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The efficacy of recombinant turkey herpesvirus vaccines targeting the H5 of highly pathogenic avian influenza virus from the 2014–2015 North American outbreak

Charles L. Balzli [a], Kateri Bertran [a], Dong-Hun Lee [a], Lindsay Killmaster [a], Nikki Pritchard [b], Perry Linz [c], Teshome Mebatsion [c], David E. Swayne [a,*]

[a] Exotic and Emerging Avian Viral Disease Research Unit, Southeastern Poultry Research Laboratory, U.S. National Poultry Research Center, Agricultural Research Service, U.S. Department of Agriculture, 934 College Station Road, Athens, GA 30605, USA
[b] Merial Select Inc., 1112 Airport Parkway, Gainesville, GA 30503, USA
[c] Merial, Inc., 1730 Olympic Drive, Athens, GA 30601, USA

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ABSTRACT

The outbreak of highly pathogenic avian influenza virus in North American poultry during 2014 and 2015 demonstrated the devastating effects of the disease and highlighted the need for effective emergency vaccine prevention and control strategies targeted at currently circulating strains. This study evaluated the efficacy of experimental recombinant turkey herpesvirus vector vaccines with three different inserts targeting the hemagglutinin gene of an isolate from the recent North American influenza outbreak. White leghorn chickens were vaccinated at one day of age and challenged with A/Turkey/Minnesota/12582/2015 H5N2 at 4 weeks of age. Birds were analyzed for survival, viral shedding at two and four days after infection, and specific antibody prior to challenge and from surviving birds. The three experimental vaccines demonstrated 100%, 45% and 15% survival with the most effective vaccine significantly reducing oral and cloacal viral shedding compared to all other groups and generated specific antibody prior to challenge with highly pathogenic avian influenza virus. More studies are needed using diverse H5Nx highly pathogenic virus isolates to fully determine the breadth of coverage against possible exposure strains, as well as possible impact of maternally derived antibody on protection and vaccine efficacy.
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1. Introduction

Since 1996, multiple outbreaks of highly pathogenic avian influenza (HPAI) have occurred throughout Southeast Asia, the Middle East, Europe and Africa. In late 2014 through mid-2015, the U.S. experienced an epidemic of HPAI in birds throughout the Northwest and Midwest. The outbreak involved a Eurasian H5N8 HPAI virus from the A/goose/Guangdong/1/1996 clade 2.3.4.4 lineage and related reassortants [1]. The reassortants contained multiple gene segments from the Eurasian strain, including the H5 hemagglutinin (HA) gene, as well as gene segments from North American low pathogenic avian influenza (LPAI) strains [2–4]. The new HPAI viruses circulated in wild birds and infected back yard poultry as point source introductions mainly along the Pacific flyway, and commercial poultry facilities in the Midwest as secondary spread from common sources, predominantly H5N2 virus. The devastation was particularly extensive in the Midwest where turkey production and chicken layer facilities had 7.4 million and 43 million birds (respectively) that succumbed to disease, or were culled for disease control purposes. The cost to the federal government was $850 million to eradicate the disease through a stamping out program [5].

Several H5 and H7 avian influenza vaccines are currently licensed in the U.S. and available for use in poultry. These vaccines include a recombinant fowl poxvirus or a recombinant turkey herpesvirus (rHVT) with H5 inserts as *in vivo* vectors, as well as inactivated whole virus vaccines [6]. However, such vaccines have not been used in the U.S. since 2003 [7]. Other disease control methods have thus far been effective for control and eradication of historical HPAI outbreaks in the U.S. In the past, vaccination has been implemented with success in conjunction with other strategies on a time-limited and regional basis in some U.S. LPAI outbreaks [8]. Although HPAI vaccination strategies have never

* Corresponding author.
E-mail address: David.Swayne@ars.usda.gov (D.E. Swayne).

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been implemented in the U.S. including during outbreaks, the need for effective vaccines against HPAI may arise considering the possibility of future recurrence and persistence of HPAI in North America and the U.S.

The development of an *in vivo* vector using rHVT that targets avian influenza has demonstrated promising results in poultry in recent studies [9–12]. The rHVT vaccine has been engineered using a genetic insert from the HA gene of clade 2.2 HPAI virus A/swan/Hungary/4999/2006 which is expressed for an extended period by a turkey herpesvirus (HVT). This technology is capable of partially overcoming maternally derived antibody (MDA) interference to elicit a protective antibody response in younger birds. Furthermore, rHVT vaccines show a marked decrease in mortality upon challenge with HPAI virus in laboratory and field testing as well as decreased viral shedding. However, recent reports have demonstrated variable protection with the rHVT vaccine in chickens when tested against genetically divergent North American clade 2.3.4.4 HPAI [13,14].

The goal of these studies was to develop and evaluate the efficacy of the rHVT vaccines with one of three new H5 clade 2.3.4.4 inserts each given as a single dose to one day old white leghorn chickens, to simulate field hatchery vaccination. Vaccinated birds were then challenged at four weeks of age with the HPAI A/-turkey/Minnesota/12582/2015 H5N2 clade 2.3.4.4, an isolate of the Midwest H5N2 cluster.

2. Materials and methods

2.1. Vaccines and challenge virus

To generate rHVT-based experimental vaccines, two codon-optimized synthetic HA genes corresponding to modified influenza A/chicken/Washington/61-9/2014 (H5N2, GenBank accession: KP739381.1) sequences were chemically synthesized (GenScript). The HA cleavage sites of both synthetic genes were altered to match a low pathogenic cleavage site sequence, by deleting 3 arginine residues and altering a lysine residue to threonine (K325T, Fig. 1B). One of the synthetic HA genes containing only the modification at the cleavage site was designated LPC-HA and used for the generation of rHVT-501 and rHVT-502, whereas the synthetic gene inserted into rHVT-503 contained 3 additional alterations at residues S120N, D155N and S223N and designated as LPCmut-HA (Fig. 1A and B). The synthetic HA genes were incorporated into donor plasmids containing flanking arms encompassing a nonessential intergenic loci, IG1, to facilitate insertion into the corresponding region of HVT genome by homologous recombination [15]. The respective expression cassettes contain Simian Virus 40 (SV40) promoter, LPC-HA and a poly A tail for the donor plasmids p501 and p502; SV40, LPCmut-HA and polyA for the donor plasmid p503 (Fig. 1A). The only difference between the donor plasmids p501 and p502 is the presence of a human Herpesvirus Type III glycoprotein B promoter (HHV3gB promoter) in reverse orientation, just before the SV40 promoter in p502, to facilitate expression of another foreign gene for future use (Fig. 1A).

The donor plasmids were verified by PCR and sequencing and a standard homologous recombination procedure was followed by co-electroporation of one of the donor plasmids and viral DNA isolated from HVT FC126 into secondary chicken embryo fibroblast (CEF) cells [15]. Electroporation was performed using 1×10^7 secondary CEF in 300 μl Opti-MEM and shocked at 150 volts with 950 uF in a 2 mm electroporation cuvette. Cells were grown in 96-well plate for 4 days and duplicated into two 96-well plates and incubated for 3 more days. One set of 96-well plates was used for an immunofluorescence assay (IFA) using chicken polyclonal sera against avian influenza H5N2 to identify positive wells containing recombinants and another set of 96-well plates was used for recovering the infected cells from the corresponding positive wells. After approximately three rounds of purification, rHVT expressing the HA gene were isolated and the purity of the respective recombinant viruses was tested by IFA and PCR to confirm the absence of parental virus.

The HPAI isolate A/turkey/Minnesota/12582/2015 (H5N2) was used to challenge birds. The virus was isolated from a turkey farm in Minnesota on April 18th, 2015 and is representative of the Midwest H5N2 cluster by phylogenetic analysis [16]. The virus was propagated and titrated by allantoic sac inoculation of 9–10 day old embryonating chicken eggs by standard methods.

2.2. Animals

In house, white leghorn, specific pathogen free, mixed sex chicks (n = 100) were used in these studies. Birds were housed under ABSL-2 conditions in cages with feed and water provided *ad libitum* during the vaccination period (until four weeks of age), when they were tagged and transferred into HEPA filtered, negative pressure isolators in ABLS-3 enhanced facilities prior to challenge with HPAI virus. All procedures were performed in accordance with institutional animal care and use committee approved protocols.

2.3. Expression analysis

For IFA, appropriate dilutions of the stock virus were mixed with 1×10^7 CEF cells and then seeded onto a 96-well plate. The plates were incubated for 3 days at 37 °C in the presence of 5% CO_2 or until viral plaques were visible. Cells were fixed with 95% ice-cold acetone for three minutes and rinsed gently three times with PCR grade, nuclease free water. Chicken anti-sera against avian influenza H5N2 (lot#J0210, Charles Rivers Laboratory) at 1:500 and HVT monoclonal antibody L78 (lot#072103, Merial) at 1:3000 was added and the plates were incubated at 37 °C for 45 min. After incubation, plates were washed three times with phosphate-buffered saline (PBS) and incubated with fluorescein isothiocyanate (FITC) anti-chicken (cat# F8888, Sigma) at 1:500 and tetramethyl rhodamine isothiocyanate (TRITC) anti-mouse (cat#A10037, Life Technologies) at 1:300 at 37 °C for 45 min. After incubation, cells were rinsed three times with PBS and visualized with a fluorescent microscope using FITC and TRITC filters.

2.4. Experimental design

Due to bird availability, experiments were performed with one vaccine group and a matching sham control group followed at a later time by the second and third vaccine groups with a matching sham control group (n = 20/group). At one day of age, chicks were vaccinated subcutaneously (0.2 ml/bird) with one of the three vaccines, or Marek's diluent for sham control groups. At four weeks of age, blood was collected for antibody titrations, and birds were inoculated intra-choanally with 0.1 ml of approximately $1 \times 10^{7.5}$ mean embryo infectious dose (EID_{50})/ml of A/turkey/Minnesota/12582/2015 H5N2 HPAI virus. The birds were monitored daily for clinical signs and any deaths were recorded. Oral and cloacal swabs were collected at 2 days post infection (DPI) and 4 DPI, placed in brain heart infusion broth (Becton, Dickinson and Company, Sparks, MD) supplemented with 1% antibiotic/antimycotic solution (Hyclone Laboratories Inc., Logan, UT), and stored at -80 °C. Birds with severe clinical symptoms were euthanized and their death recorded as the following day. Blood was collected from surviving birds at 14 DPI prior to euthanasia. Researchers in these studies were blind to the identity of the vaccine candidates

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