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Assessment of Reduced Vaccine Dose on Efficacy of an Inactivated Avian Influenza Vaccine Against an H5N1 High-Pathogenicity Avian Influenza Virus[1]

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Primary Audience: Poultry Veterinarians, Poultry Producers, Public Health Officials, Poultry Health Researchers

SUMMARY

Avian influenza (AI) vaccines are a viable emergency tool for use in a comprehensive strategy for dealing with high-pathogenicity AI in developed countries. However, the available doses of inactivated AI vaccine are limited to national vaccine banks and inventory stocks of some commercial biologics manufacturers. To determine if the available vaccine doses could be stretched by using reduced vaccine dose but maintain adequate efficacy, a study was conducted to determine if 3-wk-old specific pathogen-free White Leghorn chickens vaccinated with full, ½, ¼, and 1/10 doses of an inactivated H5N9 AI vaccine would be protected against a high-dose challenge of H5N1 highly pathogenic AI virus given 4 wk later. At all 4 AI vaccine doses, the AI-vaccinated chickens were protected from disease and death, but all the sham-vaccinated chickens developed clinical signs and died. There were no differences between the full, ½, and ¼ dose AI vaccine groups for serological titers at 7 wk of age, or for cloacal and oropharyngeal titers of challenge virus shed at 2 d postchallenge. However, the 1/10 dose group had significantly reduced hemagglutination inhibition titers at 7 wk compared with ¼ dose, and the 1/10 dose group had more chickens shedding challenge virus from the oropharynx than the full dose group. Most importantly, the mean protective dose was 1/50 dose, and using the international regulatory standard of 50 mean protective doses for Newcastle disease vaccine as a guide, the full dose of the H5N9 AI vaccine would be the minimum dose acceptable for use in the field. Use of the full vaccine dose is especially important, because protection in commercial chickens in the field is typically less than seen in experimental studies in specific pathogen-free chickens in the laboratory.

Key words: avian influenza, high-pathogenicity avian influenza, H5N1, vaccine

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DESCRIPTION OF PROBLEM

High-pathogenicity (**HP**) avian influenza (**AI**) virus of the H5N1 subtype has caused an unprecedented epizootic in domestic and wild birds in 60 Asian, African, and European countries since it was first reported in 1996 [1]. The occurrence of H5N1 HPAI viruses represents substantial economic losses to poultry producers and presents the risk of worldwide spread of these viruses. Vaccination has emerged as a tool for use in controlling H5N1 HPAI virus infections in view of future eradication [2]. Immunization with inactivated H5 vaccine has provided protection against clinical signs and death, as well as decreased virus replication and shedding in infected birds, thereby reducing environmental contamination and transmission [3–7].

Currently, the USDA vaccine bank contains 70 million doses of inactivated H5 vaccine in reserve for potential use in controlling an outbreak of H5 HPAI in the United States. In addition, several manufacturers have experimental or licensed vaccines available for use [3, 8].

Because of limited vaccine availability, this study was conducted to determine if currently available inactivated AI vaccine supplies could be extended while maintaining acceptable protection when administered to young chickens.

MATERIALS AND METHODS

Dose Comparison Experimental Design

To evaluate protection by reduced vaccine dose, 3-wk-old specific pathogen-free (**SPF**), mixed-sex White Leghorn chickens (*Gallus domesticus*) [9] were divided into 5 groups of 10 birds each. Blood was collected from the brachial veins to verify by agar gel precipitation (**AGP**) and hemagglutination inhibition (**HI**) tests that they were naive to type A influenza virus infections [10]. Chickens in 1 group received a mineral oil emulsion blank (sham) vaccine, whereas chickens in the second group were vaccinated with a full dose of the H5N9 AI vaccine in 0.5 mL [11]. Chickens in groups 3, 4, and 5 were immunized with 0.5 mL of ½, ¼, or 1⁄10 dose of the inactivated H5N9 vaccine, respectively, which was produced by dilution of the full-strength AI vaccine with the sham vaccine. All vaccine was administered s.c. in the nape of the neck. Each group of birds was housed separately in isolation cabinets maintained under continuous lighting in a USDA-certified biosafety level 3 agriculture facility. The cabinets were ventilated under negative pressure with high-efficiency particulate air-filtered air, and the chickens were allowed free access to feed and water.

Four weeks after immunization, the chickens were bled for serum and challenged intranasally with 0.1 mL containing 10^6 mean embryo infectious doses (**EID_{50}**) of H5N1 HPAI virus. The chickens were observed daily for clinical signs and mortality. On 2 d postchallenge (**DPC**), which corresponded to the peak time of virus shedding, swabs from the cloaca and oropharynx were collected, stored, and processed. At approximately 8.5 wk of age (10 DPC), blood was collected for serology, and the birds were euthanatized and necropsied. Mean death time in DPC was calculated.

Vaccine Potency Testing. To determine the vaccine potency, the mean protective dose (**PD_{50}**) was determined. Animal housing and care procedures were the same as those described previously. Groups of ten 3-wk-old SPF chickens [9] each received a 0.5-mL dose of undiluted, 1⁄10, 1⁄100, or 1⁄1,000 vaccine dose s.c. in the nape of the neck. At 7 wk of age, the birds were challenged intranasally with 10^6 EID_{50} of H5N1 HPAI virus. The PD_{50} was calculated using the method of Spearman and Kärber using mortality as the end point [12].

Challenge Virus

The challenge virus was second chicken embryo passage of A/chicken/Yamaguchi/7/04 (H5N1) HPAI virus [13].

Serological and Virological Assays

At 2 DPC, swabs of the cloaca and oropharynx were collected and stored at −70°C until tested for virus using standard chicken embryo virus isolation and titration procedures utilizing 3 eggs per swab [10]. Titers were expressed as the EID_{50} per milliliter of swab fluid with the lowest detectable titer of $10^{0.97}$ EID_{50}/mL of media.

Sera were tested for the presence of antibodies against the influenza A nucleoprotein/matrix protein by AGP test and H5 hemagglutinin by

the HI test [10]. For HI tests, inhibition at a 1:8 or higher dilution was considered positive.

Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction for the Quantification of AI Virus in Vaccines

The RNA was extracted from the vaccine with a method developed for oil emulsion vaccines as previously described [3]. The same virus isolate used to make the vaccine was used to produce the standard curve as follows: allantoic fluid virus stocks were diluted in brain heart infusion broth [14] and titrated in embryonating chicken eggs as per standard methods at the time of dilution [10]. Whole-virus RNA was extracted for the standard curve along with the vaccine samples by the same method, and quantitative real-time reverse transcription-polymerase chain reaction (**qRRT-PCR**) targeting the influenza matrix gene was performed [15]. The RNA was extracted from the sham vaccine as a negative control. Each sample was extracted and run on real-time reverse transcription-polymerase chain reaction in a replicate of 12. The cycle threshold values of the standard curve were used to approximate the titer of the virus in the vaccine in EID_{50}.

Statistics and Data Analysis

Frequency of morbidity, mortality, virus isolation, and detection of antiinfluenza virus antibodies were analyzed for significance ($P < 0.05$) by Fisher's exact test on personal computer-based software [16]. Virus isolation and HI serological titers were tested for normal distribution. Normally distributed data sets were further tested by parametric 1-way ANOVA. Data that failed a normality test were analyzed by a nonparametric ANOVA test (Kruskal-Wallis), and when significant differences were noted in the groups ($P < 0.05$), a Dunn's multiple comparison test was performed. Normality, ANOVA, Kruskal-Wallis, and Dunn's tests were performed on personal computer-based software [16].

The minimum virus titer detectable by virus isolation procedures in this study was estimated to be $10^{0.97}$ EID_{50}/mL. Thus, for statistical purposes, all oropharyngeal and cloacal swabs from which virus was not isolated were given a numeric value of $10^{0.90}$ EID_{50}/mL, which represents the lowest detectable level of virus if the virus isolation procedures were modified to use 4 instead of 3 embryonating chicken eggs per sample.

RESULTS AND DISCUSSION

Various criteria can be used to assess AI vaccine protection in chickens. The HI or neutralizing serological response can be indirect measures of protection, whereas direct measures of protection can be prevention of morbidity and mortality, reduction in the number of birds shedding challenge virus, reduction in the titer of challenge virus shed, and assessment of PD_{50} vaccine dosage. Before the vaccination, the chickens lacked serological evidence of infection with influenza A viruses. Four weeks after vaccination, all AI-vaccinated chickens had AGP and HI antibodies, whereas the sham-vaccinated chickens lacked antibodies against influenza A proteins (Table 1). The HI titers for chickens receiving the full, ½, and ¼ doses were not significantly different from each other but were significantly different from the sham-vaccinated birds (Table 1). The mean HI titer of the 1⁄10 dose group was less than half the level of the other 3 groups and significantly different from ¼ dose (Table 1). The presence of measurable HI antibodies in vaccinated birds has been associated with effective immunization and protection from virulent challenge by an AI virus within the same hemagglutinin subtype [17]. After challenge in the current study, there were no significant differences in the HI titers of the 4 AI vaccine groups, although the mean titer in the 1⁄10 vaccine dose group doubled compared with prechallenge titers, suggesting less of a protective response by vaccination (Table 1).

After challenge, all sham vaccine group chickens became sick and died, whereas all chickens in the 4 AI vaccine groups were protected (Table 1). Protection from clinical signs and death are important assessments of vaccine effectiveness against HPAI viruses, but other criteria such as challenge or field virus replication and shedding are also important, because their presence could indicate a source of environmental contamination and potential transmission [18]. In the current study, significantly fewer

Table 1. Morbidity, mortality, serological, and virological data[1] from 3-wk-old specific pathogen-free chickens vaccinated with inactivated avian influenza (AI) vaccine and challenged at 7 wk of age with a H5N1 high-pathogenicity AI virus

Vaccine group	Morbidity and mortality (MDT)[4]	Serology[2]						Virus isolation-2 DPC ($\log_{10}$ EID_{50}/mL)[3]	
		AGP			HI (GMT)				
		3 wk	7 wk	8.5 wk	3 wk	7 wk	8.5 wk	Oral	Cloacal
Sham	$10/10^{a}$ (2.0)	$0/10^{a}$	$0/10^{b}$	NS[5]	$0/10^{a}$	$0/10^{b}$ ($<8^{C}$)	NS	$10/10^{a}$ (6.76^{A})	$10/10^{a}$ (5.82^{A})
1 × AI vaccine	$0/10^{b}$	$0/10^{a}$	$10/10^{a}$	$10/10^{a}$	$0/10^{a}$	$10/10^{a}$ (832^{AB})	$10/10^{a}$ (724)	$2/10^{c}$ (1.17^{B})	$0/10^{b}$ ($\leq 0.90^{B}$)
½ × AI vaccine	$0/10^{b}$	$0/10^{a}$	$10/10^{a}$	$10/10^{a}$	$0/10^{a}$	$10/10^{a}$ (891^{AB})	$10/10^{a}$ (776)	$3/10^{bc}$ (1.14^{B})	$0/10^{b}$ ($\leq 0.90^{B}$)
¼ × AI vaccine	$0/10^{b}$	$0/10^{a}$	$10/10^{a}$	$10/10^{a}$	$0/10^{a}$	$10/10^{a}$ (955^{A})	$10/10^{a}$ (549)	$5/10^{bc}$ (1.82^{B})	$2/10^{b}$ (1.01^{B})
1/10 × AI vaccine	$0/10^{b}$	$0/10^{a}$	$10/10^{a}$	$10/10^{a}$	$0/10^{a}$	$10/10^{a}$ (416^{BC})	$10/10^{a}$ (955)	$8/10^{ab}$ (3.04^{AB})	$0/10^{b}$ ($\leq 0.90^{B}$)
Statistical analyses									
Normality	—	—	—	—	—	Failed $P < 0.001$ KW[6]	Passed $P = 0.041$ ANOVA[7]	Failed $P < 0.001$ KW	Failed $P < 0.001$ KW
1-way ANOVA[6,7]	—	—	—	—	—	$H^{8} = 39.693$ df = 4 $P < 0.001$	$F^{9} = 2.673$ df = 3 $P = 0.062$	H = 42.948 df = 4 $P < 0.001$	H = 32.933 df = 4 $P < 0.001$

[a,b]Different superscript lowercase letters indicate significant difference in frequency of positive results between individual vaccine groups ($P < 0.05$).
[A–C]Different superscript uppercase letters indicate significant differences between vaccine groups (Dunn's method; $P > 0.05$).
[1]Frequency data are reported as number of chickens positive (morbidity, mortality, antibody, or virus)/total number in group.
[2]AGP = agar gel precipitation; HI = hemagglutination inhibition. GMT = geometric mean titer.
[3]2 DPC = 2 d postchallenge; EID_{50} = 50% embryo infectious dose.
[4]All chickens with clinical signs and lesions died. MDT = the mean death time in days postchallenge.
[5]NS = no survivors.
[6]KW = nonparametric Kruskal-Wallis.
[7]ANOVA = parametric 1-way ANOVA.
[8]H = Kruskal-Wallis H test.
[9]F = ANOVA F test.

full, ½, ¼, and 1/10 dose vaccinated chickens shed challenge virus from the digestive tract (i.e., cloaca) as compared with the sham-vaccinated chickens, and the virus titers shed by each of the 4 AI vaccine groups were significantly less than the sham group but were not different among the 4 vaccine doses (Table 1). For the respiratory tract, significantly fewer full, ½, and ¼ dose vaccinated chickens shed challenge virus from the respiratory tract (i.e., oropharynx), and the titers shed were significantly less as compared with the sham group (Table 1). However, significantly more chickens in the 1/10 dose vaccine group shed challenge virus than the full dose vaccine group, and the virus titers shed were higher in the 1/10 dose group, although the mean titers were not significantly different (Table 1). Therefore, some criteria of protection, such as morbidity, mortality, and digestive tract shedding, were not different among the 4 AI vaccine doses, but for 1 criterion, virus shed from the respiratory tract, the 1/10 AI vaccine dose was less effective than the full dose. Similarly, in other studies, efficacious inactivated AI vaccine at full dose has reduced virus shed from the respiratory and digestive tracts in addition to providing protection from morbidity and mortality [3, 7, 17].

A quantitative measure of vaccine potency is the PD_{50} (i.e., the dose of vaccine that protects 50% of the birds from challenge) [17]. In this study, the PD_{50} for the H5N9 AI vaccine, using

Table 2. Data supporting mean protective dose (PD_{50}) determination for chickens using mortality as measure of protection in 3-wk-old specific pathogen-free chickens vaccinated with 1, 0.1, 0.01, and 0.001 doses of inactivated avian influenza (AI) vaccine and challenged at 7 wk of age with a H5N1 high-pathogenicity AI virus[1]

Vaccine dose	Mortality[2]	PD_{50}
1	0/10	0.02
0.1	0/10	
0.01	8/10	
0.001	10/10	

[1]The PD_{50} was calculated using the method of Spearman and Kärber [12].
[2]Number dead/total in the group.

mortality as the metric, was 0.02 or 1:50 of a full dose (Table 2). With inactivated Newcastle disease vaccine, the World Organization for Animal Health recommends 50 PD_{50} [19] per dose as a minimum and if a lower confidence limit is used, a minimum of 35 PD_{50}. The ideal dose of vaccine should provide protection in 90 to 100% of the chickens and also provide an additional safety factor to ensure proper immunity will develop under a variety of field conditions. If we use the World Organization for Animal Health Newcastle disease vaccine standards as a guide, the H5N9 AI vaccine minimum dose would be 50 PD_{50} or the full vaccine dose, and for this vaccine, a reduced dosage would not be acceptable for field use. In addition, use of the full vaccine dose is especially important, because protection in commercial chickens in the field is typically less than that seen in experimental studies using SPF chickens in the laboratory [20]. A recent comparative AI vaccine study in Indonesia using 7 of the available commercial inactivated vaccines demonstrated variations in PD_{50} for different vaccines (PD_{50} from 1:6 to 1:147), and only 5 of 7 vaccines met or exceeded the minimum 50 PD_{50} per vaccine dose to be acceptable for field usage.

A more precise method of standardizing AI vaccines is to quantify the amount of hemagglutinin protein in each dose [17, 21]. Hemagglutinin is the primary protein that elicits a protective immune response against influenza A viruses. This quantification can be based on various assays including the following: 1) measurement of infectious titer of the virus before inactivation of the vaccine virus, 2) measurement of the hemagglutinin protein by using a hemagglutination assay or radial immunodiffusion test after inactivation, or 3) quantification of viral RNA copies in the final emulsified vaccine preparation and conversion to hemagglutinin protein quantity [3, 17, 21]. In the current study, qRRT-PCR was performed on 12 replicates (SD of 1.2 cycles; CV = 3.6%), and the extrapolated vaccine titer was $10^{3.5}$ EID_{50}/mL. However, the measured preinactivation titer of vaccine was $10^{8.5}$ EID_{50} per dose, and postactivation titer was 154 hemagglutination (**HA**) units, which based on prior titers [17] would equate to 10 to 33 μg of hemagglutinin protein per dose. In a previous study using the H5N9 vaccine strain and a generic mineral oil emulsion, the PD_{50} was 0.006 μg of hemagglutinin protein and 0.3 μg of hemagglutinin for 50 PD_{50}, which was equivalent to 4 HA units or $10^{6.45}$ EID_{50}/dose [17]. Therefore, the current vaccine hemagglutinin titer based on qRRT-PCR was lower than calculated based on preinactivation infectious titer or postinactivation HA titers. Based on the latter 2 criteria, the vaccine had sufficient antigen to produce a protective immune response. An underestimation of the hemagglutinin in the vaccine by qRRT-PCR is probably due to inefficient RNA extraction; the protocol was developed with a different formulation of oil emulsion that may not have been appropriate for the vaccine used in this study. Additionally, with any vaccine, degradation of the RNA during the inactivation process is a concern, although the effect of this is minimized, because the real-time reverse transcription-polymerase chain reaction target is only about 100 bp in length. The discrepancy seen in this study demonstrates that the qRRT-PCR method needs additional modification and validation before being accepted as a reliable method for quantifying the hemagglutinin protein content of oil emulsion vaccines.

CONCLUSIONS AND APPLICATIONS

1. An inactivated H5N9 oil-emulsified vaccine derived from A/Turkey/Wisconsin/68 (H5N9) low-pathogenic AI virus provided protection in young chickens against a 2004 H5N1 HPAI virus isolated from layer chickens in Japan.

2. Full, ½, ¼, and 1⁄10 doses of this vaccine protected all chickens from lethal H5N1 challenge based on prevention of morbidity, mortality, and reducing challenge virus shedding from the digestive tract.
3. The full dose was more effective at reducing the number of chickens shedding virus from the respiratory tract than the 1⁄10 dose.
4. The PD_{50} was 1⁄50 of a full vaccine dose and, using the international regulatory standard of 50 PD_{50} for Newcastle disease vaccine as a guide, the minimum field dose would be equivalent to 1 full dose of this commercial H5N9 AI vaccine. With this AI vaccine, using less than the full dose is not recommended to extend vaccine stocks. However, with some vaccines that contain greater than 50 PD_{50} per manufacturer's dose, reduced doses could potentially be used if the final administered dose contains 50 or more PD_{50}.

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