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Impact of vaccination on infection with Vietnam H5N1 high pathogenicity avian influenza virus in hens and the eggs they lay

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ABSTRACT

High pathogenicity avian influenza virus (HPAIV) infections in chickens negatively impact egg production and cause egg contamination. Previously, vaccination maintained egg production and reduced egg contamination when challenged with a North American H5N2 HPAIV. However, Asian H5N1 HPAIV infection has some characteristics of increased pathogenicity compared to other H5 HPAIV such as more rapid drop and complete cessation in egg production. Sham (vaccinated at 25 and 28 weeks of age), inactivated H5N1 Once (1X-H5-Vax; vaccinated at 28 weeks of age only) and inactivated H5N1 Twice (2X-H5-Vax; vaccinated at 25 and 28 weeks of age) vaccinated adult White Leghorn hens were challenged intranasally at 31 weeks of age with 6.1 $\log_{10}$ mean embryo infectious doses (EID_{50}) of clade 2.3.2.1a H5N1 HPAIV (A/chicken/Vietnam/NCVD-675/2011) which was homologous to the inactivated vaccine. Sham-vaccinated layers experienced 100% mortality within 3 days post-challenge; laid soft and thin-shelled eggs; had recovery of virus from oral swabs and in 53% of the eggs from eggshell surface (35%), yolk (24%), and albumin (41%); and had very high titers of virus (average 7.91 $\log_{10}$ EID_{50}/g) in all segments of the oviduct and ovary. By comparison, 1X- and 2X-H5-Vax challenged hens survived infection, laid similar number of eggs pre- and post-challenge, all eggs had normal egg shell quality, and had significantly fewer contaminated eggs with reduced virus quantity. The 2X-H5-Vax hens had significantly higher HI titers by the day of challenge (304 GMT) and at termination (512 GMT) than 1X-H5-Vax hens (45 GMT and 128 GMT). The current study demonstrated that AIV infections caused by clade 2.3.2.1a H5N1 variants can be effectively controlled by either double or single homologous vaccination.

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1. Introduction

Since its emergence in 1996 in Guangdong, China, H5N1 highly pathogenic avian influenza (HPAI) virus (HPAIV) has spread widely across Asia, Europe, and Africa, infecting domestic and wild birds and occasionally spilling over into mammals including humans [1,2]. Over time, the H5N1 HPAIV has continuously diverged into multiple phylogenetically distinct lineages based on the H5 hemagglutinin (HA) gene [3,4]. The predominant H5N1 lineage currently circulating in South East Asia is clade 2.3.2 [3]. In Vietnam in particular, since 2004 an influenza surveillance program has been implemented by the Department of Animal Health (DAH), the National Centre for Veterinary Diagnostics (NCVD), and regional DAH laboratories. This program, which entailed the utilization of stamping-out for infected flocks, and selection of vaccine seed strains and their application in a vaccination program, sought to identify and characterize H5N1 viruses and was coupled with systematic active surveillance and outbreak response programs in affected regions [5–7]. Such measures have significantly reduced the number of outbreaks from 2003 to 2011 [8]. However, vaccine pressure, together with trade of infected poultry and the close proximity to foreign borders where other clades continue to circulate, may have facilitated a rapid evolution of co-circulating H5N1 HPAIV into new variants with distinct antigenic properties and virulence, like clade 2.3.2.1 lineage which dominates in Vietnam and continues to evolve into viruses that replicate systemically in domestic ducks and have more shedding potential [7,9].

HPAIV infections produce systemic fatal disease in chickens, commonly alongside severe drops in egg production and decreased egg quality as reported after natural and experimental infections [10–15]. In both natural infections [14,16–19] and experimental studies [15,20–23], viral replication occurs systemically, with HPAIV being isolated in multiple internal organs of chickens [17–20] including the reproductive tract [17,19,23], on the eggshell surface [14–16,18,22], and from the yolk [15,16,20–22] and albumin [14–16,20–22]. On one hand, these findings indicate that

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contaminated eggs via reused egg flats and equipment could represent a transmission route between chicken flocks, as already observed in turkeys [24]. On the other hand, consumption of raw eggs carrying HPAIV could represent a potential risk factor for human infections.

Typically, mortality and viral shedding have been the main parameters in poultry to assess protection through vaccination [25]. Some studies have also evaluated vaccine efficacy using metrics of prevention of egg production drops and/or decreased virus contamination of eggshell and egg internal contents. Previously, multiple dose vaccination with a commercial H5N2 vaccine was able to decrease clinical signs and mortality of broiler breeder chickens upon challenge with HPAIV of classic Egyptian clade 2.2.1 [22]. Even if the amount of virus shed was reduced in vaccinated birds, considerable amounts of viral RNA were still detected throughout the experiment in all birds' swab samples as well as on eggs. On the contrary, no protective effects were seen against a challenge virus of the variant 2.2.1 lineage, being the virus consistently detected in swabs and eggs (shell and albumin samples) [22]. In our recent study, layer chickens immunized once or twice with an inactivated H5N9 vaccine prevented not only mortality but also declines in egg production after challenge with a Pennsylvania 1983 H5N2, and reduced number of and virus quantity in contaminated eggs (both eggshell and internal contents) [15].

Asian H5N1 HPAIV possess an increased pathogenicity in birds and mice compared to other H5 HPAIV [26,27]. In addition, the long-term circulation of H5N1 HPAIV has led to the emergence of variant viruses which have increased virulence in chickens (i.e. shortened mean death times [MDT]), but with unknown changes on pathogenesis of virus infection, especially in reproductive tract. In addition, the ability of inactivated vaccines to protect the reproductive tract against increased virulent viruses is unknown. The present study was conducted to assess how Asian clade 2.3.2.1 H5N1 HPAIV affect egg production in White Leghorn (WL) hens, determine the presence and quantity of virus located on or within eggs, assess virus replication in the reproductive tract, and determine the effect of once or twice vaccination as a mitigation strategy.

2. Materials and methods

2.1. Virus

The clade 2.3.2.1a H5N1 HPAIV isolate from Vietnam (A/chicken/Vietnam/NCVD-675/2011) (VN/11) was used as the vaccine seed strain and the challenge virus. The virus was propagated and titrated by allantoic sac inoculation of 9 day-old embryonating chicken eggs (ECE) by standard methods [28].

2.2. Vaccine

Infectious allantoic fluid containing VN/11 HPAIV was inactivated with 0.1% β-propiolactone (BPL, Sigma-Aldrich, St. Louis, MO) and used to prepare an oil-in-water emulsion vaccine as previously described [29–31]. Briefly, one part (10 ml) of aqueous antigen was emulsified in four parts (40 ml) oil phase, which contained 36 ml Drakeol 6 VR pharmaceutical grade mineral oil (Penreco, Karns City, PA), 3 ml Arlacel 80 (7.5% sorbitan mono-oleate, Sigma Aldrich), and 1 ml Tween 80 (2.5% polysorbate, Sigma Aldrich). Vaccines were prepared 2 days prior to administration and homogenized using a Waring blender (Fisher Scientific International Inc., Hampton, NH) with the following mixing speeds: 1 min on low speed, 1 min rest, 1 min on low speed, 1 min rest, 30 s on high speed [32]. Vaccines were incubated for 1 h at 37 °C and stored at 4 °C until use. Vaccines were subcutaneously administered in the nape of the head in a dose of 512 HA units in 0.5 ml per hen. Sham-vaccinated birds received the vaccine containing sterile, non-infected specific-pathogen-free (SPF) allantoic fluid.

2.3. Animals and housing

Reproductively active, SPF WL hens (23 weeks of age) from *in house* flocks were provided feed and water *ad libitum* and 16 h of daily light. Hens were individually housed in layer cages contained within a HEPA-filtered vinyl enclosure (Class Biologically Clean, Ltd., Madison, WI) located within an animal biosafety level 3 enhanced (ABSL-3E) facility. All procedures were performed according to the requirements of the protocol approved by the Institutional Laboratory Animal Care and Use Committee.

2.4. Experimental design and sampling

Thirty-four hens were randomly distributed into three groups: 10 sham-vaccinated hens (group 1), 12 H5-vaccinated-Once hens (group 2, 1X-H5-Vax), and 12 H5-vaccinated-Twice hens (group 3, 2X-H5-Vax). 1X-H5-Vax hens were vaccinated at 28 weeks of age, while 2X-H5-Vax hens were vaccinated at 25 and 28 weeks of age. Negative control hens received a sham vaccine at both time points. At 31 weeks of age, all hens were bled and intranasally inoculated with approximately 10^6 mean chicken embryo infectious doses (EID_{50}) of VN/11 HPAIV in a volume of 0.1 ml. The inoculum titer was subsequently verified as $10^{6.1}$ EID_{50}/0.1 ml by back titration in ECE.

Hens were monitored daily for clinical signs and mortality. Egg production was daily recorded pre-challenge (−6 to 0 dpc) and post-challenge (1 to 14 dpc). Individual eggs were collected daily from 1 to 14 dpc for virus isolation. Each egg's entire surface area was wet swabbed before being handled and each swab was placed in 1.0 ml brain heart infusion (BHI) media (Becton, Dickinson and Company, Sparks, MD) with penicillin (2000 units/ml; Sigma Aldrich), gentamicin (200 μg/ml; Sigma Aldrich) and amphotericin B (5 μg/ml; Sigma Aldrich), mixed thoroughly by agitation, and stored at −70 °C. For yolk and albumin samples, each egg was cracked and the content was carefully placed in a petri dish, avoiding the yolk to break and mix with the albumin. Albumin was mixed thoroughly and a 2.0 ml sample was removed using a serological pipet. Egg yolk was mixed thoroughly, and a 1.0 ml sample was removed using a displacement pipet and diluted 1:2 in BHI because of high viscosity. Egg samples were stored at −70 °C. Oropharyngeal swabs were collected in BHI on any euthanized or dead birds, and up to two hens per group were necropsied. Ovary, infundibulum, magnum, isthmus, uterus (or shell gland), and vagina were collected in 10% neutral buffered formalin (Thermo Fisher Scientific, Waltham, MA) and routinely processed to paraffin embedded sections to assess histopathologic changes and to identify the site of virus replication in each portion of the reproductive tract and the affected cell types. Heart, kidney, duodenum, and pancreas were collected to confirm systemic infection. All sections of the reproductive tract were also collected in BHI. Virus isolation and titration in ECE was attempted from oropharyngeal swabs, yolk, albumin, and shell surface swab samples as previously described [28], and from reproductive sections in BHI as described elsewhere [33]. Surviving birds at 14 dpc were bled and euthanized using cervical dislocation.

2.5. Serology

Blood samples were taken at time of vaccination (i.e., at 25 weeks of age in sham-vaccinated and 2X-H5-Vax hens, and at 28 weeks of age in all hens), challenge (0 dpc), and termination (14 dpc). Hemagglutinin inhibition (HI) assays were carried out using H5 antigen specific for the vaccine seed virus. The