including torticollis and tremors. For vaccinated birds the protection % in the vaccinated groups with local vaccine was 0%, 20%, and 86% in groups 1, 2, and 3; respectively. Also, protection % in the vaccinated groups with imported vaccine was 0%, 20%, and 86% in groups 4, 5, and 6; respectively.

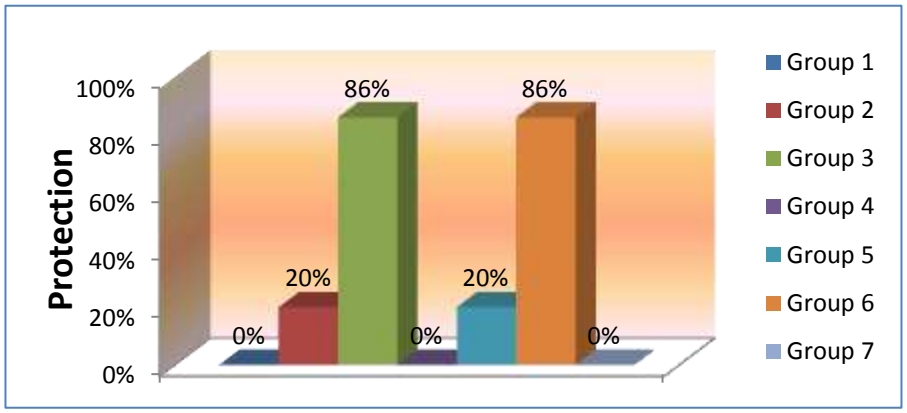

Figure (2): Protection% and challenge via infection intra-nasally with 106 EID50 of HPAIV/bird (A/Duck/Egypt/CLEVB-24-N00238/2015) for local and imported300 HAU Inactivated AIV (H5N1) groups at different ages.

Group1: vaccinated with local vaccine at 1day, Group2: vaccinated with local vaccine at 5days, Group 3: vaccinated with local vaccine at10 days, Group4: vaccinated with imported vaccine at 1day, Group5: vaccinated with imported vaccine at 5days, Group 6: vaccinated with imported vaccine at 10 days.

Virus shedding

Virus shedding titers could be detected by both rRT-PCR and challenge virus re-isolation in ECE for tracheal swabs on days 3, 5, 7, and 10 post challenge. There were a statistical significant difference among groups, in the 3rd day results revealed a higher rate of virus shed in groups 1, 4, 5 and 7, the virus shedding decreased in group 2 lower titers recorded in group 3 and 6. High titers of challenge virus could be detected from tracheal swabs in the SPF ECE, the results were 100% in groups 1, 2, 4 and 5, while it was 80% in group 3, whereas it was 60% in group 6. In the 5th day post challenge, there were higher virus shedding titer in groups 1, 2, 3, 4, and 5, lower shedding titers detected in group 6. However, it found by virus isolation the results were 100% in groups1, 2, 4, and 5 and reduced in group 3 recording 20%, and for group 6 was 0%. By the day 7, the rRT-PCR results were significantly different with high titer in groups 1, 2, 4 and 5 with no shedding in group 3 and 6. Virus re-isolation results were 100% in groups 1, 2, and 4, 80% in group 5, and 0% in group 3 and 6. At the 10th day post challenge, significant high titers by rRT-PCR were recorded in groups 1, 2, 4 and 5, and no shedding in group 3and 6. On

the other hand, for virus isolation results were 100% in groups 1 and 4, 80% in group 2, 60% in group 5, 0% in groups 3, and 6 (Table 2).

Table 2: Results of rRT- PCR and virus isolation in SPF ECE for tracheal swabs collected from chickens vaccinated with local or imported Inactivated AIV (H5N1) vaccines containing 500HAU.

Groups	Days post challenge							
	3rd		5th		7th		10th	
	Virus titer (Log10)							
	rRT-PCR	Isolation	rRT-PCR	Isolation	rRT-PCR	Isolation	rRT-PCR	Isolation
Group 1	4.7 ± 0.3^{b}	100%	4.4 ± 0.7^{bc}	100%	4.8 ± 0.5^{b}	100%	4.8 ± 0.5^{b}	100%
Group 2	3.8 ± 0.5^{cd}	100%	3.9 ± 0.7^{bc}	100%	4.6 ± 0.3^{b}	100%	3.5 ± 0.5^{c}	80%
Group 3	3.1 ± 0.4^{c}	80%	3.2 ± 1.1^{bd}	20%	0^{c}	0%	0^{a}	0
Group 4	4.7 ± 0.2^{b}	100%	4.5 ± 0.5^{c}	100%	4.8 ± 0.3^{b}	100%	4.5 ± 0.3^{bd}	100%
Group 5	4.0 ± 0.5^{b}	100%	3.8 ± 0.8^{bc}	100%	4.3 ± 0.4^{b}	80%	3.9 ± 0.7^{cd}	60%
Group 6	3.2 ± 0.4^{c}	60%	2.3 ± 0.3^{d}	0	0^{a}	0	0^{a}	0
Group 7	4.2 ± 0.2^{bd}	NS	NS	NS	NS	NS	NS	NS
Group 8	0^{a}	0^{a}	0^{a}	0^{b}	0^{a}	0^{a}	0^{a}	0^{a}

Means with different superscript letters (a, b, c, d) within the same column are significantly different at P value < 0.05 between chicken groups. Group1: vaccinated with local vaccine at 1day, Group2: vaccinated with local vaccine at 5days, Group 3: vaccinated with local vaccine at10 days, Group4: vaccinated with imported vaccine at 1day, Group5: vaccinated with imported vaccine at 5days, Group 6: vaccinated with imported vaccine at 10 days. Group 7: positive challenge control. Group 8: negative non-vaccinated control. NS: non survival.

For cloacal swabs virus shedding titers also could be detected by both rRT-PCR and challenge virus re-isolation in ECE. There was a statistical significant difference among groups detected by rRT-PCR in the 3rd day results revealed a higher rate of virus shed in groups 1, 4, 5 and 7, lower virus shedding titers were in group 2, lower virus titers recorded in groups 3 and 6. On the other hand virus isolation results were 100% in groups 1, 2, 4 and 5, 80% in group 3, while it was 60% in group 6. Testing of swabs collected at 5th days revealed significant higher virus shed in groups 1, 2, 3, 4 and 5, the virus shedding was lower in group 6. However, virus isolation from such swabs was 100% in groups 1, 2, 4 and 5, 20% in group 3, while there was no shedding in group 6. By the day 7 post challenge, the rRT-PCR results recorded significantly high titer in groups 1, 2, 4 and 5, no shedding in groups 3 and 6. The virus isolation results were 100% in groups 1, 2 and 4, 80% in group 5, and negative results in groups 3 and 6. Testing of swabs collected at 10th day post challenge by rRT-PCR revealed significant high virus shed in groups 1, 2, 4 and 5, and no shedding in groups 3 and 6 and the virus isolation results was 100% for groups 1 and 4, 80% in group 2, 60% in group 5, for both groups 3, and 6 there was 0% (Table 3).

Table3: Results of rRT- PCR and virus isolation in SPF ECE for cloacal swabs collected from chickens vaccinated with local or imported Inactivated AIV (H5N1) vaccines containing 500HAU.

Groups	Days post challenge							
	3rd		5th		7th		10th	
	Virus titer (Log10)							
	rRT-PCR	Isolation	rRT-PCR	Isolation	rRT-PCR	Isolation	rRT-PCR	Isolation
Group 1	4.8±0.7 [a]	100%	5.1±0.5 [a]	100%	4.7±0.7 [a]	100%	4.9±0.2 [a]	100%
Group 2	4.2±0.5 [abc]	100%	4.0±0.4 [bd]	100%	4.8±0.5 [a]	100%	4.5±0.2 [bc]	80%
Group 3	3.8±0.3 [bc]	80%	3.3±0.6 [bc]	40%	0 [b]	0%	0 [d]	0%
Group 4	4.9±0.5 [a]	100%	4.9±0.5 [a]	100%	5.1±0.2 [a]	100%	4.7±0.1 [ab]	100%
Group 5	4.6±0.8 [ab]	100%	4.7±0.1 [ad]	100%	4.6±0.3 [a]	100%	4.2±0.1 [c]	100%
Group 6	3.4±0.5 [c]	60%	3.0±0.6 [c]	20%	0 [b]	0%	0 [d]	0%
Group 7	5.0±0.1 [a]	100%	NS	NS	NS	NS	NS	NS
Group 8	0[d]	0[a]	0[e]	0[b]	0[b]	0[a]	0[d]	0[a]

Means with different superscript letters (a, b, c, d) within the same column are significantly different at P value < 0.05 between chicken groups. Group1: vaccinated with local vaccine at 1day, Group2: vaccinated with local vaccine at 5days, Group 3: vaccinated with local vaccine at10 days, Group4: vaccinated with imported vaccine at 1day, Group5: vaccinated with imported vaccine at 5days, Group 6: vaccinated with imported vaccine at 10 days. Group 7: positive challenge control. Group 8: negative non-vaccinated control. NS: non survival.

DISCUSSION

The aim of this study is to evaluate the efficacy of a prepared H5N1 AIV vaccine containing at least 500 HA units of the antigen under experimental conditions in commercial broiler chickens. MDA in unvaccinated chicks declined gradually starting from 7 days and disappeared or being undetectable at 28 days of age reflecting a good vaccination of the dams. In general, maternally derived antibodies (MDA) can provide protection in young chicks against viral diseases (Nemeth and Brown, 2007). It was previously reported that maternally derived antibodies recorded the highest titer directly after hatching, gradually decreases within 3 to 4 weeks to score zero (Maas *et al.,* 2011). Recent data indicates that vaccination of maternal antibody-positive chicks 10 days post-hatch or later may be the best approach due to the interfering effect with vaccine immune response as late as 3 weeks post-hatch (De Vriese *et al.,* 2010; Khedr *et al.,* 2018). In mean time, the maternal immunity detected in experimental broiler chickens that were vaccinated at 10 days of age and challenged at day 34 were protected against H5N1 virus (De Vriese *et al.,* 2010). Also, the passive transfer of H5N1 antibodies to young chicks decreases the efficiency of subsequent active vaccination (Kim *et al.,* 2010). It is also important to continue to explore methods to overcome and circumvent maternal antibody interference through better vaccination strategies. The live H5 fowl pox-vectored vaccine can be used effectively and recommended in the first days of chicks as it is not inhibited or interfered by maternal antibodies and does not interfere with routine serological surveillance; however, its efficacy seems to be compromised by active immunity, and strict measures of biosecurity must be taken during this period to prevent avian influenza virus infection (Bublot *et al.,* 2006). Recently, prime boost vaccination strategy with vector or even inactivated mucosal vaccines as priming followed by boosting with the inactivated H5N1 vaccines demonstrated enhance protection and reduce shedding of the challenge virus (Ismail *et al*., 2018 a; Ismail *et al*., 2018 b). It was reported that it can be predicted at what age young chicks can be vaccinated based on

antibody titers (Solano *et al.,* 1986). In the present study, results revealed that both vaccination at 1 and 5 days old post hatching failed to produce protective level of antibody titers compared to vaccination of chicks at 10-day old age (table 1). At the beginning of application of the vaccination strategy against H5N1in Egypt, vaccination at one days old was applied and by the time several grandparent and breeder flocks were extensively vaccinated with H5 vaccines resulting in the high level of maternal antibodies (unpublished data). Therefore, most of the chickens in the field currently vaccinated between 5 and 10 days. In the present study, the HI results revealed variations in titers induced by vaccines and at 4 weeks post vaccination, it is proved that the vaccines with antigenic content of 500HA units induced statistical higher titers than those applied at 1 and 5 days (Table 1). Indeed, the time of vaccination in the field where many factors affect the outcome of the immune response is very and greatly affects the success of vaccination strategy. Periodical information is needed about the efficacy of vaccination in a variety of different avian species, bearing in mind the diverse farming systems used in developed and developing countries (Capua and Marangon, 2006). Protective HI antibody titer against disease and virus shedding in chickens are important and arguable as it depends on several factors including antigen content of the vaccine, vaccine preparation and formulation, age of chicken flocks at time of vaccination, and finally time between vaccination and challenge (Swayne *et al.,* 2015). Challenge test using current circulating virus is an important test as it reflects the protection % simulating the fields and farms condition. In the present study, results of protection revealed 0%) in one-day old vaccinated groups, in five-day vaccinated groups about (14%) (Figure 2), with high virus shedding titer in both 1 and 5day old vaccinated chicks till 10^{th} d.p.c. On contrast, protection % in 10-day old vaccinated chicks was (86%) which reflect the accordance of the HI and protection results in this group. Such finding was reported in other study where they found that vaccinated chickens with adequate HI antibody titers are usually provide protection against infection with HPAIV (Maas *et al.,* 2009). Both tracheal and cloacal swabs results showed positive results with significant high titers in groups vaccinated at 1 and 5 days old in both rRT- PCR and virus isolation in SPF ECE 3 ,5 ,7 , and 10 days post challenge for both vaccines, however in 10 days old vaccinated chicks showed significant reduced titers at 3, and 5 days post challenge and no shedding at 7, and 10 days post challenge for both vaccines in both rRT- PCR and virus isolation in SPF ECE which were mentioned in tables (3 and 4). Virus shedding not prevented completely but decreased to its minimal level which was satisfactory and it is very important in controlling the spread of virus infection among neighboring farms. It was recently published that antigen content of 512 HAU/chicken can help to prevent morbidity and mortality and reduce virus shedding of HPAI H7N3 virus (Spackman *et al.,* 2014). In addition, a single vaccination of chickens with an inactivated H5N3 oil emulsion vaccine containing as low as 0.25 mg HA protein was highly effective against H5N1 challenge (Webster *et al.,* 2006). Reduction of virus shedding in case of HPAI is depend on the virus load in the farms and environment which play a great role in direct virus transmission to other birds or in environment. The shedding pattern of HPAI is depends on the circulating virus in many of wild bird's population, persistence in water or environment (Henaux and Samuel, 2011). It was found that HPAI viruses have short virus shedding duration (mostly 15 days), therefore the amount of virus shed from the individual bird is important and constitute a basic component in the spread of infection to other bird (Henaux and Samuel, 2011). Selection of potent vaccines capable of reducing virus shedding is a primary factor in controlling the transmission of HPAI in Egypt. Indeed, routine evaluation of the currently used H5 vaccine is important especially after the reporting of new strains of H5N8 belonging the clade 2.3.4.4 which are extensively circulation in poultry population not only in Egypt but also in many countries. Recent reports

indicated the circulation of mutants of H5N8 causing breaks and mortalities in H5 vaccinated flocks (manuscript in preparation).

CONCLUSION

These differences in antigenic mass in H5 inactivated vaccine is critical and greatly related to the reduction of virus shedding. The study demonstrates that 500 HA units of the vaccine seed antigen is enough to induce the protection above 86% and reduce the virus shedding with almost no shedding at 7 and 10 days post challenge. Indeed, the study highlight the importance of both time of vaccination and antigenic content of the inactivated H5 vaccines currently used in Egypt. Also, the study compares the efficacy of the prepared and imported vaccines and concluded that the routine testing of the utilized vaccines in the field is fundamental taking in consideration changing the challenge H5N1 virus by selecting the prevalent strains and updating the challenge virus.

AUTHOR DETAILS

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RECEIVED: May 2018 ; **ACCEPTED**: June 2018; **Published:** July 2018

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Cite this article as:
Hussein Aly ***et al.,*** **(2018**): Antigenic content of 500 HA units in H5N1 reassortant AI vaccine enhances protection and reduces shedding of HPAI H5N1 clade 2.2.1.2 in broiler chickens., *J. of Virol. Sci.,* Vol. 4: 33-43, 2018.