A Dose-Response Study of Inactivated Low Pathogenic Avian Influenza H9N2 Virus in Specific-Pathogen-Free and Commercial Broiler Chickens

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SUMMARY. Since the first report of low pathogenic avian influenza (LPAI) H9N2 virus in Egypt in 2011, the Egyptian poultry industry has suffered from unexpected economic losses as a result of the widespread spread of LPAI H9N2. Hence, inactivated H9N2 vaccines have been included in the vaccination programs of different poultry production sectors. The optimal antigen content of avian influenza virus vaccines is essential to reach protective antibody titers. In this study, the correlation between antigen content (based on hemagglutinating units [HAU]) and postvaccination (PV) antibody response of H9N2 inactivated vaccine was studied. Five different vaccine antigen loads (128, 200, 250, 300, and 350 HAU/dose) were investigated in commercial broiler and specific-pathogen-free (SPF) chickens. Vaccine safety and PV antibody responses were monitored. At the fourth week PV only SPF vaccinated groups (128, 200, 250, and 300 HAU/dose) were challenged using LPAI H9N2 (A/Ck/EG/114940/NLQP/11) virus with 10^5 EID50/bird. Oropharyngeal swabs were used to monitor virus shedding at 2, 4, 6, and 10 days postchallenge. Results showed that all vaccine formulations were well tolerated, and the highest antibody titers were observed in birds vaccinated with higher HAU. Vaccines containing 128 and 200 HAU/dose did not induce the required protective HI titers by 3 wk PV. Meanwhile, the challenge experiment in SPF chickens showed that 250 and 300 HAU vaccine doses were required to reduce the level and duration of virus shedding. Study results thus suggest that inactivated H9N2 vaccines containing at least 250 HAU/dose will induce the optimal protective titers and minimize virus shedding in SPF chickens.

RESUMEN. Estudio de dosis-respuesta en aves libres de patógenos específicos y en pollos de engorde comerciales contra un virus de influenza aviar de baja patogenicidad H9N2 inactivado.

Desde el primer informe del virus H9N2 de influenza aviar de baja patogenicidad (LPAI) en Egipto en el año 2011, la industria de las aves avícolas de Egipto ha sufrido pérdidas económicas inesperadas como resultado de la amplia propagación del virus de baja patogenicidad H9N2. Por lo tanto, se han incluido vacunas inactivadas H9N2 en los programas de vacunación en los diferentes sectores de la producción avícola. El contenido óptimo de antígeno de las vacunas contra el virus de la influenza aviar es esencial para alcanzar los títulos de anticuerpos protectores. En este estudio, se analizó la correlación entre el contenido de antígeno (basado en unidades hemaglutinantes [HAU]) y la respuesta de anticuerpos después de la vacunación (PV) inducida por la vacuna inactivada H9N2. Se investigaron cinco cargas antígenicas vacunales diferentes (128, 200, 250, 300 y 350 HAU/dosis) en pollos de engorde comerciales y en aves libres de patógenos específicos (SPF). Se determinaron la seguridad de las vacunas y las respuestas de anticuerpos después de la vacunación. En la cuarta semana después de la vacunación se desafiaron únicamente los grupos con aves libres de patógenos específicos vacunadas (128, 200, 250, y 300 unidades hemaglutinantes por dosis) usando un virus de baja patogenicidad H9N2 (A/pollo/EGipto/114940/NLQP/11) con 10^5 dosis infectantes para embrión de pollo 50% (EID50) por ave. La eliminación viral se determinó mediante hisopos orofaríngeos a los 2, 4, 6, y 10 días después del desafío. Los resultados mostraron que todas las formulaciones de vacunas se toleraron bien y los títulos de anticuerpos más altos se observaron en las aves vacunadas con las mayores unidades hemaglutinantes. Las vacunas que contenían 128 y 200 unidades hemaglutinantes por dosis no indujeron por tres semanas después de la vacunación, los títulos protectores de inhibición de la hemaglutinación requeridos. Mientras tanto, el experimento de desafío en aves libres de patógenos específicos mostró que se necesitaban 250 y 300 unidades hemaglutinantes por dosis de vacuna para reducir el nivel y la duración de la eliminación del virus. Por tanto, los resultados del estudio sugieren que las vacunas inactivadas H9N2 que contienen al menos 250 unidades hemaglutinantes por dosis inducirán títulos protectores óptimos y minimizarán la diseminación del virus en aves libres de patógenos específicos.

Key words: inactivated vaccine, avian influenza, H9N2, chickens

Abbreviations: AI = avian influenza; DPC = days postchallenge; EID50 = 50% embryo infective dose; HA = hemagglutinin; HAU = hemagglutinating units; HI = hemagglutination inhibition; LPAI = low pathogenic avian influenza; MEVAC = Middle East Company for Veterinary Vaccine; NA = neuraminidase; NLQP = National Laboratory for Veterinary Quality Control on Poultry Production; OIE = Office International des Epizooties; PV = postvaccination; SPF = specific-pathogen-free

Avian influenza (AI) viruses belong to the family Orthomyxoviridae, genus influenza virus A (4). To date, 16 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes have been identified (7). Though H9N2 viruses are low pathogenic avian influenza (LPAI), they induce significant disease in poultry and are occasionally accompanied by considerable mortality (5). The H9N2 viruses have become endemic among both Asian and Middle Eastern poultry, with a rise in Middle Eastern countries of four distinct and cocirculating genetic groups. These have been provisionally named A, B, C, and D, all belonging to the G1 lineage (8,10).
The virus H9N2 AI was first reported in Egypt in a commercial quail farm (6). It was suggested that the H9N2 virus population circulating in the countries bordering Egypt might be considered the main source for viruses detected in the country (14). Since then, H9N2 viruses have spread in Egyptian poultry, producing significant morbidity and high mortality, especially with coinfection with other pathogens such as infectious bronchitis viruses. This has resulted in great economic losses. Though human infection is relatively rare and frequently fatal (16), the fact that both AI viruses of H5N1 and H9N2 subtypes are now endemic in Egypt has raised concern over potential reassortment and has complicated the needed control measures.

Vaccination against AI remains the main control measure against morbidity and mortality, and it was added as a control tool in addition to stamping out in AI control strategy (17,22). Vaccinated birds have shown reduced virus shedding, increased resistance to infection, and subsequently reduction of contact transmission of AI in the field (2). Vaccination in Egypt is currently the only effectively implemented tool for AI control (11). Presently, in the broiler production sector in Egypt, different AI vaccination programs involve vaccination at 7 to 10 days of age (12). Furthermore, an intensive vaccination program (four to six inoculations) using different inactivated vaccines has been adopted in other production sectors (layer, breeder, and grandparent flocks). This intensive program works as follows: The first shot is administered at 10–14 days of age; the second shot at 6–8 wk of age; then other refreshment boosters inoculated every 3–4 mo. The AI vaccinations against both H5N1 and H9N2 are mainly a precautionary measure taken by the producers to preclude any risk for their business at such critical production phases (12).

An effective vaccine must provide a high and long-lasting immune response at a relatively low antigen dose. Therefore, formulation of vaccines using adjuvants to stimulate a robust immune response is an important approach because it helps reduce the antigen dose. Though quantification of viral antigens through a radial immunodiffusion test is an accurate method, indirect methods, such as the HA titer and EID50, are more frequently used (22). 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 quantified serologically (23).

In this study, the efficacy of inactivated H9N2 AI vaccine formulated using the Egyptian strain was evaluated, and the optimal antigen content based on hemagglutinating units (HAU) that provides optimal antibody response was investigated in both commercial and specific-pathogen-free (SPF) broiler chickens.

**Materials and Methods**

**Virus and vaccines.** The avian (A/Ck/EG/114940v/NLQP/11 [H9N2]) virus (GenBank accession no. JQ440373) used in this study was isolated from a broiler flock in Menoufia governorate in Egypt in 2011 at the National Laboratory for Veterinary Quality Control on Poultry Production (NLQP), Animal Health Research Institute, Dokki, Giza, Egypt. The selected virus was propagated using SPF embryonated chicken eggs (Nike SPF eggs, Koom Oshiem, Fayoum, Egypt) that were confirmed to be free of other biological contaminants, including avian influenza H5N1, avian leukosis subgroup J, infectious bursal disease virus, avian reovirus, infectious bronchitis virus, Newcastle disease virus, chicken infectious anemia, avian encephalomyelitis virus, Adeno viruses, EDS76 virus, avian mycoplasma, and Salmonella spp.

**Virus inactivation and vaccine formulation.** The different vaccine formulas were prepared according to the Office International des Epizooties (OIE) manual of diagnostic tests and vaccines for terrestrial animals (15). Briefly, the virus was inactivated using 0.2% formalin (Sigma Chemical Co., St. Louis, MO). The hemagglutination activity of the inactivated virus harvest was calculated using hemagglutination test. Antigen loads of 128, 200, 250, 300, and 350 HAU/dose were then mixed with MONTANIDE™ISA 70 VG adjuvant (SEPPIC SA, Puteaux, France). The antigen aqueous phase was preemulsified with ISA 70 at a 30:70 ratio (w/w) at 1000 rpm using the Silverson L5M high-shear laboratory mixer (Silverson Machines Inc., Buckinghamshire, United Kingdom). The speed of the mixer was then increased to 3000 rpm and maintained for 30 min for the emulsification of 10,000 doses of the vaccine. During the emulsion formulation, the temperature of the mixture was maintained between 18 and 22 C. Prepared emulsions were then tested for viral and bacterial sterility before being used in animal experiments.

**Bird immunization.** Sixty commercial broiler (Cobb 500) and 60 SPF (white leghorn) chickens 3 wk of age were used in this immunization study. Both the commercial broiler and SPF chickens were allotted into six groups (10 birds/group). Birds in groups 1 to 5 were vaccinated via the intramuscular (thigh muscle) route with 128, 200, 250, 300, and 350 HAU, respectively (in 0.5 ml doses of the prepared vaccine formulas). Group 6 was kept as an unvaccinated negative control. Serum samples were collected on a weekly basis for 4 wk postvaccination (PV). The hemagglutination inhibition (HI) test was carried out to monitor the postvaccination humoral immune response for each vaccine formula using the homologous (A/Ck/EG/114940v/NLQP/11[H9N2]) HA antigen (15). HI reactivity was determined using a 1% suspension of chicken red blood cells, and the seroconversion rate was estimated as the proportion of birds with $\geq 2^3$ HI titers.

**Challenge experiment.** Experiments were conducted in biosafety level III chicken isolators at the Middle East Company for Veterinary Vaccine (MEVAC), Egypt. The experiment was conducted according to MEVAC guidelines on research ethics in animals. Because of a limited number of available isolators and based on the HI test results, challenge experiments were conducted only on SPF chicken groups, which were vaccinated with antigen loads of 128, 200, 250, and 300 HAU/doses. SPF vaccinated chicken groups and the unvaccinated control group were challenged using the LPAI (A/Ck/EG/114940v/NLQP/11[H9N1]) virus strain with a dose of 10$^6$ EID50/ml/bird via the ocular-nasal route at 4 wk PV. Challenged birds were monitored on a daily basis for 10 days postchallenge (DPC) for clinical signs, morbidity, and mortality. Oropharyngeal swabs were collected from surviving birds at 2, 4, 6, and 10 DPC to monitor number of shedders and measure virus shedding titers. The collected swab samples were vortexed in 1 ml of phosphate buffered saline and then centrifuged at 2000 rpm for 10 minutes at 4 C to pellet the debris. The supernatants were then used for virus titration in 10-day SPF embryonated chicken eggs and EID50/ml was calculated (18).

**Statistical analysis.** One-way ANOVA with Tukey’s posttest was used to estimate differences between HI antibody titers and virus shedding titers. A two-sided $\alpha$ level of 0.05 was used to determine significance. Correlation between HI antibody titers and antigen load/dose was determined using the Spearman correlation test via GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA; www.graphpad.com).

**Results**

**Antibody response in commercial broiler chickens.** In commercial broiler chickens, detectable antibody response and seroconversion were discerned by the first week PV in all groups except birds that
had received the 128 and 200 HAU doses (Table 1). The mean HI log₂ titers (3.1 ± 0.7) and seroconversion rate (80%) were significantly higher in birds that had received the 250 HAU doses compared with those that had received the 300 and 350 HAU doses. At the second week PV, the HI antibody titers in the groups that had received 250, 300, and 350 HAU were significantly higher (4.3 ± 0.4, 4.2 ± 0.6, 4.5 ± 0.5, respectively) than those of birds that had received 128 and 200 HAU (2.9 ± 0.7 and 3.5 ± 0.8, respectively). Birds that had received 300 and 350 HAU showed significantly (*P < 0.01*) higher antibody titers at 3 and 4 wk PV compared with other groups. Also, groups that had received 300 and 350 HAU doses reached protective titers (6 log₂) by the third week PV, while other groups reached this titer by the fourth week PV (Table 1). The PV seroconversion rate (titers ≥ 3 log₂) was 100% in the groups vaccinated with the 250, 300, and 350 HAU doses starting from the second week PV.

Antibody response in SPF chickens. In SPF chickens, about 20% of birds that had received 128 and 200 HAU doses showed positive (≥3 log₂) antibody titers. Comparatively, more than 50% of birds in the other groups were seropositive at 1 wk PV (Table 1). There were no statistical differences between the mean HI titers of birds that had received the 250, 300, or 350 HAU doses during all weeks PV. However, these groups’ titers were significantly higher than those of two groups that had received the 128 and 200 HAU doses. The same trend was observed for the seroconversion rates. All birds of different groups showed 100% seroconversion rate by the second week PV (Table 1).

Correlation between the mean HI antibody titers and antigen loads. In general, the postvaccination humoral antibody responses in SPF vaccinated groups were significantly higher than their levels (1–2 log₂) in the commercial broiler vaccinated groups in all weeks. The postvaccination HI antibody response of different vaccine formulas in both commercial broilers and SPF chickens showed positive correlation with the antigen load/dose. Nevertheless, a clear statistically significant correlation was observed in SPF vaccinated groups at 2 and 4 wk PV (Spearman correlation coefficient of 0.97; *P = 0.02*) (Fig. 1).

Virus shedding in SPF chickens. Only the SPF bird groups vaccinated with the 128, 200, 250, and 300 HAU doses were challenged in order to discern the optimal HAU load required/dose. The 350 HAU dose was excluded because it did not show significant differences when compared with the groups vaccinated with both the 250 and 300 HAU doses.

In general, all challenged birds in vaccinated groups did not show any clear clinical signs. However, the unvaccinated control challenged group showed mild respiratory signs. No mortalities were recorded in all challenged groups. As shown in Table 2, the amount of virus shedding titers was significantly lower in all vaccinated groups compared with the unvaccinated challenge control group in all testing time points.

The number and shedding rate during the second DPC were 10/10 (100%) of the 128 HAU vaccinated group. However, we have recorded a 5/10 (50%) shedding rate in the groups that had received 200 and 250 HAU doses. Only 2/10 (20%) of birds that had received the 300 HAU doses were positive shedders at the second DPC. By the fourth DPC only 10% of birds that had received the 300 HAU doses, 20% of birds that had received the 200 and 250 HAU doses, 50% of birds that had received the 128 HAU dose, and 70% of birds in the unvaccinated challenge control group were positive shedders. At the sixth DPC, virus shedding diminished in groups that had received the 250 and 300 HAU dose. Only 10% and 30% of birds maintained virus shedding in the 200 and 128
HAU vaccinated groups with mean titer of 1.7 ± 0.3 and 2.0 ± 0.4 EID₅₀/ml, respectively. Also 30% of birds in unvaccinated challenge control groups were positive shedders with significantly higher mean virus titer of 3.57 ± 0.5 EID₅₀/ml (Table 2). In general both the 250 and 300 HAU doses were capable of providing proper protection and stopping virus shedding by 6 DPC.

**DISCUSSION**

Since the first H9N2 LPAI outbreak in late 2011 (6), several outbreaks have been reported in Egypt. H9N2 infection has become one of the greatest challenges to the Egyptian poultry industry. Therefore, in Egypt the use of LPAI H9N2 vaccine began in 2012 using the local strain (A/Ck/EG/114940v/NLQP/11) and became the only implemented control tool against LPAI H9N2 infection. For an effective vaccination strategy, it is necessary to select a vaccine strain that has a genetic and antigenic relationship to the circulating field viruses (26). Therefore, the selected vaccine strain virus (A/Ck/EG/114940v/NLQP/11 H9N2) was isolated in 2012. It belongs to the G1 lineage (1,6). Studies of H9N2 AI viruses in Egypt showed minor evolution with no significant differences in the HA gene compared with the recent Egyptian isolates (data not shown).

The quantity of viral antigen in the vaccine is known to be important in protecting from morbidity and mortality (24,26). In this study, the efficacy of different inactivated H9N2 vaccines containing different HAU doses was evaluated in commercial broilers and SPF chickens at 3 wk of age. Concentrations of these HAU doses could be approximately estimated to be about 10, 12, 16, 20, and 24 μg/dose based on previous studies that highlighted the relationship between HAU and HA protein concentration (13). The current study aimed
to test the 128, 200, 250, 300, and 350 HAU doses, all per 0.5 ml. The PV humoral antibody response of different vaccine formulas in both commercial broilers and SPF chickens showed positive correlation to the antigen load in all study weeks. The same results of positive correlation between the HI antibody responses and HPAI vaccines antigen levels were also reported (20).

Detectable antibody titers were noticed by the first week PV with the exception of commercial broilers that had received the 128 HAU/dose. Though the same trend of increasing HI antibody titers was observed in both SPF and commercial broilers, the commercial broilers showed relatively lower HI titers compared to SPF chickens. This difference was expected due to type of birds bred for meat and layer types and also possibly due to the interference of maternal antibodies (19). Protective HI antibody titer against disease and virus shedding in chickens is arguable (13,24) because it depends on several factors, including vaccine antigen content, vaccine preparation, age of chickens at vaccination, and time between vaccination and challenge (9,24). In this study, based on both seroconversion and vaccine efficacy testing, a protective HI titer of $\geq 4 \log_2$ was proposed (20). This protective HI antibody titer was achieved as early as the second week PV in SPF birds that had received 250, 300, and 350 HAU and by the third week PV in the remaining groups. These results further confirm that a rapid and high-level immune response against HI could be achieved using higher amounts of antigen (20). However, high antigen concentration requires an increased vaccine production cost. Therefore, in the current study, the optimal antigen content determination was not dependent only on serological response but also on maximal effectiveness against the challenge virus.

There was no significant difference between the 300 and 350 HAU groups’ results at different time points and between 250, 300, and 350 HAU/dose groups by 3 wk PV. Based on these results the 350 HAU/dose group was excluded from the challenge test. Virus shedding titers via oropharyngeal swabs were significantly lower in all vaccinated groups compared with the unvaccinated challenge control. The virus shedding was minimal (20%) of birds with low titers in birds that had received the 200, 250, and 300 HAU doses, and it was diminished in groups that had received the 250 and 350 HAU doses by the sixth DPC.

These results also support that the minimal HAU required to induce earlier and protective HI antibody titers, to provide clinical protection, and to reduce virus shedding of H9N2 AI virus was the 250 and 300 HAU doses (approximately 16–20 μg/dose). Previous studies using 128 or 200 HAU/doses of inactivated H9N2 AI virus inducted high antibody titers, but the challenge virus shedding could not be prevented (3). Conversely, studies on HPAI vaccines were variable where, for example, an antigen dose of 512 HAU/chicken can completely prevent morbidity and mortality and reduce virus shedding of HPAI H7N3 virus (21). In another study, a single vaccination of chickens with an inactivated H5N3 oil emulsion vaccine containing as low as 0.25 μg HA protein was highly effective against H5N1 challenge (25). The difference in antigen mass measurement methods, vaccines, and challenge virus strains used may explain the different study outcomes.

In conclusion, a dose of 250 HAU seems to be the minimal essential dose in the induction of a protective postvaccination humoral immune response in SPF chickens with maximal reduction in virus shedding. Meanwhile, at least 300 HAU/dose is needed to induce better HI antibody response in broiler chickens. The importance of the vaccine antigen dose optimization is not simply for clinical protection but also extends to the prevention of virus circulation.

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