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Protection of White Leghorn chickens by recombinant fowlpox vector vaccine with an updated H5 insert against Mexican H5N2 avian influenza viruses

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ABSTRACT

Despite decades of vaccination, surveillance, and biosecurity measures, H5N2 low pathogenicity avian influenza (LPAI) virus infections continue in Mexico and neighboring countries. One explanation for tenacity of H5N2 LPAI in Mexico is the antigenic divergence of circulating field viruses compared to licensed vaccines due to antigenic drift. Our phylogenetic analysis indicates that the H5N2 LPAI viruses circulating in Mexico and neighboring countries since 1994 have undergone antigenic drift away from vaccine seed strains. Here we evaluated the efficacy of a new recombinant fowlpox virus vector containing an updated H5 insert (rFPV-H5/2016), more relevant to the current strains circulating in Mexico. We tested the vaccine efficacy against a closely related subcluster 4 Mexican H5N2 LPAI (2010 H5/LP) virus and the historic H5N2 HPAI (1995 H5/HP) virus in White Leghorn chickens. The rFPV-H5/2016 vaccine provided hemagglutinin inhibition (HI) titers pre-challenge against viral antigens from both challenge viruses in almost 100% of the immunized birds, with no differences in number of birds seroconverting or HI titers among all tested doses (1.5, 2.0, and 3.1 $\log_{10}$ mean tissue culture infectious doses/bird). The vaccine conferred 100% clinical protection and a significant decrease in oral and cloacal virus shedding from 1995 H5/HP virus challenged birds when compared to the sham controls at all tested doses. Virus shedding titers from vaccinated 2010 H5/LP virus challenged birds significantly decreased compared to sham birds especially at earlier time points. Our results confirm the efficacy of the new rFPV-H5/2016 against antigenic drift of LPAI virus in Mexico and suggest that this vaccine would be a good candidate, likely as a primer in a prime-boost vaccination program.

Abbreviations: 1995 H5/HP, A/chicken/Queretaro/14588-19/1995 H5N2 HPAI virus; 2010 H5/LP, A/chicken/Aguascalientes/IA13/11/2010 H5N2 LPAI virus; AI, avian influenza; ABSL-2, animal biosafety level 2; ABSL-3E, animal biosafety level 3 enhanced; BSL-3E, biosafety level 3 enhanced; BIAH, Boehringer Ingelheim Animal Health USA Inc; BHI, brain heart infusion; CEF, chicken embryo fibroblasts; CL, cloacal; dpc, days post-challenge; ECE, embryonated chicken eggs; GMT, geometrical mean titers; HA, hemagglutinin; HI, hemagglutinin inhibition; HPAI, high pathogenicity avian influenza; HVT, herpesvirus turkey; IFA, immunofluorescence antibody; LPAI, low pathogenicity avian influenza; ML, maximum-likelihood; MDT, mean death time; MOI, multiplicity of infection; OP, oropharyngeal; PBS, phosphate buffered saline; qRRT-PCR, quantitative real-time reverse transcriptase polymerase chain reaction; rFPV, recombinant fowlpox virus; rFPV-H5/2016, recombinant fowlpox virus with H5 insert from the A/chicken/Mexico/P-14/2016; $TCID_{50}$, mean tissue culture infectious doses; SPF, specific pathogen free; USNPRC, U.S. National Poultry Research Center.

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1. Introduction

The use of avian influenza (AI) vaccines for control of high pathogenicity avian influenza (HPAI) was first reported in 1995 in Mexico during the H5N2 HPAI epizootic [1]. Vaccination together with other control measures were associated with the eradication of the H5N2 HPAI virus by mid-1995, but the predecessor H5N2 low pathogenicity avian influenza (LPAI) virus has continued to circulate in Mexico and spread to some Central American countries [2,3]. Enzootic H5N2 LPAI infections of poultry in Mexico and Central America cause a significant financial burden on poultry production and are still controlled by routine vaccination and secondary pathogen management control programs [4].

Despite decades of vaccination, surveillance, and biosecurity measures, H5N2 LPAI is enzootic in large geographic areas of Mexico and neighboring countries [5–7]. One explanation for tenacity of H5N2 LPAI is antigenic drift of circulating viruses compared to vaccine seed strains [8,9]. Since 1994, inactivated vaccine seed strains have been changed twice because of antigenic drift of field viruses and loss of vaccine protection. Initially in 1994, an inactivated oil-emulsified AI vaccine using the officially authorized vaccine seed strain A/chicken/Mexico/CPA-232/1994 (H5N2) provided protection in chickens against the 1995 HPAI field virus and the early H5N2 LPAI field viruses [1,9,10]. However, the 1994 vaccine strain was no longer protective against two later lineages of H5N2 LPAI viruses [9] and, starting in 2009, it was replaced by A/chicken/Mexico/VacunaCPA/2005 (H5N2) [11]. Recently, A/chicken/Mexico/VacunaCPA/2005 (H5N2) has been replaced by A/chicken/Guanajuato/CPA-20966-15-VS/2015 (H5N2) vaccine seed strain [11]. In addition to inactivated vaccines, H5 recombinant vectors have been used since 1998 [1,12]: recombinant fowlpox virus (FPV) (rFPV) with H5 insert of A/turkey/Ireland/1378/1983 (rFPV-H5/1983), recombinant Newcastle disease with H5 insert of A/chicken/Mexico/435/2005, and recombinant herpesvirus turkey (HVT) with H5 insert of clade 2.2 A/swan/Hungary/4999/2006 [11]. These recombinant vectors are used in 1-day-old chicks in the hatchery [6] to induce earlier protection than can be achieved by inactivated vaccines typically given at 10–14 days of age. However, for synergism, the live vectored vaccines can be used as a priming vaccine followed by a boost with inactivated vaccine in the field [1,11,12].

The continuing antigenic drift of H5N2 LPAI virus in H5-vaccinated farms [8,9] suggests need for proactive development of an updated H5 insert for current and future use. The objective of this study was to evaluate the efficacy of an experimental rFPV-H5 vaccine containing an updated H5 insert, more closely related to the current strains circulating in Mexico, and to provide experimental protection against a closely related H5N2 subcluster 4 LPAI virus and the regulatory-required 1995 H5N2 HPAI virus.

2. Materials and methods

2.1. Vaccine and viruses

A construct of live rFPV vaccine containing an H5 LPAI virus gene insert from the A/chicken/Mexico/P-14/2016 (GenBank accession number MF280172) (rFPV-H5 3006, hereafter rFPV-H5/2016) was constructed the same way as the licensed rFPV-H5/1983 (TROVAC-H®, Boehringer Ingelheim Animal Health USA Inc. [BIAH], Athens, GA, USA) and tested in this experiment. The FPV vector for rFPV-H5/1983 was derived from the vaccine strain contained in the DIFTOSEC® FPV vaccine (BIAH). The rFPV-H5/1983 received license in the United States in 1998 and has since been used commercially in Mexico, Guatemala, El Salvador, and Vietnam [6].

The rFPV-H5/2016 vaccine was characterized via immunofluorescence antibody (IFA) assay, polymerase chain reaction (PCR), and sequencing as described below. For IFA, chicken embryo fibroblasts (CEF) were infected with rFPV-H5/2016 at a multiplicity of infection (MOI) of 0.1 or 0.01. Uninfected CEF were included as a negative control. After 40-hour incubation, the media was removed and CEF were fixed with 95% ice cold acetone and washed once with deionized water. The fixed CEF monolayer was incubated with chicken anti-H5N2 (Charles River, Norwich, CT, USA) as primary antibody diluted 1:300 in phosphate buffered saline (PBS) for 1 h at 37 °C. The CEF were washed three times with PBS before incubation with rabbit anti-chicken IgG-FITC (Sigma Aldrich, St Louis, MO, USA) as secondary antibody diluted 1:500 in PBS for 1 h at 37 °C. The CEF were washed three times with PBS. Cultures were examined for hemagglutinin (HA) expression using fluorescent microscopy.

The PCR was performed to ensure both the absence of parental virus within rFPV-H5/2016 and the presence of the expression cassette within the targeted recombination region. Platinum PCR SuperMix High Fidelity (Thermo Scientific, Waltham, MA, USA) was used for all reactions. In the first PCR, primers Fowlpox probe F (5′ AATATCCGGTCTTAAAGAAGTCGCGG 3′) and Fowlpox probe R (5′ TTCCATAGAGGATCATGAGTTTCCGC 3′) were used to confirm the absence of any parental virus in the purified recombinant material. These primers bind to a region of the FPV genome which is deleted during recombination and, as such, only amplify parental virus. The amplicon produced by parental virus is 819 base pairs in size. The thermal profile for this reaction was as follows: denaturation at 94 °C for 2 min; 35 cycles at 94 °C for 30 sec, 56 °C for 30 sec, 68 °C for 1 min; elongation at 68 °C for 5 min; 4 °C hold. In the second PCR, primers 11339CXL (5′ GTAGTGATCAAAATACAGAACCAT 3′) and 11340CXL (5′ GAATCCGTCATTCAACTTTCTAGT 3′) were used to amplify the entire expression cassette. These primers lie just outside of the recombination arm. Although they amplify both recombinant and wild type FPV, the amplicon produced differ in size: 4989 base pairs for rFPV-H5/2016 and 4457 base pairs for parental virus. The thermal profile for this reaction was as follows: denaturation at 94 °C for 2 min; 35 cycles at 94 °C for 30 sec, 49.5 °C for 30 sec, 68 °C for 5 min; elongation at 68 °C for 10 min; 4 °C hold. The PCR products were examined using 1% agarose gel electrophoresis.

Finally, the recombination region of rFPV-H5/2016 was sequenced to confirm that its integrity had been maintained throughout the purification and scale up process. Primers 11339CXL and 11340CXL were again used to amplify the expression cassette which was sequenced by Eurofins Genomics (Louisville, KY, USA). The candidate vaccine rFPV-H5/2016 was administered at 1 day of age by the subcutaneous route at the target doses of 1.5, 2.0, and 3.1 $\log_{10}$ mean tissue culture infectious doses ($TCID_{50}$) in 0.2 ml per bird.

The influenza A isolates A/chicken/Aguascalientes/IA13/11/2010 H5N2 LPAI virus (2010 H5/LP) (GenBank accession numbers KM657856 to KM657863) and A/chicken/Queretaro/14588-19/1995 H5N2 HPAI virus (1995 H5/HP) (GenBank accession numbers AB558474 and CY015097 to CY015103) were used as challenge viruses. The 2010 H5/LP virus was chosen because of the epidemiological relevance of LPAI viruses in broiler premises in Mexico, which currently account for the majority of H5N2 LPAI cases in broilers and broiler breeders [2,3]. The 1995 H5/HP virus was chosen because it is the mandatory reference isolate for testing and licensing H5 influenza vaccines by the Mexican veterinary authorities. Working stocks were prepared and titrated in embryonated chicken eggs (ECE) using standard methods [13]. Stocks were diluted to the target dose with brain heart infusion (BHI) broth (Becton, Dickinson and Company, Sparks, MD, USA) with penicillin (2000 units/ml; Sigma Aldrich), gentamicin (200 ug/ml; Sigma

Aldrich) and amphotericin B (5 ug/ml; Sigma Aldrich). The studies were performed in biosafety level 3 enhanced (BSL-3E) facilities in accordance with procedures approved by the U.S. National Poultry Research Center (USNPRC) Institutional Biosecurity Committee.

2.2. HA sequence analysis

A total of 125 H5 HA sequences (nucleotide sequence length > 900) originated from Mexico and other Central American countries were downloaded from the National Center for Biotechnology Information Influenza Virus Resource database in May 2019 and added to sequence alignments. Retrieved sequences were aligned with other strains used in this study (n = 3) using Multiple Alignment with Fast Fourier Transformation in Geneious v8.1.2 program and trimmed to remove nucleotides that were outside the HA1 coding region. Phylogenetic relationships of 128 sequences were inferred from maximum-likelihood (ML) phylogeny with RAxML [14] using the general time reversible nucleotide substitution model and visualized with MEGA 7 software. Bootstrap support values were generated using 1,000 rapid bootstrap replicates and shown next to the branches (>70). Bayesian relaxed clock phylogenetic analysis of HA1 region was done using BEAST v1.8.4 [38]. We applied an uncorrelated lognormal distribution relaxed clock method, the Hasegawa-Kishino-Yano nucleotide substitution model, and the Bayesian skyride coalescent prior. A Markov Chain Monte Carlo method to sample trees and evolutionary parameters was run for 30 million generations. At least three independent chains were combined to ensure adequate sampling of the posterior distribution of trees (effective sampling size>200). BEAST output was analyzed with TRACER v1.7.1 with 10% burn-in. A maximum clade credibility tree was generated for each data set using TreeAnnotator in BEAST. FigTree 1.4.4 (http://tree.bio.ed.ac.uk/) was used for visualization of trees. The nucleotide and amino acid sequence identities between complete HA sequences of vaccine and challenge strains were calculated using Geneious v8.1.2 program.

The molecular characterization of H5 HA of the experimental vaccine (rFPV-H5/2016) and the challenge LPAI (2010 H5/LP) and HPAI (1995 H5/HP) viruses was performed as described below. Briefly, the HA sequences were aligned, and residues analyzed in the Lasergene 12 using Clustal W, MegAlign software (DNA STAR, Madison, WI, USA). After sequence alignment, the HA antigenic sites were identified as previously published [15,16]. The potential N-glycosylation sites were predicted using NetNGlyc server 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc). The HA results were numbered according to the H5 subtype conversion obtained in the Influenza Research Database (https://www.fludb.org).

2.3. Animals and housing

Two hundred specific pathogen free (SPF) White Leghorn chickens (BIAH) were randomized into groups (Table 1). For the vaccination period, each group was housed separately in negative pressured HEPA-filtered isolators within the animal biosafety level 2 (ABSL-2) facilities of BIAH. For the challenge period, the birds were transferred to negative pressured HEPA-filtered isolators within the animal biosafety level 3 enhanced (ABSL-3E) facilities of the USNPRC and allowed to acclimate for 3 days before challenge. Birds had *ad libitum* access to feed and water throughout the experiment. This study was reviewed and approved by the USNPRC Institutional Animal Care and Use committee.

Table 1
Experimental design.

Group	N	rFPV-H5/2016 vaccine dose (log_{10} $TCID_{50}$)[1]	Challenge virus[2]
1	20	Sham-vaccinated	2010 H5/LP
2	20	Low dose (1.5)	2010 H5/LP
3	20	Medium dose (2.0)	2010 H5/LP
4	20	High dose (3.1)	2010 H5/LP
5	30	Sham-vaccinated	1995 H5/HP
6	30	Low dose (1.5)	1995 H5/HP
7	30	Medium dose (2.0)	1995 H5/HP
8	30	High dose (3.1)	1995 H5/HP

[1] The rFPV-H5/2016 vaccine was administered at 1 day old by the subcutaneous route at the indicated target doses in 0.2 ml per bird.
[2] Challenge viruses were A/chicken/Aguascalientes/IA13/11/2010 H5N2 LPAI virus (2010 H5/LP) and A/chicken/Queretaro/14588-19/1995 H5N2 HPAI virus (1995 H5/HP).

2.4. Experimental design and sampling

Day-old chickens were either vaccinated with rFPV-H5/2016 vaccine at various doses or sham-vaccinated with sterile HVT vaccine diluent (BIAH) only (Table 1). Three weeks post-vaccination (21 days of age), all birds were challenged by the intra-choanal route with estimated 6 log_{10} EID_{50}/0.1 ml of either 2010 H5/LP or 1995 H5/HP. The inoculum titers were subsequently verified as 5.9 and 5.7 log_{10} EID_{50}, respectively, by back titration in ECE. All the birds were monitored daily for clinical signs and mortality for 2 weeks following challenge. Severely sick birds were euthanized and counted as dead for the next day in mean death time (MDT) calculations. Oropharyngeal (OP) and cloacal (CL) swabs were collected at 2, 4, and 7 days post-challenge (dpc) from 2010 H5/LP challenged groups, and at 2 dpc from 1995 H5/HP challenged groups and placed in 1.5 ml of BHI with antibiotics and antifungal. Serum samples were collected pre-challenge (18 days post-vaccination) and at termination (14 dpc). At 14 dpc, surviving birds were euthanized by cervical dislocation.

2.5. Determination of virus from swabs

Swab samples in BHI were processed for quantitative real-time reverse transcriptase PCR (qRRT-PCR) [17] with modifications [18] to determine viral RNA titers. The standard curves for viral RNA quantification were established with RNA extracted from dilutions of the same titrated stocks of the challenge virus. This is a standard protocol among published veterinary influenza vaccine studies given the high correlation between the quantity of RNA determined by qRRT-PCR and the EID_{50} determined by ECE titration when the same challenge virus stock is used to generate the standard curve [19]. The limit of detection was 1.9 log_{10} EID_{50}/ml for 2010 H5/LP and 1.5 log_{10} EID_{50}/ml for 1995 H5/HP.

2.6. Serology

Hemagglutinin inhibition (HI) assays were performed using H5 antigens specific for each corresponding challenge antigen. The antigens were prepared as previously described [20] and the HI assays were performed according to standard procedures [21]. Titers were expressed as log_2 geometrical mean titers (GMT). GMT included only positive serum samples. Samples with titers below 3 log_2 GMT were considered negative and then assigned as 2 log_2 GMT for statistical purposes.

2.7. Statistical analysis

Statistical analyses were performed using Prism 7 (GraphPad software, San Diego, CA, USA). Survival curves were compared

using the Mantel-Cox Log-rank test. Fisher's exact test was used to analyze statistical significance of virus shedding. Mean virus shedding titers and HI antibody titers had a non-parametric distribution and were analyzed with Kruskal–Wallis test and Dunn's Multiple Comparison test. A P-value of < 0.05 was considered to be significant. P values in figures are represented as: * for $p < 0.05$; ** for $p < 0.01$; *** for $p < 0.001$; and **** for $p < 0.0001$.

3. Results

3.1. Characterization of rFPV-H5/2016

Expression of HA, PCR purity, and sequencing of rFPV-H5/2016 were confirmed. The anti-H5N2 polyclonal antibody reacted strongly with rFPV-H5/2016, while no binding was detected in the uninfected control (Fig. S1). The PCR specific to non-recombinant FPV confirmed the absence of parental virus within rFPV-H5/2016 (Fig. S2a). The PCR binding to the recombination arms of rFPV-H5/2016 confirmed the presence of a single band of the expected size (Fig. S2b). Sequencing of this amplicon confirmed the intended sequence for recombination arms, promoter, and gene (data not shown).

3.2. HA sequence analysis

To understand the role of HA in the protection of chickens against challenge with Mexican H5N2 LPAI and HPAI viruses, we compared the amino acid and nucleotide identity for complete HA, the HA antigenic sites, and the predicted N-glycosylation sites between the experimental rFPV-H5/2016 vaccine seed strain and the challenge strains.

The HA sequences of the experimental rFPV-H5/2016 vaccine seed strain (A/chicken/Mexico/P-14/2016), the two experimental challenge viruses (A/chicken/Aguascalientes/IA13/11/2010 and A/chicken/Queretaro/14588–19/1995), and the 5 vaccine seed strains used in Mexico from 1995 to 2017 were compared with each other and with other Mexican and Central American HA sequences (Table 2, Fig. 1, and S3). The Mexican and Central American HA sequences formed a well-supported monophyletic group with four distinct phylogenetic subclusters defined by high posterior probabilities in Bayesian phylogeny (>95) and bootstrap values in ML phylogeny (>80) (Fig. 1). The vaccine seed strain A/chicken/Mexico/CPA-232/1994 and the 1995 H5/HP isolate belonged to subcluster 3, while the experimental rFPV-H5/2016 vaccine seed strain, A/chicken/Mexico/VacunaCPA/2005, A/chicken/Mexico/435/2005, and the 2010 H5/LP isolate belonged to subcluster 4. The HA sequences of the rFPV-H5/2016 vaccine seed strain and the 2010 H5/LP isolate clustered together phylogenetically (Fig. 1 and S3). The A/turkey/Ireland/1378/1983 and A/mute_swan/Hungary/4999/2006 vaccine seed strains belonged to a different genetic lineage (the latter was not included in the trees due to its large phylogenetic distance with Mexican and Central American HA sequences) (Fig. 1 and S3) and shared the lowest ($\leq$82.2%) full-length HA sequence identities with the 2010 H5/LP challenge virus (Table 2). In contrast, the HA sequence of the experimental rFPV-H5/2016 vaccine seed strain had 96.3% amino acid and nucleotide sequence identities with the 2010 H5/LP isolate (Table 2), and 88.7% (amino acid) and 89.2% (nucleotide) sequence identity with the 1995 H5/HP isolate (Table 2). The HA sequence of the other 3 vaccine seed strains (A/chicken/Mexico/CPA-232/1994, A/chicken/Mexico/435/2005, and A/chicken/Mexico/VacunaCPA/2005) had 88.3–94.0% sequence identities with the 2010 H5/LP isolate (Table 2).

Both the amino acid alignment and the analysis of the antigenic sites showed a few amino acid differences between the HA sequence of the experimental vaccine strain and the challenge strains (data not shown). Some of these amino acid substitutions resulted in potential new predicted glycosylation sites in the experimental rFPV-H5/2016 vaccine seed strain and the 2010 H5/LP strain, which were not detected in the 1995 H5/HP strain (data not shown). In particular, these two more recent strains, i.e. the experimental vaccine strain and the 2010 H5/LP virus, obtained additional potential glycosylation sites in positions 84, 126, and 163 of the HA, which are found within antigenic sites A, D, and E, respectively.

3.3. Efficacy of rFPV-H5/2016 on protection against 2010 H5/LP virus

Clinical protection. All birds survived 2010 H5/LP challenge, except for one sham-vaccinated bird that died at 7 dpc from unrelated causes and was excluded from the study (Fig. 2a). Sham-vaccinated and vaccinated birds lacked clinical signs from rFPV-H5/2016 vaccination or following challenge with 2010 H5/LP virus.

Serology. None of the sham-vaccinated birds had detectable HI antibody titers before challenge (Fig. 3a). In contrast, 18/20 birds of each vaccinated group had antibodies detected when using 2010 H5/LP challenge virus as test antigen on serum samples collected prior to challenge, with GMT ranging 3.4 to 4.2 $\log_2$ (Fig. 3a). At termination, 100% of the sham-vaccinated and rFPV-H5/2016 vaccinated birds had similarly high HI antibody titers. An anamnestic response was observed after challenge for the sham-vaccinated group (6.8-fold increase) and all vaccinated groups (5.4-to-6-fold increase) (Fig. 3a).

Table 2
Nucleotide and amino acid full-length HA sequence similarities among vaccine and challenge H5 AI isolates.

	Nucleotide sequence identity matrix							
Virus strain	Ire/83	Hungary	232	435	VaCPA	Mx16	H5/HP	H5/LP
A/turkey/Ireland/1378/1983 – vax	–	87.2	81.4	79.2	79.1	79.2	81.9	79.4
A/mute_swan/Hungary/4999/2006 – vax	90.1	–	79.9	78.0	77.6	77.2	80.1	77.3
A/chicken/Mexico/CPA-232/1994 – vax*	87.6	87.0	–	92.0	91.3	90.3	97.6	90.5
A/chicken/Mexico/435/2005 – vax	83.9	83.1	90.8	–	93.6	90.3	91.6	90.8
A/chicken/Mexico/VacunaCPA/2005 – vax	83.6	82.4	89.7	94.7	–	90.8	90.9	92.0
A/chicken/Mexico/P-14/2016 – exp vax	82.7	82.0	88.7	92.0	93.6	–	89.2	96.3
A/chicken/Queretaro/14588–19/1995 – 1995 H5/HP	88.7	87.5	97.5	90.8	89.4	88.7	–	89.5
A/chicken/Aguascalientes/IA13/11/2010 – 2010 H5/LP	82.2	81.0	88.3	92.4	94.0	96.3	88.3	–
	Amino acid sequence identity matrix							

Vax, vaccine seed strains; exp vax, experimental vaccine seed strain tested here; 1995 H5/HP, H5N2 HPAI virus challenge strain; 2010 H5/LP, H5N2 LPAI virus challenge strain.

* The full-length HA gene of A/chicken/Hidalgo/28159-232/1994(H5N2) virus was used instead, which is found to be similar to A/chicken/Mexico/CPA-232/1994 [9].

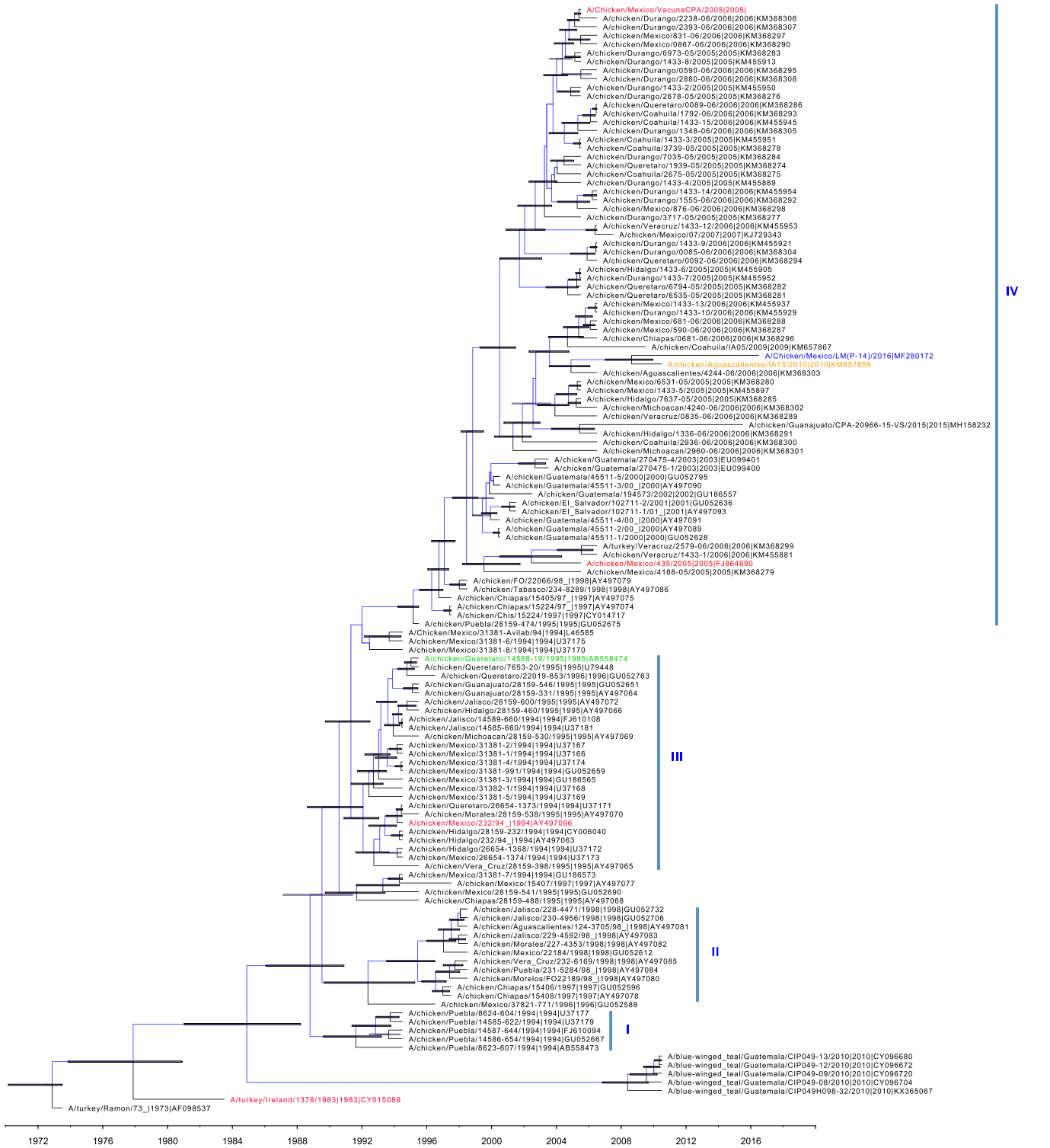

Fig. 1. Relaxed clock molecular phylogenetic tree for H5 gene from Mexico and other Central American countries. The phylogenetic relationships have been estimated by Bayesian molecular clock analysis. The branches colored with blue identify high posterior probabilities >95%. The brackets represent the genetic subclusters supported by high values of posterior probabilities in Bayesian phylogeny (>95) and bootstrap values in maximum-likelihood phylogeny (>80). The following taxa are highlighted: the 5 vaccine seed strains used in Mexico from 1995 to 2017 (red), the experimental vaccine seed strain (blue), and the challenge viruses 2010 H5/LP (orange) and 1995 H5/HP (green) used in this study. A/mute_swan/Hungary/4999/2006 was not included due to its large phylogenetic distance with Mexican and Central American HA sequences.

Viral shedding. All the sham-vaccinated birds (20/20) excreted high virus titers in the oropharynx at 2 and 4 dpc (mean 5.8 and 5.7 $\log_{10}$ EID_{50}/ml, respectively), but fewer birds (9/20) and lower titers (2.3 $\log_{10}$ EID_{50}/ml) were shed via the OP route at 7 dpc (Fig. 4a). Low virus titers (mean 2.2–2.5 $\log_{10}$ EID_{50}/ml) were detected in the cloaca of 12/20 (2 dpc), 11/20 (4 dpc), and 8/20 (7 dpc) sham-vaccinated birds (Fig. 4b). Mean OP and CL shedding titers of the three vaccine-dose groups were statistically lower than those of sham-vaccinated controls at 2 dpc, but number of birds shedding was significantly different only for CL shedding

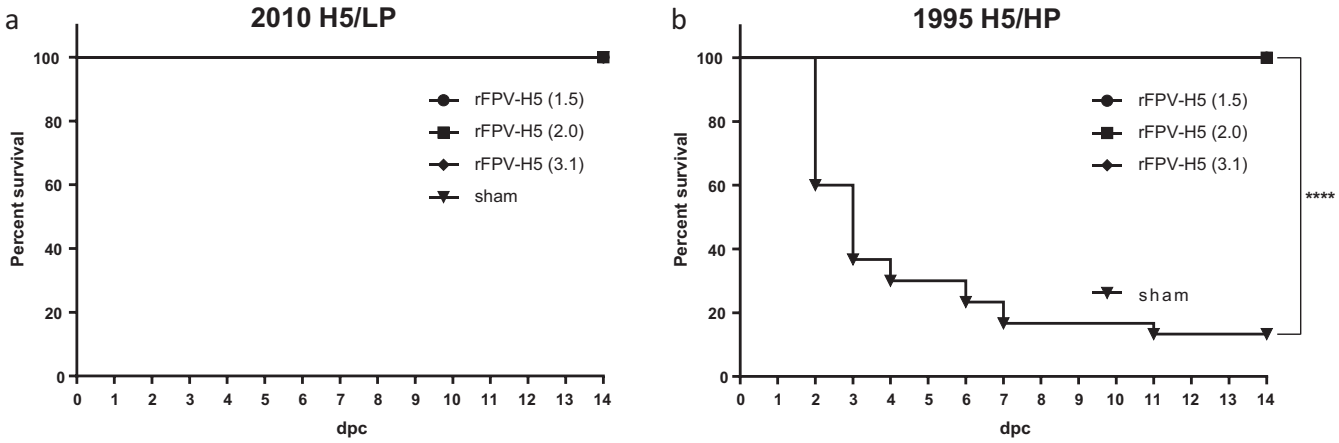

Fig. 2. Survival curves of chickens vaccinated with rFPV-H5/2016 vaccine at various doses or sham-vaccinated and challenged with **a.** 2010 H5/LP virus or **b.** 1995 H5/HP virus. P values in figures are represented as: * for $p < 0.05$; ** for $p < 0.01$; *** for $p < 0.001$; and **** for $p < 0.0001$.

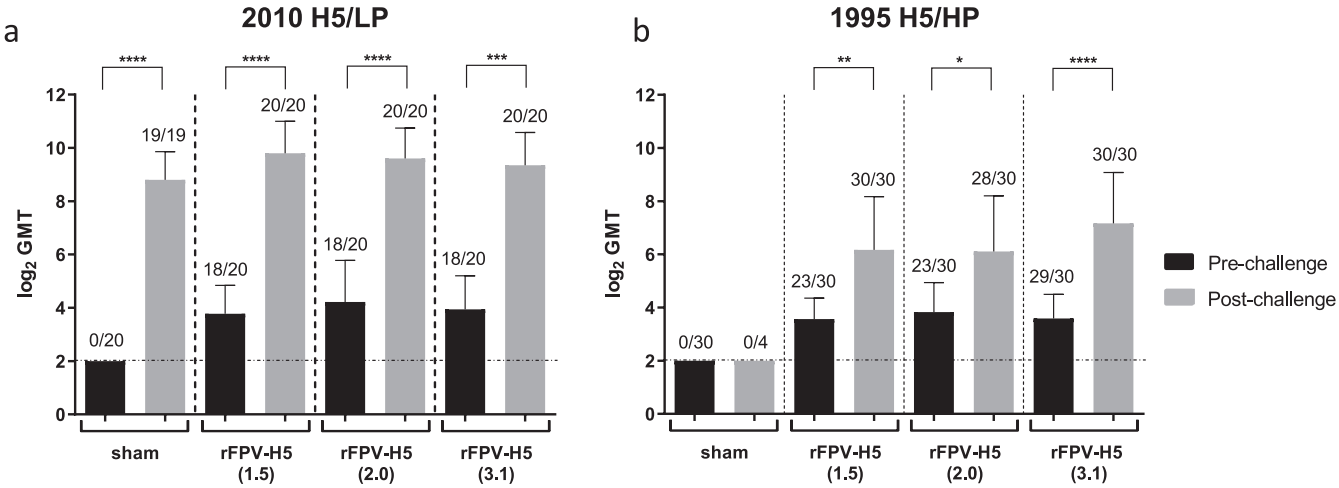

Fig. 3. Serology from chickens sham-vaccinated or vaccinated with rFPV-H5/2016 vaccine at various doses and challenged with **a.** 2010 H5/LP virus or **b.** 1995 H5/HP virus. HI titers pre- and post-challenge against corresponding challenge viruses as test antigens. Titers are expressed as $\log_2$ GMT. GMT includes only positive serum samples. Samples with titers below 3 $\log_2$ GMT were considered negative. Ratios above the bars indicate the number of birds with HI titers from the total number of birds. P values in the figure are represented as: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$.

(Fig. 4a and b). Birds vaccinated with 2.0 and 3.1 $\log_{10}$ $TCID_{50}$ (but no 1.5 $\log_{10}$ $TCID_{50}$) had significantly lower OP and CL shedding titers than sham-vaccinated controls at 4 dpc, and no significant differences were observed at 7 dpc (Fig. 4a and b). No significant differences were observed between the three vaccine-dose groups regarding shedding titers or number of birds shedding at any time point (Fig. 4a and b).

3.4. Efficacy of rFPV-H5/2016 on protection against 1995 H5/HP virus

Clinical protection. After 1995 H5/HP challenge, 26/30 (87%) sham-vaccinated birds showed acute severe clinical disease and death, with a MDT of 3.5 dpc (Fig. 2b). All the vaccinated birds remained clinically healthy during the observation period (14 dpc), with no clinical signs from the rFPV-H5/2016 vaccination or following challenge with 1995 H5/HP virus.

Serology. None of the sham-vaccinated birds had detectable HI antibody titers before challenge (Fig. 3b). In contrast, 23/30 to 29/30 birds of the vaccinated groups had antibodies detected when using the 1995 H5/HP challenge virus as test antigen on serum samples collected prior to challenge, with GMT ranging 3.6 to 3.8 $\log_2$ (Fig. 3b). At termination, 93–100% of the vaccinated birds had similarly moderate-to-high HI antibody titers. All vaccinated groups had an anamnestic response (2.3–3.6-fold increase) after challenge (Fig. 3b).

Viral shedding. High virus titers were detected at 2 dpc in the oropharynx (mean 4.1 $\log_{10}$ EID_{50}/ml) and cloaca (mean 3.4 $\log_{10}$ EID_{50}/ml) of 25/30 and 19/30 sham-vaccinated birds, respectively (Fig. 4c and d). Mean OP and CL shedding titers of vaccinated birds were statistically lower than those of sham-vaccinated controls, as well as number of birds shedding (Fig. 4c and d). No significant differences were observed between vaccine-dose groups regarding OP and CL shedding titers and number of birds shedding (Fig. 4c and d).

4. Discussion

After two decades of vaccination and use of other disease control tools, H5N2 LPAI still affects many farms in Mexico and neighboring countries [5–7]. Antigenic drift of circulating field viruses away from vaccine strains is the most likely explanation for the presence of enzootic infections [8,9]. Here we evaluated the efficacy of experimental rFPV-H5/2016 vaccine containing an updated H5 insert to provide protection against a recent Mexican H5N2 LPAI virus from 2010 and the original type strain of H5N2 HPAI virus from 1995.

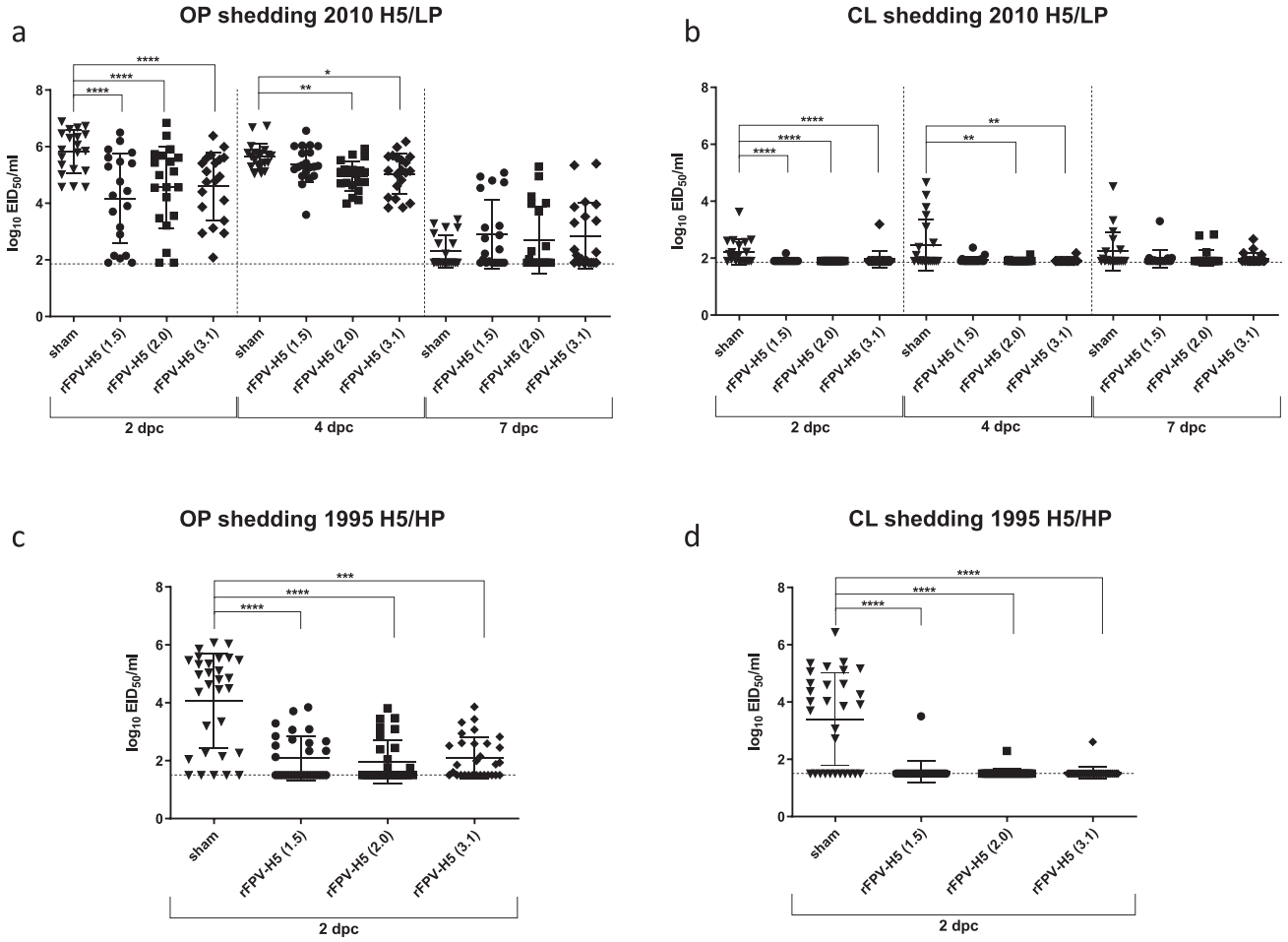

Fig. 4. Scatter plot of oropharyngeal (OP) and cloacal (CL) shedding from chickens sham-vaccinated or vaccinated with rFPV-H5/2016 vaccine at various doses and challenged with 2010 H5/LP virus or 1995 H5/HP virus. Shedding titers are expressed as $\log_{10}$ EID_{50}/ml with error bars included. The limit of detection of the qRRT-PCR was 1.9 $\log_{10}$ EID_{50}/ml for 2010 H5/LP and 1.5 $\log_{10}$ EID_{50}/ml for 1995 H5/HP. P values in the figure are represented as: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$.

Our ML and Bayesian phylogenetic analyses of the HA gene segments show the divergence of H5N2 viruses in Mexico into multiple subclusters. However, only a single lineage of virus evolved and maintained in Mexican poultry, eventually giving rise to subcluster 4. As previously suggested [9], our data indicates that the H5N2 LPAI viruses circulating in Mexico and neighboring countries since 1994 have undergone antigenic drift away from vaccine seed strains used in this country. Lee et al. (2004) conducted cross-HI tests with 8 isolates representative of different Mexican H5N2 virus sublineages and found that the viruses belonging to subcluster 2 (A/chicken/Aguascalientes/124-3705/1998 and A/chicken/Puebla/231-5284/1998) and subcluster 4 (A/chicken/Puebla/28159-474/1995, A/chicken/El_Salvador/102711-1/2001, and A/chicken/Guatemala/194573/2002) in our phylogenetic tree are antigenically different from the A/chicken/Mexico/CPA-232/1994 vaccine strain and from each other [9]. Similarly, Escorcia et al. (2010) performed phylogenetic and serological analysis on H5N2 LPAI field viruses isolated in Mexico between 1994 and 2008 and revealed that molecular drift in HA gene follow a yearly trend, suggesting gradually cumulative sequence mutations [22]. In particular, sequence similarities to the vaccine strain A/chicken/Mexico/CPA-232/1994 were 94.5–98% for 1994–1996 isolates, but dropped to 90–91% for 2006–2008 isolates [22]. Interestingly, the authors also showed that in HI tests using antiserum produced with antigen A/chicken/Mexico/CPA-232/1994, HI titers were significantly higher in subcluster 4 viruses isolated in 2002 compared to subcluster 4 viruses isolated between 2006, 2007, and 2008 [22]. In our study, sequence similarities between A/chicken/Mexico/CPA-232/1994 and the 1995 H5/HP challenge isolate (subcluster 3) or the 2010 H5/LP challenge isolate (subcluster 4) were <91% and >97%, respectively, in line with Escorcia et al. (2010) [22]. Our results confirm that H5N2 LPAI viruses have been drifting away from vaccines used in Mexico, and that some currently used vaccines may no longer be sufficiently protective against circulating viruses, which could explain their continuing diagnosis in clinical cases of respiratory disease [5–7]. In addition, the continued use of inadequate vaccines may have an effect on the evolution of AI virus [9], which is concerning due to the possibility of mutation again to the HP form of the virus [23–26]. Collectively, our results confirm the need to update the vaccines used in Mexico.

The H5 insert candidate tested here had very high genetic similarity with the 2010 H5/LP challenge virus, but <90% genetic similarity with the 1995 H5/HP challenge virus, the reference isolate for vaccine licensing in Mexico. Nonetheless, the tested rFPV-H5/2016 vaccine provided protective HI titers pre-challenge against both challenge antigens in almost 100% of the immunized birds, with no differences in number of birds seroconverting or HI titers among tested doses. The vaccine conferred clinical protection against 1995 H5/HP virus challenge, and a significant decrease in virus shedding on both OP and CL swabs compared to sham controls at all tested doses. It is worth mentioning that two out of the 4 sham controls that survived 1995 H5/HP challenge were not infected

based on lack of virus shedding and lack of detectable HI antibodies at termination, resulting in 93% infection and mortality rates and thus complying with the acceptance criteria for vaccine efficacy studies of ≥90% mortality in shams [11,27]. The reason why the rFPV-H5/2016 vaccine conferred protection against 1995 H5/HP challenge despite these isolates being <90% similar could be that, although the insert (as well as the 2010 H5/LP virus) had acquired 3 potential N-glycans in antigenic sites A, D, and E that are not found in the 1995 H5/HP virus, these N-glycans did not physically block access to antigenic sites in the 1995 H5/HP virus, likely allowing the vaccine-induced antibody-mediated and/or cell-mediated response to effectively neutralize the virus [28–32].

Virus shedding titers from vaccinated 2010 H5/LP virus challenged birds significantly decreased compared to sham birds especially at earlier time points. However, virus titers were still relatively high and significant virus titer reduction was less likely over time. Albeit vaccination does not completely prevent virus replication [2], the shedding of H5 LPAI viruses is of concern because of the potential for transmission to naïve flocks and the possibility of mutation to the HP form of the virus [9,23–26]. Eggert et al. (2010) [33] evaluated the efficacy of 10 commercially available H5 vaccines against challenge with H5N2 LPAI viruses isolated from Latin America in 2003 [33]. 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 respiratory shedding titers, but these were still relatively high (4 and 4.4 $\log_{10}$ EID_{50}/ml). In addition, an rFPV containing a more distantly related Eurasian lineage H5 gene insert significantly reduced respiratory shedding as compared to shams, but mean titers among shedders were 3.9 $\log_{10}$ EID_{50}/ml [33]. Collectively, these findings indicate that single vaccinations may not provide optimal immunity capable of preventing all H5 LPAI virus replication and shedding, but the goal of vaccination is to reduce virus replication and ultimately reduce transmission [2]. Therefore, further studies aimed at optimizing the efficacy of rFPV-H5/2016 are needed, such as increasing the dose or using it as a priming vaccine in the hatchery followed by inactivated AI virus vaccine boost in the field.

The choice to update a live virus vector vaccine insert as opposed to an inactivated whole-virus vaccine responds to the following: (i) vector vaccines allow for rapid insertion of any influenza HA (or other) gene, making them attractive platforms due to constant antigenic shift and drift among AI viruses [34,35]; and (ii) vector vaccines can stimulate humoral and cellular immunity when given parenterally and, if they replicate at a mucosal site, induce mucosal immunity, thus reducing shedding [1]. The mentioned advantages of vector vaccines, together with the efficacy of the updated rFPV-H5/2016 vaccine tested here against both 2010 H5/LP and 1995 H5/HP Mexican viruses, suggest this vaccine would be a good candidate for licensing in Mexico, likely as a primer at the hatchery followed by an inactivated adjuvanted vaccine on the farm [36,37].

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author Contributions

Conceptualization and Funding acquisition: MSS, JW, NP, TM, and DES. Data curation, Formal analysis, Methodology: KB, MC, DHL, LK, MSS, EL, and EA. Writing original draft: KB, MC, DHL, and DES. Writing, review, and editing: all authors.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.11.072.

References

[1] Swayne DE, Kapczynski DR. Vaccines, vaccination and immunology for avian influenza viruses in poultry. In: Swayne DE, editor. Avian influenza. Ames, IA: Blackwell Publishing; 2008. p. 407–51.
[2] Swayne DE, Pavade G, Hamilton K, Vallat B, Miyagishima K. Assessment of national strategies for control of high-pathogenicity avian influenza and low-pathogenicity notifiable avian influenza in poultry, with emphasis on vaccines and vaccination. Rev Sci Tech Off Int Epiz 2011;30:839–70.
[3] Perez DR, de Wit JJ. Low-pathogenicity avian influenza. In: Swayne DE, editor. Animal influenza. Ames, IA: Wiley Blackwell; 2016. p. 271–301.
[4] Swayne DE. Avian influenza control strategies. In: Swayne DE, editor. Avian influenza. Ames, IA: Blackwell Publishing; 2008. p. 287–97.
[5] Villarreal-Chavez C, Rivera-Cruz E. An update on avian influenza in Mexico. Avian Dis 2003;47(3 Suppl):1002–5.
[6] Bublot M, Pritchard N, Swayne DE, Selleck P, Karaca K, Suarez DL, et al. Development and use of fowlpox vectored vaccines for avian influenza. Ann NY Acad Sci 2006;1081:193–201.
[7] World Organisation for Animal Health (OIE), Update on avian influenza in animals (types H5 and H7), https://www.oie.int/animal-health-in-the-world/update-on-avian-influenza/.
[8] Forrest HL, Garcia A, Danner A, Seiler JP, Friedman K, Webster RG, et al. Effect of passive immunization on immunogenicity and protective efficacy of vaccination against a Mexican low-pathogenic avian H5N2 influenza virus. Influenza Other Respir Viruses 2013;7(6):1194–201.
[9] Lee CW, Senne DA, Suarez DL. Effect of vaccine use in the evolution of Mexican lineage H5N2 avian influenza virus. J Virol 2004;78:8372–81.
[10] Garcia A, Johnson H, Srivastava DK, Jayawardene DA, Wehr DR, Webster RG. Efficacy of inactivated H5N2 influenza vaccines against lethal A/Chicken/Queretaro/19/95 infection. Avian Dis 1998;42(2):248–56.
[11] Swayne DE, Sims LD. Avian influenza vaccines. In: Metwally S, Werner B, editors. Veterinary Vaccines for Livestock. Rome: Food and Agriculture Organization; 2020. in press.
[12] Villarreal C. Experience in control of avian influenza in the Americas. Dev Biol (Basel) 2007;130:53–60.
[13] Spackman E, Killian ML. Avian influenza virus isolation, propagation, and titration in embryonated chicken eggs. Methods Mol Biol 2014;1161:125–40.
[14] Stamatakis A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 2014;30(9):1312–3.
[15] Stray SJ, Pittman LB. Subtype- and antigenic site-specific differences in biophysical influences on evolution of influenza virus hemagglutinin. Virol J 2012;9(91).
[16] Yang H, Carney PJ, Mishin VP, Guo Z, Chang JC, Wentworth DE, et al. Molecular characterizations of surface proteins hemagglutinin and neuraminidase from recent H5Nx avian influenza viruses. J Virol 2016;90(12):5770–84.
[17] Spackman E, Senne DA, Myers TJ, Bulaga LL, Garber LP, Perdue ML, et al. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. J Clin Microbiol 2002;40(9):3256–60.
[18] Slomka MJ, Densham AL, Coward VJ, Essen S, Brookes SM, Irvine RM, et al. Real time reverse transcription (RRT)-polymerase chain reaction (PCR) methods for detection of pandemic (H1N1) 2009 influenza virus and European swine influenza A virus infections in pigs. Influenza Other Respir Viruses 2010;4(5):277–93.
[19] Lee CW, Suarez DL. Application of real-time RT-PCR for the quantitation and competitive replication study of H5 and H7 subtype avian influenza virus. J Virol Methods 2004;119:151–8.
[20] Abbas MA, Spackman E, Fouchier R, Smith D, Ahmed Z, Siddique N, et al. H7 avian influenza virus vaccines protect chickens against challenge with antigenically diverse isolates. Vaccine 2011;29:7424–9.

[21] Pedersen JC. Hemagglutination-inhibition assay for influenza virus subtype identification and the detection and quantitation of serum antibodies to influenza virus. In: Spackman E, editor. Animal influenza virus. New York, NY: Springer; 2014. p. 11–26.
[22] Escorcia M, Carrillo-Sanchez K, March-Mifsut S, Chapa J, Lucio E, Nava GM. Impact of antigenic and genetic drift on the serologic surveillance of H5N2 avian influenza viruses. BMC Vet Res 2010;6(57).
[23] Garcia M, Crawford JM, Latimer JW, Rivera-Cruz E, Perdue ML. Heterogeneity in the haemagglutinin gene and emergence of the highly pathogenic phenotype among recent H5N2 avian influenza viruses from Mexico. J Gen Virol 1996;77(7):1493–504.
[24] Horimoto T, Rivera E, Pearson J, Senne D, Krauss S, Kawaoka Y, et al. Origin and molecular changes associated with emergence of a highly pathogenic H5N2 influenza virus in Mexico. Virology 1995;213(1):223–30.
[25] Kawaoka Y, Naeve CW, Webster RG. Is virulence of H5N2 influenza viruses in chickens associated with loss of carbohydrate from the hemagglutinin? Virology 1984;139(2):303–16.
[26] Perdue ML, Garcia M, Beck J, Brugh M, Swayne DE. An Arg-Lys insertion at the hemagglutinin cleavage site of an H5N2 avian influenza isolate. Virus Genes 1996;12(1):77–84.
[27] OIE. Manual of diagnostic tests and vaccines for terrestrial animals. Avian Influenza; 2016 [chapter 2.3.4].
[28] Tate MD, Job ER, Deng YM, Gunalan V, Maurer-Stroh S, Reading PC. Playing hide and seek: how glycosylation of the influenza virus hemagglutinin can modulate the immune response to infection. Viruses 2014;6(3):1294–316.
[29] Herve PL, Lorin V, Jouvion G, Da Costa B, Escriou N. Addition of N-glycosylation sites on the globular head of the H5 hemagglutinin induces the escape of highly pathogenic avian influenza A H5N1 viruses from vaccine-induced immunity. Virology 2015;486:134–45.
[30] Wu CY, Lin CW, Tsai TI, Lee CD, Chuang HY, Chen JB, et al. Influenza A surface glycosylation and vaccine design. Proc Natl Acad Sci USA 2017;114(2):280–5.
[31] Zost SJ, Parkhouse K, Gumina ME, Kim K, Diaz Perez S, Wilson PC, et al. Contemporary H3N2 influenza viruses have a glycosylation site that alters binding of antibodies elicited by egg-adapted vaccine strains. Proc Natl Acad Sci USA 2017;114(47):12578–83.
[32] Criado MF, Bertran K, Lee DH, Killmaster L, Stephens CB, Spackman E, et al. Efficacy of novel recombinant fowlpox vaccine against recent Mexican H7N3 highly pathogenic avian influenza virus. Vaccine 2019;37(16):2232–43.
[33] Eggert D, Thomas C, Spackman E, Pritchard N, Rojo F, Bublot M, et al. Characterization and efficacy determination of commercially available Central American H5N2 avian influenza vaccines for poultry. Vaccine 2010;28:4609–15.
[34] Bosworth B, Erdman MM, Stine DL, Harris I, Irwin C, Jens M, et al. Replicon particle vaccine protects swine against influenza. Comp Immunol Microbiol Infect Dis 2010;33(6):e99–e103.
[35] Vander Veen RL, Mogler MA, Russell BJ, Loynachan AT, Harris DL, Kamrud KI. Haemagglutinin and nucleoprotein replicon particle vaccination of swine protects against the pandemic H1N1 2009 virus. Vet Rec 2013;173(14):344.
[36] Suarez DL, Pantin-Jackwood MJ. Recombinant viral-vectored vaccines for the control of avian influenza in poultry. Vet Microbiol 2017;206:144–51.
[37] Bertran K, Lee DH, Criado MF, Balzli CL, Killmaster LF, Kapczynski DR, et al. Maternal antibody inhibition of recombinant Newcastle disease virus vectored vaccine in a primary or booster avian influenza vaccination program of broiler chickens. Vaccine 2018;36(43):6361–72.
[38] Drummond AJ, Rambaut A. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol Biol 2007;7(214).