Characterization and efficacy determination of commercially available Central American H5N2 avian influenza vaccines for poultry

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A B S T R A C T

A poultry vaccination program was implemented in Central America beginning in January 1995 to control both H5N2 low (LPAI) and high pathogenicity avian influenza. This study was conducted to identify seed strain composition and the efficacy of 10 commercially available H5 vaccines against challenge with H5N2 LPAI viruses isolated from Latin America in 2003. The original 1994 vaccine seed virus in commercial inactivated vaccines did not significantly reduce challenge virus shed titers. However, two seed strains of inactivated vaccines, genetically more closely related to the challenge virus, did significantly reduce titers of challenge virus shed from respiratory tract. In addition, a live recombinant fowlpox virus vaccine containing a more distantly related Eurasian lineage H5 gene insert significantly reduced respiratory shedding as compared to sham vaccinates. These results demonstrate the feasibility of identifying vaccine seed strains in commercial finished products for regulatory verification and the need for periodic challenge testing against current field strains in order to select efficacious vaccine seed strains.

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1. Introduction

Antigenic drift has been observed in type A influenza viruses resulting from point mutations which ultimately change the hemagglutinin (HA) protein epitope structure [1]. These changes allow the virus to evade the host’s acquired immunity regardless if the acquired immunity is from vaccination or natural infection, and such viruses can spread in a functionally naïve population. Therefore, every year human seasonal influenza vaccine formulation requires reevaluation by comparison with the major currently circulating strains and, periodic replacement of older strains with new variant strains to maintain efficacious human vaccines [2,3]. In contrast, broad immunity (i.e. immunity to variant strains of the same subtype as measured by reduction in viral titers and shed) to high pathogenicity avian influenza (HPAI) has been observed from avian influenza (AI) vaccination in chickens even when the HA protein sequences have as little as 86% nucleotide (or amino acid) identity [4,5]. This broad immunity might be attributed to the oil adjuvant systems used in inactivated vaccines to produce robust humoral response and the cell-mediated immunity from

live recombinant virus vaccines. Prior to 1995, antigen drift of field viruses was of low concern because of the lack of endemicity of AI in most countries’ commercial poultry and long term vaccination programs have historically been rare therefore, selective immune pressure on field viruses has been minimal [6,7].

H5N2 low pathogenicity avian influenza (LPAI) virus was first identified in Mexico in May 1994 and by November 1994 had mutated into HPAI virus. In January 1995, emergency eradication activities were initiated and included depopulation of affected farms, cleaning and disinfection of facilities, use of sentinel birds and vaccination [8,9]. The government of Mexico declared Mexico HPAI free in May 1995. However, the vaccination program was continued with the intent for vaccine use as a tool towards the eradication of H5N2 LPAI. More than 1 billion doses of inactivated emulsified vaccines were used between 1995 and 2001 and 459 million doses of recombinant fowlpox virus-vectored H5 AI vaccine were used between 1998 and 2001 [10]. However, after more than 14 years and 2 billion doses, H5N2 LPAI is still present in some parts of Mexico and genetically related viruses have periodically spread to Guatemala, El Salvador, Dominican Republic and Haiti, although no additional HPAI outbreaks have occurred [11]. One study phylogenetically analyzed the H5 HA gene from 52 LPAI virus isolates isolated 1993–2002 and concluded that most viruses isolated after the introduction of vaccine did not belong to the vaccine
seed strain lineage and antigenic drift had occurred resulting in the lack of optimal protection observed in challenge studies when using an experimental, non-commercial inactivated oil-emulsion vaccine containing the original 1994 H5N2 vaccine seed strain [6]. Presently, there are concerns about viral antigenic drift and viral evolution due to selection pressure created by vaccination and how this could affect which seed strains of virus are used in commercial vaccines as well as their potency.

This study was conducted to identify the seed strain composition and evaluate the efficacy of commercial H5N2 vaccines used in some areas of Latin America following challenge with recent H5N2 LPAI field viruses. Our study demonstrates that 3 different inactivated seed strains were used in current commercially available inactivated vaccines as well as a live recombinant fowlpox virus vaccine with a Eurasian AI H5 gene insert (rFPV-AIV-H5). The inactivated vaccines using the original 1994 virus as the seed strain did not protect chickens against H5N2 LPAI virus challenge as evidenced by similar levels of respiratory viral replication and shedding as sham vaccinated chickens. By contrast, two inactivated vaccines using seed strains more closely related to the 2003 challenge virus and the live rFPV-AIV-H5 vaccine provided better protection based on significant reduction in respiratory virus shed.

2. Materials and methods

2.1. Viruses

Two closely related viruses from the same premises were selected as challenge viruses because they represent the most recent H5N2 avian influenza viruses available from Latin America to international reference laboratories. Virus stocks of A/chicken/Guatemala/270475-1/03 and A/chicken/Guatemala/270475-4/03 which share 98.9% identity in hemagglutinin protein were grown by passage in 9-day-old embryonating chicken eggs twice. Allantoic fluid was removed and diluted in brain-heart infusion (BHI) medium to a final titer of 10⁶ 50% embryo infectious doses (EID₅₀) per 0.1 ml as previously described [12].

2.2. Vaccines

Commercially available inactivated H5 whole virus vaccines, adjuvanted, in proprietary oil emulsions were used. A rFPV-AIV-H5 genetically engineered to contain the HA gene insert from A/turkey/Ireland/84 (H5H9) (rFPV-AI-H5) was also used.

2.3. Vaccine seed strain identification

The most closely related isolate (MCRI) to the seed strain (and thus likely the seed strain) used to produce each vaccine was identified by sequencing. Total RNA was extracted from the inactivated, oil adjuvant vaccines by adding 100 μl of virus to 900 μl TRIzol® LS reagent (Invitrogen Inc., Carlsbad, CA), mixing by vortexing, incubating at room temperature for 5 min, and then adding 200 μl of chloroform. The mixture was mixed by vortexing, incubated at room temperature a minimum of 10 min and centrifuged for 15 min at 12,000 x g. RNA extraction was completed with the top two phases using the MagMax™ 96 Al/ND Viral RNA isolation kit (Ambion Inc., Austin, TX) in accordance with kit instructions using the KingFisher® magnetic particle processor (Thermo Scientific, Waltham, MA). DNA was extracted from 0.5 ml of a 1:10 dilution of the rFPV-AI-H5 vaccine (vaccine J) in PBS using the DNeasy® kit (Qiagen Inc., Valencia, CA) in accordance with the manufacturer’s instructions and eluted in 50 μl of nuclease-free water. The sequencing template was prepared by using 3 μl of DNA in a 50 μl PCR reaction using 2X PCR Master Mix (Promega Inc., Madison, WI) using the same primers and PCR conditions as the other vaccines

Sequencing templates were generated from the vaccine derived RNA by RT-PCR using primers directed to conserved sequences in the HA1 region of the H5 HA gene. The forward primer was H5 F645 5'-CTACGACAACTCAGAACACCTTA-3', and the reverse primer was R1082 5'-GCTCDAAAKGCCTCTTGT-3'. This primer pair failed to amplify the material in vaccine I, therefore a second set of primers using a conserved sequence at the 5’ end of the gene [13] with a reverse primer in the HA1 region of the gene; H5 R230 5'-AGC CAT CCA GCT ACA CTA CCA-3' was used. Due to the poor yield and quality of RNA that can be obtained from inactivated, oil emulsion vaccines, we targeted relatively short segments and did not attempt to generate sequence for the full length HA genes.

A one-step RT-PCR kit (Qiagen Inc., Valencia, CA) was used in accordance with the kit instructions for a 25 μl reaction volume with 5 μl of total RNA template. Thermal cycling conditions were a reverse transcription step of one cycle of 50°C for 30 min and 95°C for 15 min then 35 cycles of 95°C for 45 s, 53°C for 1 min and 72°C for 1 min. The RT-PCR products were visualized by agarose gel electrophoresis. Products of the expected size (485 bp) were excised, purified with the QIAquick® gel extraction kit (Qiagen Inc.) and directly sequenced with the primers used to produce the template. The sequencing template for the rFPV-AI-H5 vaccine was generated with the same primers and PCR cycling conditions listed above using the Promega 2X PCR MasterMix (Promega Inc.).

Cycle sequencing was performed with the BigDye® terminator kit (Applied Biosystems, Foster City, CA) and run on an ABI 3730 (Applied Biosystems). Contigs were assembled with SeqMan® 7.1 (DNASTar, Madison, WI) and were initially characterized by BLASTSM search to find the most similar sequences in GenBankSM. Phylogenetic analysis was performed by aligning the deduced amino acid sequences with Clustal V (Lasergene® 7.1, DNASTar, Madison, WI). Trees were constructed with merged duplicate runs with BEAST v. 1.4.8 [14] using Blossum62 substitution, Gamma + invariant site heterogeneity model, relaxed lognormal clock, Yule Process tree prior with default operators with UPGMA starting tree and MCML length of 10⁶.

2.4. Animals and housing

Animals were cared for and housed in accordance to an Institutional Animal Care and Use Committee approved animal use protocol at the Southeast Poultry Research Laboratory (SEPRL), Agricultural Research Service (ARS), United States Department of Agriculture (USDA), Athens, GA, USA. Experiments were performed in the USDA-certified Biosafety Level 3-enhanced facility [15]. Birds had ad libitum access to feed and water.

2.5. Experimental design: experiment 1, inactivated vaccines

Twelve-day-old specific pathogen free (SPF) White Plymouth Rock chickens from in-house flocks were bled for serum then vaccinated with the appropriate commercial vaccine subcutaneously in the nape of the neck as per manufacturer’s instructions with 0.5 ml for vaccines A–H or 0.2 ml for vaccine I. At 3 weeks post-vaccination, birds were banded for identification, bled for serum and challenged intranasally (i.n.) with 10⁶ EID₅₀ per bird of A/chicken/Guatemala/270475-4/03 (H5N2). The sham vaccinated group received a vaccine prepared with non-infectious allantoic amniotic fluid mechanically emulsified in 4 parts of oil phase as previously described [16,17].

2.6. Experiment 2, recombinant fowlpox virus vaccine (rFPV-AI-H5)

Chickens were inoculated at 1 day of age, subcutaneously in nape of the neck with 0.2 ml per bird as per the manufac-
The percent aa identity for the entire HA gene of the MCRI with the challenge virus is also provided.

The percent closely related influenza isolate (MCRI) to each vaccine based on BLAST search of partial HA1 region of the HA gene and the percent amino acid (aa) identity. The percent Table 1

**Table 1**

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Most closely related isolate (MCRI)</th>
<th>%aa identity of vaccine with MCRI&lt;sup&gt;a&lt;/sup&gt;</th>
<th>%aa identity of vaccine with the closest strain in SEPRL repository&lt;sup&gt;b&lt;/sup&gt;</th>
<th>%aa identity of vaccine with the challenge strain&lt;sup&gt;c&lt;/sup&gt;</th>
<th>%aa identity of vaccine with most recent H5N2 from Central America&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>A/Chicken/Hidalgo(Mexico)/28159-232/94 (H5N2)</td>
<td>98.5</td>
<td>98.5&lt;sup&gt;e&lt;/sup&gt;</td>
<td>89.6</td>
<td>86.9</td>
</tr>
<tr>
<td>B</td>
<td>A/Chicken/Hidalgo(Mexico)/28159-232/94 (H5N2)</td>
<td>100</td>
<td>100&lt;sup&gt;f&lt;/sup&gt;</td>
<td>89.3</td>
<td>79.9</td>
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<td>C</td>
<td>A/Chicken/Hidalgo(Mexico)/28159-232/94 (H5N2)</td>
<td>98.4</td>
<td>98.4&lt;sup&gt;g&lt;/sup&gt;</td>
<td>88.4</td>
<td>87.5</td>
</tr>
<tr>
<td>D</td>
<td>A/Chicken/Hidalgo(Mexico)/28159-232/94 (H5N2)</td>
<td>98.5</td>
<td>98.5&lt;sup&gt;h&lt;/sup&gt;</td>
<td>89.6</td>
<td>86.9</td>
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<tr>
<td>E</td>
<td>A/Chicken/Hidalgo(Mexico)/28159-232/94 (H5N2)</td>
<td>100</td>
<td>100&lt;sup&gt;i&lt;/sup&gt;</td>
<td>88.5</td>
<td>84.5</td>
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<tr>
<td>F</td>
<td>A/Chicken/Hidalgo(Mexico)/28159-232/94 (H5N2)</td>
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<td>98.3&lt;sup&gt;j&lt;/sup&gt;</td>
<td>87.4</td>
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<td>A/Chicken/Guatemala/45511-5-00 (H5N2)</td>
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<td>97.7&lt;sup&gt;k&lt;/sup&gt;</td>
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<td>H</td>
<td>A/Chicken/Guatemala/45511-5-00 (H5N2)</td>
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<td>98.4&lt;sup&gt;l&lt;/sup&gt;</td>
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<td>I</td>
<td>A/Chicken/Ibaraki(Japan)/1/05 (H5N2)</td>
<td>99.1</td>
<td>98&lt;sup&gt;m&lt;/sup&gt;</td>
<td>94.8</td>
<td>94.9</td>
</tr>
<tr>
<td>J</td>
<td>A/Turkey/Ireland/1378/83</td>
<td>100</td>
<td>100&lt;sup&gt;n&lt;/sup&gt;</td>
<td>90.4</td>
<td>80.9</td>
</tr>
</tbody>
</table>

<sup>a</sup> Based on a pair-wise alignment (Lipman–Pearson) of 95 amino acids (aa) from 232 to 327 for vaccines A through H, or aa positions 10–114 for vaccine I.

<sup>b</sup> Closest related H5N2 low pathogenicity virus of Central American lineage in SEPRL repository and was used as HI antigen in cross HI testing.

<sup>c</sup> Based on ClustalV<sup>®</sup> multiple alignments. Challenge strains A/chicken/Guatemala/270475-4/03 (H5N2) for vaccine groups A–I and A/chicken/Guatemala/270475-1/03 (H5N2) for vaccine group J.

<sup>d</sup> Hemagglutinin amino acid identity of vaccine seed strains and most recently released virus from Central America (A/chicken/Mexico/435/2005 (H5N2)).

<sup>e</sup> A/Chicken/Hidalgo(Mexico)/28159-232/94 (H5N2).

<sup>f</sup> A/Chicken/Guatemala/194573/02 (H5N2).

<sup>g</sup> A/Chicken/Guatemala/45511-5/00 (H5N2).

<sup>h</sup> A/Chicken/Guatemala/194573/02 (H5N2).

<sup>i</sup> A/Chicken/Hidalgo(Mexico)/28159-232/94 (H5N2).

<sup>j</sup> A/Chicken/Guatemala/194573/02 (H5N2).

<sup>k</sup> A/Chicken/Hidalgo(Mexico)/28159-232/94 (H5N2).

<sup>l</sup> A/Chicken/Hidalgo(Mexico)/28159-232/94 (H5N2).

<sup>m</sup> A/Chicken/Guatemala/194573/02 (H5N2).

<sup>n</sup> A/Turkey/Ireland/1378/83.

...turer's instructions. Three weeks post-vaccination, the chickens were challenged by the i.n. route with 10<sup>6</sup>EID<sub>50</sub> per bird of A/Chicken/Guatemala/270475-1/03. The sham vaccinated received sterile diluent.

To evaluate virus shed for both experiments 1 and 2, oropharyngeal swabs were collected 3 days post-challenge (d.p.c.). At 14 d.p.c. birds were bled for serum and humanely euthanized.

### 2.7. Quantification of oropharyngeal virus shedding

Oropharyngeal swabs were collected at 3 d.p.c. and were stored at −70°C until RNA isolations could be carried out. RNA was extracted by adding 750 µl TRIzol<sup>®</sup> LS (Invitrogen Inc., Carlsbad, CA) to 250 µl swab material. The swab material–TRIzol<sup>®</sup> LS material was mixed by vortexing and incubated at room temperature for 5 min, then 200 µl of chloroform was added. The material was mixed by vortexing, incubated at room temperature a minimum of 10 min and centrifuged for 15 min at 12,000 × g. Instead of precipitation with 2-propanol according to the manufacturer's instructions, the RNA extraction was completed by binding and eluting the RNA from the aqueous phase using the MagMAX<sup>™</sup> 96 Al/ND Viral RNA isolation kit (Ambion Inc., Austin, TX) in accordance with kit instructions using the KingFisher<sup>®</sup> magnetic particle processor (Thermo Scientific, Waltham, MA).

Quantitative RT-PCR (qRT-PCR) which targets the influenza M gene [18] was performed using the SmartCycler<sup>®</sup> 2 (Cepheid Inc., Sunnyvale, CA) and the OneStep RT-PCR kit (Qiagen, Valencia, CA) in accordance with the U.S. National Veterinary Services Laboratories protocol AVSOP1521.01. The standard curve for virus quantification was established with RNA extracted from dilutions of the same titrated stock of the challenge virus run in duplicate and reported as relative equivalent units [REU] as EID<sub>50</sub>/ml.

qRT-PCR detection limit was 10<sup>4.1</sup>EID<sub>50</sub>/ml or 10<sup>1.9</sup>EID<sub>50</sub> per reaction for A/chicken/Guatemala/270475-4/03 and 10<sup>3.7</sup>EID<sub>50</sub>/ml or 10<sup>1.3</sup>EID<sub>50</sub> per reaction for A/chicken/Guatemala/270475-1/03. If viral RNA was not detected from a sample then a number 10<sup>6.1</sup>EID<sub>50</sub>/ml lower than the respective lower detection limit was used for statistical analysis (i.e. 10<sup>4.0</sup>EID<sub>50</sub>/ml for A/chicken/Guatemala/270475-4/03 and 10<sup>3.8</sup>EID<sub>50</sub>/ml for A/chicken/Guatemala/270475-1/03).

### 2.8. Serology

Hemagglutination inhibition assays were performed to quantify antibody response to vaccine antigen and challenge virus antigen with serum collected pre- and post-challenge as previously described [12] using 0.5% suspension of chicken erythrocytes in phosphate buffered saline and most HI antigen from most closely related influenza isolate (MCRI) in our repository. For inactivated vaccine groups, cross HI tests were performed using antisera from a single chicken per group with 128-512 GMT to MCRI in our SEPRL avian influenza repository; i.e. either A/Chicken/Hidalgo(Mexico)/28159-232/94 (H5N2) or A/Chicken/Guatemala/194573/02 (H5N2). The antisera were also tested against the challenge strains A/chicken/Guatemala/270475-4/03 and 10<sup>3.8</sup>EID<sub>50</sub>/ml for A/chicken/Guatemala/270475-1/03.

### 2.9. Statistical analysis

Statistical significance between groups was analyzed by ANOVA for both serological and virological testing. Pearson’s correlation coefficient analysis was also carried out using pre-challenge vaccine strain antigen GMT or pre-challenge challenge strain antigen GMT and the respective vaccine group's titer for further statistical...
analysis of serological data. All statistical analyses were carried out using Excel. Differences were considered statistically significant at 

\( p < 0.05 \).

3. Results

3.1. Vaccine seed strain identification

RNA was isolated from the commercial inactivated oil emulsified vaccines and DNA from the recombinant fowlpox virus vectored AI vaccine, the HA1 region of the HA gene was sequenced and phylogenetically analyzed to evaluate protein sequence identity between vaccines and challenge virus and to identify the most closely related virus isolate. The AIV isolate with the most closely related HA gene to the sequence from each vaccine as determined by BLAST search and its identity to the challenge virus is provided in Table 1. Protein sequence comparison between the vaccine seed strains and the challenge virus A/chicken/Guatemala/270475-4/03 yielded percent identity ranging between 87.4 and 89.6 for vaccines A, B, C, D, E and F while analysis revealed vaccines G, H and I seed strains were more closely related to the challenge virus yielding 95.3%, 94.8% and 98.2% protein identity, respectively.

The most closely related virus isolate to the vaccines A, B, C, D, E, and F with 98.3–100% protein identity, was A/chicken/Hidalgo(Mexico)/232/94 (H5N2) which is the official H5N2 AI vaccine seed strain in Mexico. Vaccines G and H seed strains were most closely related to A/chicken/Guatemala/45511-5/00 (H5N2) with 96.9% and 97.0% protein identity, respectively. The most closely related strain to vaccine I was A/chicken/Ibaraki(Japan)/1/05 (H5N2) with 99.1% protein identity. Most closely related strain in our repository to vaccines G, H and I was A/Chicken/Guatemala/194573/02 (H5N2) with 97.7, 98.4 and 98.0% amino acid identity. The recombinant fowl pox-vectored AI vaccine (vaccine J) was verified to have the HA gene inserted from A/turkey/Ireland/1378/83 (H5N9) with 100% protein identity (Fig. 1).

Antigenic relatedness of vaccine seed strain to challenge virus were determine by HI tests. Sera from vaccines A–F had a 3–4 log\(_2\) reduction in HI titer when using challenge virus verses MCRI strain as test antigens (Table 2). However, vaccines G–I only had a 0–2 log\(_2\) reduction in titer when using challenge virus verses MCRI in the repository as test antigen (Table 2). This indicates vaccine seed strains A–F were antigenically more distantly related to the challenge virus while vaccine seed strains G–I were more closely related to the challenge virus.

3.2. Mortality and virus isolation from challenged birds

In experiment 1, only two birds died from the sham group on days 4 and 9. All chickens were tested for viral shedding on 3 d.p.c. from oropharyngeal swabs by qRRT-PCR. Chickens vaccinated with vaccines H or I showed both statistically significant decreases (\( P \leq 0.05 \)) in the quantity of virus shed (vaccine H and I) and the number of birds per group shedding virus (vaccine H only) when compared to the sham group. In the sham group, 10 of 10 birds were positive by qRRT-PCR average REU 10\(^{7.4}\) EID\(_{50}\)/ml. In vaccine groups H and I, 0 of 10 birds and 6 of 10 (10\(^{4.4}\) EID\(_{50}\)/ml average REU) birds were positive with qRRT-PCR, respectively. Chickens in vaccine groups A–G were positive (10 of 10 in each group) with qRRT-PCR average REU ranging between 10\(^{6.3}\) and 10\(^{7.2}\) EID\(_{50}\)/ml which were not significantly different when compared to the sham vaccinated group (Table 3).

In experiment 2, the efficacy of vaccine J was examined. The challenge virus used was Chicken/Guatemala/270475-1/03 which had 98.9% identity in hemagglutinin gene with Chicken/Guatemala/270475-4/03. No morbidity or mortality was observed for either vaccinated group or sham group after challenge. Viral shedding was detected and quantified by qRRT-PCR from RNA extracted from 3 d.p.c. oropharyngeal swabs. In the sham group, 8 of 10 birds were positive with a qRRT-PCR average REU 10\(^{6.2}\) EID\(_{50}\)/ml. Only 2 of 10 vaccinated birds were positive with an average REU of 10\(^{3.3}\) EID\(_{50}\)/ml which was significantly reduced in both number of
birds shedding and the average titer of virus shed as compared to the sham group ($P \leq 0.05$).

### 3.3. Serology

Testing for H5 antibodies was conducted using hemagglutinin inhibition (HI) assays on serum. In experiment 1, all birds were negative for H5 antibodies pre-vaccination. Sham groups were negative for H5 antibodies both pre- and post-vaccination. Antibody response to each H5 vaccine is presented in Fig. 2. Respective vaccine specific antibodies were detected in all vaccinated groups post-vaccination and had geometric mean titers (GMT) ranging between 147 for vaccine groups A, B and F to 1351 in vaccine group I. Post-challenge vaccine specific antibody responses ranged between a GMT of 416 for vaccine group F and 4705 for vaccine group I (Fig. 2A). Post-vaccination antibody response using the challenge strain, chicken/Guatemala/270475-1/03. Post-challenge vaccine specific antibodies were detected in all vaccinated groups (3 d.p.i.) and mean relative equivalent units [REU] of 7.4±0.58 for vaccine group A, 7.4±0.35 for vaccine group B, 7.5±0.19 for vaccine group C, 7.4±0.33 for vaccine group D, 7.2±0.33 for vaccine group E, 7.0±1.24 for vaccine group F, 7.3±0.41 for vaccine group G, 6.3±0.81 for vaccine group H, and 4.4±1.96 for the sham group (Fig. 2B).

In experiment 2, sham vaccinated animals were negative for H5 antibodies at both pre- and post-vaccination. Post-vaccination, vaccine group J induced an antibody response (49 GMT) to the vaccine strain, however there were no detectable antibodies specific for the challenge virus, chicken/Guatemala/270475-1/03. Post-challenge animals in vaccine group J had a GMT of 13 to the challenge strain.

### 4. Discussion

Early studies with Latin American H5N2 LPAI viruses identified two sublineages of the HA gene: Puebla and Jalisco [19]. The original seed strain for inactivated vaccine used in Mexico was A/chicken/Mexico(Hidalgo)/232/94 (H5N2) and is from the Jalisco sublineage. A study conducted on viral isolates by Lee et al. [6] found both original sublineages had disappeared and been replaced with new sublineages, A and B. The study indicated sublineage A may have evolved from the Jalisco sublineage and another strain may have been the progenitor strain for sublineage B which includes viruses isolated from Guatemala and El Salvador [6]. Both genetic and antigenic drift of field viruses away from the original vaccine seed strain was demonstrated. In another study 18 AIV isolates collected between 2002 and 2006 from 9 regions in Mexico were analyzed and shown to be related to sublineage B and therefore more distantly related to the official vaccine strain [20]. These data support continued genetic and antigenic drift of the field viruses away from the historical vaccine seed strain from H5N2 poultry vaccines. One of the reasons for the observed antigenic drift could have been vaccine pressure, although the presence of immunity from infection of non-vaccinated flocks by H5N2 LPAI field viruses may also have contributed.

A critical question in ongoing evaluations of H5N2 LPAI vaccination programs is which seed strain or strains are used in commercial vaccines applied in the field. In our study, based on sequencing of short RNA fragments, we observed that inactivated H5N2 vaccines A, B, C, D, E, and F used the official 1994 Mexican seed strain. Vaccines G and H were produced with seed strains that were more recent and closely related to A/chicken/Guatemala/45511-5/00 (H5N2). Unexpectedly, the vaccine I seed strain was most closely related to an outbreak virus from Japan, A/chicken/Ibaraki(Japan)/1/05 (H5N2) (99.1% similarity in hemagglutinin), which is a North American lineage H5 virus and not an Asian lineage H5 virus. This later finding supports the possibility of the introduction of the North American lineage H5N2 virus into Asia via human activity since this virus lineage has not been identified in wild birds, but only in poultry of Latin America and the Caribbean Islands [21]. Vaccine J was a recombinant fowlpox vaccine and confirmed to contain an HA insert from A/turkey/Ireland/1378/83 (H5N9) as claimed by the manufacturer.

Potency is a measure of sufficient antigenic mass in the vaccine to produce a desired immune response, usually set by individual country licensing regulations to a minimal protective level. Potency can be directly evaluated with challenge studies which are both time consuming and expensive, requiring inoculation of birds with various doses of vaccine and looking at the prevention of mortality, reduction in challenge virus shed or serological response [7]. Because consistent induction of clinical signs and mortality are not usually observed in experimental infections with LPAI viruses, additional testing methods have been employed to discern if vaccines are potent. Commonly, HI assay was used to indirectly assess vaccine potency [7]. Previously for inactivated AI vaccines, protection of chickens from illness and/or death and reduction or elimination of challenge virus shed from the respiratory and alimentary tracts has been associated with an average HI titer at or above 139 to the challenge virus [22] or HI titer at or above 32 to protection from death [23]. All of the vaccines we tested produced a pre-challenge group average HI titer above 139 when using the vaccine seed strain as antigen indicating all the vaccines met minimum requirements for potency. However, based on the HI
titers using the challenge virus as antigen, none of the groups had a pre-challenge average HI titer above 84, raising questions concerning of inadequate protection, especially with groups A–F which had average HI titers below 32. Furthermore, analysis of individual antisera confirmed the lower antigenic relatedness of vaccines A–F seed strains as evident by 3–4 log₂ drop in HI titer between MCRI strain and challenge virus when used as HI antigen, and higher antigenic relatedness of vaccines G–J as evident by 0–2 log₂ drop in HI titer between MCRI and challenge strain when used as HI antigens.

In our study, the commercially available LPAI vaccines from Latin America were tested in vivo for efficacy by challenge with virus A/chicken/Guatemala/270475-4/03 (H5N2) or A/chicken/Guatemala/270475-1/03 (H5N2) which were originally isolated from the same farm and had 98.8% protein identity in the hemagglutinin proteins. Since LPAI virus do not produce consistent morbidity and mortality in experimental challenge, also confirmed in our study, we looked at alternative measures of protection, qRT-PCR or virus isolation with titration have been used to measure reductions in LPAI and HPAI challenge virus replication and shedding from oropharyngeal and/or cloacal swabs for quantification of protection as previously described [24–26]. In our study, a significant decrease in challenge virus shedding based on viral RNA in oropharyngeal swabs at 3 d.p.c. was observed for vaccines H, I and J indicating less respiratory viral shedding. The other inactivated vaccines failed to significantly lower challenge virus shedding when compared to the sham group although the group G had a numeric 1 log₁₀₁₀ reduction in viral shedding that was not statistically significant. This indicates for the inactivated virus vaccines (vaccines A–I) that only the seed strains most closely related to the challenge virus were protective, sharing >95% hemagglutinin similarity and producing no more than 2 log₂ drop in HI titer between vaccine seed and challenge virus when used as antigen. However, the live recombinant fowl pox with HA hemagglutinin insert still provided protection as measured by significant reduction in challenge virus replication in respiratory tract, but this vaccine only had 83% hemagglutinin similarity with the challenge virus and HI testing demonstrated a 4 log₂ drop in HI titer (data not shown). This suggests that use of HI serological testing can be helpful in predicting antigenic relatedness and protection from challenge LPAI virus replication and shedding, but such serology may not be predictive of protection for live vectored vaccines.

The situation in Latin America provides a unique opportunity in regards to viral drift, with vaccine pressure as one contributing factor. Mexico and Pakistan are the only two countries which have utilized an AI vaccine program for over a decade. However, since 2002, several countries in Asia have adopted vaccine use to control H5N1 HPAI, as it is considered more cost-effective and feasible than traditional stamping out/eradication policy in countries with endemic infections [27]. In association with such broad vaccine use there has been emergence of field strains that are resistant to immunity induced by classical vaccine seed strains in China, Egypt and Indonesia [28]. In experimental studies in Indonesia, the classic H5 inactivated vaccines were very potent, i.e. produced high HI titers in vaccinated birds, but failed to protect from morbidity following Indonesian H5N1 HPAI virus challenge. In another recent experimental study, inactivated H5 reverse genetic AI vaccine seed strain using a North American HA produced significantly less reduction in oropharyngeal H5N1 HPAI challenge virus shedding as compared to a Eurasian HA gene insert vaccine [29]. In our study, vaccines H, I and J resulted in statistically significant reduction in virus shedding from the respiratory tract compared to the sham group. Vaccines H and J also resulted in a statistically significant decrease in the number of birds that shed virus. Only vaccine H reduced shedding to below detection limits. The challenge viruses used in our studies were from 2003 and were the most recent available to our laboratory which raises the question of how would newer H5N2 field viruses protect in relation to the 2003 challenge viruses. We assessed the genetic relatedness of hemagglutinin amino acid sequences from the vaccines seed strains and the most recently released sequence from Mexico, A/chicken/Mexico/435/2005 (H5N2) (Table 1). This more recent virus showed similar or lesser relatedness to the vaccine seed strains than the 2003 challenge viruses and would suggest similar poor protection from vaccines A–F. Predicted protection by vaccines G–J would be unknown against this field virus without some antigen or challenge data. This findings have significant ramifications for the use of vaccine to control AI because seed strains should be evaluated and updated on a periodic basis to provide maximal reduction in replication and shedding of field viruses and thus reduce environmental contamination to prevent transmission to naive flocks as well as reduce the risk of mutation into HPAI viruses. Potent and efficacious vaccines, applied in an appropriate manner are critical to prevent shedding of virus, reduce environmental contamination and transmission and thus become an effective tool in either control or eradication programs.

This study indicates the current inactivated commercial H5N2 vaccines in Latin America use more than one seed strain. Protection from inactivated vaccines is mainly from humoral immunity such that low HI titers may indicate poor cross protection from LPAI virus replication while moderate titers are suggestive of some level of protection [30]. Inactivated vaccines that use the older and genetically more distant 1994 H5N2 seed strain were ineffective in protecting against challenge with a more recent H5N2 LPAI virus while the inactivated vaccine with the seed strain most genetically related to the challenge virus (vaccine H) was protective as indicated by reduced replication and shedding of the challenge virus. Interestingly, live vectored vaccine J was protective against the newer H5N2 challenge virus despite the HA amino acid identity being only 83.0% to the challenge strain A/chicken/Guatemala/270475-1/03 (H5N2). This disparity in lack of protection by inactivated vaccine verses a recombinant fowlpox vaccine is unknown, but potentially is a result of broader cell-mediated immunity from the recombinant fowlpox vaccine. Indeed broad cross-protection has been demonstrated against HPAI viruses in previous studies [4,5]. Regardless, an effective vaccine must induce a robust response in the host to effectively neutralize the virus. Failure to do so may alternatively contribute to antigenic drift by selecting populations to which the vaccine offers little or no protection. This study highlights the need for continued monitoring of avian influenza field viruses and the need to evaluate and update poultry vaccine seed strains on a periodic basis.

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