Vaccine 37 (2019) 2232–2243

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Efficacy of novel recombinant fowlpox vaccine against recent Mexican H7N3 highly pathogenic avian influenza virus

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ARTICLE INFO

Article history:
Received 18 October 2018
Received in revised form 1 March 2019
Accepted 3 March 2019
Available online 15 March 2019

Keywords:
Chickens
High pathogenicity avian influenza
H7N3
Immunity
Recombinant fowlpox virus vaccine
Vaccine

ABSTRACT

Since 2012, H7N3 highly pathogenic avian influenza (HPAI) has produced negative economic and animal welfare impacts on poultry in central Mexico. In the present study, chickens were vaccinated with two different recombinant fowlpox virus vaccines (rFPV-H7/3002 with 2015 H7 hemagglutinin [HA] gene insert, and rFPV-H7/2155 with 2002 H7 HA gene insert), and were then challenged three weeks later with H7N3 HPAI virus (A/chicken/Jalisco/CPA-37905/2015). The rFPV-H7/3002 vaccine conferred 100% protection against mortality and morbidity, and significantly reduced virus shed titers from the respiratory and gastrointestinal tracts. In contrast, 100% of sham and rFPV-H7/2155 vaccinated birds shed virus at higher titers and died within 4 days. Pre- (15/20) and post- (20/20) challenge serum of birds vaccinated with rFPV-H7/3002 had antibodies detectable by hemagglutination inhibition (HI) assay using challenge virus antigen. However, only a few birds (3/20) in the rFPV-H7/2155 vaccinated group had antibodies that reacted against the challenge strain but all birds had antibodies that reacted against the homologous vaccine antigen (A/turkey/Virginia/SEP-66/2002) (20/20). One possible explanation for differences in vaccines efficacy is the antigenic drift between circulating viruses and vaccines. Molecular analysis demonstrated that the Mexican H7N3 strains have continued to rapidly evolve since 2012. In addition, we identified *in silico* three potential new N-glycosylation sites on the globular head of the H7 HA of A/chicken/Jalisco/CPA-37905/2015 challenge virus, which were absent in 2012 H7N3 outbreak virus. Our results suggested that mutations in the HA antigenic sites including increased glycosylation sites, accumulated in the new circulating Mexican H7 HPAIV strains, altered the recognition of neutralizing antibodies from the older vaccine strain rFPV-H7/2155. Therefore, the protective efficacy of novel rFPV-H7/3002 against recent outbreak Mexican H7N3 HPAIV confirms the importance of frequent updating of vaccines seed strains for long-term effective control of H7 HPAI virus.

Abbreviations: Jalisco/12283/2012, A/chicken/Jalisco/CPA-12283/2012 [H7N3]; Jalisco/37905/2015, A/chicken/Jalisco/CPA-37905/2015 [H7N3]; AP, Alkaline phosphatase; ABSL-2, Animal biosafety level 2; ABSL-3E, Animal biosafety level 3 enhanced; AIV, Avian influenza viruses; CL, Cloacal swabs; dpc, Days post-challenge; DI, Defective interfering; ECE, Embryonating chicken eggs; EID_{50}, 50 percent embryo infectious doses; GMT, Geometric mean titers; HI, Hemagglutination inhibition; HA, Hemagglutinin; HPAI, Highly pathogenic avian influenza; LPAI, Low pathogenicity avian influenza; MDT, Mean death time; NA, Neuraminidase; OP, Oropharyngeal swabs; qRRT-PCR, Quantitative real-time RT-PCR; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; SEPRL, Southeast Poultry Research Laboratory; SPF, Specific-pathogen-free; SQ, Subcutaneous; TBST, Tris-buffered saline with Tween 20; WL, White Leghorn.

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https://doi.org/10.1016/j.vaccine.2019.03.009
0264-410X/Published by Elsevier Ltd.

1. Introduction

The H5 and H7 highly pathogenic (HP) avian influenza (AI) viruses (HPAIV) have been one of the most frequent causes of severe poultry outbreaks around the world [1,2]. Recently, the H7N3 HPAI in Mexico has caused dramatic negative economic and social impacts due to a high number of poultry losses from deaths and culling. First reported in June 2012, the H7N3 HPAI has affected over 22 million chickens in Jalisco State, Mexico's most important table egg producing area [2,3]. Simultaneously, an immunization campaign using H7 inactivated virus [A/cinnamon teal/Mexico/2817/2006 (H7N3) vaccine strain], and several measures were established to control the disease including quarantine of affected premises, depopulation of infected poultry, and enhanced surveillance for infection in birds [3]. The initial vaccine seed strain (A/cinnamon teal/Mexico/2817/2006 [H7N3] vaccine strain) was protective against the June 2012 outbreak virus (A/chicken/Jalisco/CPA-12283/2012 [H7N3], Jalisco/12283/2012) [4–6]. Since 2013, additional H7N3 HPAIV outbreaks have occurred and affected primarily layers, but also some broilers, breeders, and backyard poultry in the Mexico States of Jalisco, Aguascalientes, Guanajuato and Puebla [2]. Recently, two outbreaks of H7N3 HPAI in Mexico were reported by OIE (World Organization for Animal Health) demonstrating the outbreaks are continuing [7]. Previous studies have characterized the pathobiology of H7N3 HPAIV, and effectiveness of different vaccine seeds strains on protection [4–6]. The vaccine trials have shown variable efficacy, with older H7 vaccine seed strains providing poor protection due to a combination of genetic and antigenic distance and low immunogenicity [4–6]. Outbreaks of H7N3 HPAI in vaccinated flocks have been reported in Mexico since 2016 [8].

A recombinant fowlpox vaccine (rFPV) expressing HA from H5 LPAIV (A/turkey/Ireland/1378/1983) has been licensed and successfully used during the last twenty years in Mexico and other countries in Central America for immunization at 1-day-of-age [1], but there is no similarly licensed rFPV-H7 AIV available in Mexico for hatchery vaccination and induction of early immunity. An experimental vaccine, rFPV-H7/2155 was previously tested by our group and found to be protective when used alone or can enhance the licensed inactivated H7N3 AIV vaccine in a prime-boost vaccination regime [5].

Efficacious AIV vaccines have demonstrated protection against morbidity and mortality from HPAIV challenge and have reduced viral shedding from the respiratory and gastrointestinal tract of birds [1,9]. In developing countries, poultry vaccination has been an important control tool in combination with depopulation of infected poultry, biosecurity measures, education, rapid diagnostic, and surveillance to control HPAIV [1,9]. However, mutations of influenza A virus, especially in the HA gene, have resulted in loss of vaccine optimal efficacy, hindering HPAIV control [9–11]. Mutations in the AIV genomes have generated changes responsible for antigenic drift and loss of protection by some vaccine seed strains [2,12]. Furthermore, within influenza A viruses, the acquisition of N-glycosylation has prevented neutralization by antibodies directed against previous strains and escaped vaccine-induced immunity elicited by heterologous vaccines [13–16]. Therefore, the N-glycosylation of HA also can influence antigenicity and thus play an important role in candidate influenza A vaccine strain selection and vaccination effectiveness against newly emerging field viruses [15,17,18].

Our goal was to analyze the protective efficacy of two different constructs of recombinant fowlpox virus vaccine (rFPV) containing a 2002 (rFPV-H7/2155) and 2015 (rFPV-H7/3002) H7 low pathogenicity avian influenza (LPAI) virus (LPAIV) HA inserts to protect chickens against challenge with H7N3 HPAIV isolated from a recent outbreak in Mexico, A/chicken/Jalisco/CPA-37905/2015 (Jalisco/37905/2015). Protective efficacy was determined by analyzing morbidity and mortality rates, titer of challenge virus shed, and pre-challenge serum antibody levels. Phylogenetic analysis was accomplished to understand the antigenic variation in the H7 HA from Mexico in comparison to historical North American lineage H7 AIV. Molecular analysis of H7 HA, as amino acid substitutions, protein structure, and N-glycosylation were evaluated to understand the possibilities of how the HA antigenicity could have contributed to field virus escape from vaccine-induced immunity.

2. Materials and methods

2.1. Animals

One-day-old SPF White leghorn chickens were housed separately by group in negative pressure isolators with HEPA-filtered intake air in an ABSL-2 facility (Merial/Boehringer-Ingelheim) for first 3 weeks of the study, and subsequently transferred to ABSL-3 enhanced facility (Southeast Poultry Research Laboratory) for HPAIV challenge. Birds had *ad libitum* access to feed and water throughout the experiment. All procedures were performed in accordance with the protocol approved by the Institutional Laboratory Animal Care and Use Committee.

2.2. Viruses and vaccines

The H7N3 HPAIV, A/chicken/Jalisco/CPA-37905/2015 (Jalisco/37905/2015), (GenBank accession number MH342039) was isolated from a backyard chicken during 2015 in the state of Jalisco, Mexico (courtesy of MVZ Joaquín B. D. Álvarez and Mario Solís Hernández, Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria [SENASICA], Mexico). The virus was propagated and titrated by allantoic sac inoculation of 9–10 day-old embryonating chicken eggs (ECE) by standard methods [19] and used as a challenge virus in this study.

Two constructs of recombinant fowlpox virus vector each containing a single H7 LPAIV gene insert (rFPV-H7-AIV) from: (1) North American strain A/turkey/Virginia/SEP-66/2002 (H7N2, GenBank accession number AY240913) (rFPV-H7/2155) or (2) the Mexican strain A/chicken/Guanajuato/07437-15/2015 (H7N3, GenBank accession number AKL91078.1) (rFPV-H7/3002) were constructed the same way as the licensed TROVAC-H5 and tested in this experiment. The fowlpox vector for TROVAC-H5 was derived from the vaccine strain contained in the DIFTOSEC fowlpox vaccine. TROVAC-H5 received license in the United States in 1998 and has since been used in Mexico, Guatemala, El Salvador, and Vietnam [20].

2.3. Experimental design and sampling

Experiment design and sampling were done using animals, vaccination, and challenge protocols following identical conditions as previously published from our group [5]. In summary, sixty chickens were distributed into three groups of twenty birds each and were given an individual identification number. All birds were vaccinated at one-day of age by the subcutaneous (SQ) route, at a dose of $10^{3.5}$ 50 percent embryo infectious doses (EID_{50}) in 0.2 ml per bird. Groups 1 and 2 were inoculated with the rFPV-H7-AIV vaccines: rFPV-H7/3002 and rFPV-H7/2155, respectively. Group 3 (Sham) received the rFPV-H7-AIV vaccine diluent. Three-weeks post-vaccination (21-days-of-age), birds were bled to evaluate

antibody titers in serum, and challenged by the intranasal route with 10^6 EID_{50} of HPAIV H7N3, Jalisco/37905/2015, in a volume of 0.1 ml. The inoculum titer was verified as $10^{5.9}$ EID_{50}/0.1 ml by back titration in ECE.

Oropharyngeal (OP), which includes swabbing the choanal cleft, and cloacal (CL) swabs were collected at 2- and 4-days post-challenge (dpc), placed in Becton-Dickinson BBL brain heart infusion (BHI) medium with 2X antibiotics (Penicillin/Streptomycin/Fungiezone; Hyclone, Logan, UT, USA), and stored at −80 °C until tested to determine virus shed titers. All chickens were observed daily for clinical signs and mortality from 0 to 14 dpc. Chickens, which were euthanized for humane reasons when clinical symptoms as severe listlessness, neurological signs, respiratory distress, or others HPAI infection signs, were counted as dead the next day for mean death time calculations. At the end of the experiment (14 dpc, 5-weeks-of-age), surviving birds were bled to evaluate antibody titers and euthanized according to the approved IACUC protocol.

2.4. Determination of virus shedding

OP and CL swabs were processed for quantitative real-time RT-PCR (qRRT-PCR) to determine the HPAIV viral load from the oropharynx and gastrointestinal tract on 2 and 4 dpc. This methodology has been used as standard protocol among published veterinary influenza vaccine studies. As published [21], the protocol demonstrated the high correlation between qRRT-PCR quantity and infectious titers when: (a) using only influenza A challenge viruses chicken adapted and propagated in embryonating chicken eggs (ECE), (b) using low MOI to inoculate ECE in propagating the viruses to generate the challenge inoculum which minimizes defective interfering (DI) RNAs, and (c) using the same specific challenge virus stock to generate the standard curve with each dilution point directly comparing qRRT-PCR CT values and infectious titer.

Briefly, in this study the viral RNA from OP and CL swabs was extracted using MagMAXTM-96 AI/ND Viral RNA Isolation Kit® (ThermoFisher Scientific, Carlsbad, CA, USA) following the manufacturer's instruction. Further, qRRT-PCR assay was performed as previously described [22] using the AgPath-ID One-step RT-PCR kit (ThermoFisher Scientific) with primers and probe targeting the influenza matrix gene. Samples were run on the 7500 FAST Real time PCR System (Applied Biosystems, Foster City, CA, USA). For viral quantification, a standard curve was established with viral RNA extracted from 10-fold dilutions of the challenge virus [23], Jalisco/37905/2015. Results were reported as $\log_{10}$ EID_{50}/ml and the lower limit of detection was 1.1 $\log_{10}$ EID_{50}/ml; therefore qRRT-PCR negative samples were treated as 1.0 $\log_{10}$ EID_{50}/ml for statistical purposes.

2.5. Serology

The serum collected pre- and post-challenge was used in hemagglutination inhibition (HI) assay to determine the antibody levels in the vaccinated birds. The antigens were prepared as previously described [24] and the HI assays were performed according to standard protocol [25]. The challenge virus, Jalisco/37905/2015 was used as antigen in the HI with each of the 3 groups of vaccinated birds (rFPV-H7/3002, rFPV-H7/2155, and Sham). To confirm vaccination, the rFPV-H7/2155 vaccinated group was also tested with the homologous vaccine antigen, A/turkey/Virginia/SEP-66/2002 (H7N2). To observe differences in serological response we tested the sera from rFPV-H7/2155-vaccinated birds with the challenge virus, Jalisco/37905/2015, and virus from the first H7N3 outbreak in Mexico, Jalisco/12283/2012, as antigens. Titers were expressed as geometric mean titers (GMT-$\log_2$). Samples with titers below 3 $\log_2$ GMT were considered negative, and then assigned as 2 $\log_2$ GMT for statistical purpose.

2.6. Phylogenetic analysis for complete HA gene of H7 avian influenza viruses

A phylogenetic tree was created by the Maximum-likelihood (ML) algorithm for the HA gene of H7 avian influenza viruses. All available complete HA gene sequences identified from North America, South America, Asia, and Europe (n = 2135) were retrieved from the Influenza Virus Resource database (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html) on October 18, 2017. The sequences were pruned by using the software cd-hit [26] at 97% homology to filter-out 92 representative sequences. The nucleotide sequences of HA segment from 2015 to 2016 sequenced in this study (n = 5) and all Mexican H7 viruses available in the GenBank (n = 17) were added to the dataset. A total of 114 nucleotide sequences were aligned using MAFFT and manual editing of alignments were performed in Geneious 8 software [27]. The ML tree was estimated by the RAxML software [28] using the general time-reversible model of nucleotide substitution. Bootstrap support values were generated by using 500 rapid bootstrap replicates. The ML phylogenetic tree was visualized with MEGA 7 software (http://www.megasoftware.net). Bootstrap values >70% are shown at the branch nodes.

2.7. Molecular characterization of H7 hemagglutinin: residues, N-glycosylation sites, and protein structure

The molecular characterization of H7 hemagglutinin of the challenge virus (Jalisco/37905/2015), vaccines (rFPV-H7/2155 and rFPV-H7/3002) were performed using different methodologies. The virus from 2012 outbreak in Mexico, Jalisco/12283/2012, was included in the molecular analyses for comparison with the challenge virus, Jalisco/37905/2015, isolated in a recent outbreak.

The HA sequence were used for alignment and residue analysis in the Lasergene 12 using Clustal W, MegAlign software (DNA STAR, Madison, WI). The potential N-glycosylation sites were predicted using NetNGlyc server 1.0 [29,30]. The HA structure was modelling using the H7 HA template (PDB accession number, 3M5G) in the SWISS-MODEL server [31–33]. The 3D molecular HA structures were visualized using the PyMOL Molecular Graphics System (Version 2.0 Schrödinger, LLC).

2.8. Deglycosylation of hemagglutinin

A reaction for removal of glycans from the HA protein (deglycosylation) was conducted to identify the N-glycosylation sites in the HA. Samples were digested with peptide N-Glycosidase F (PNGase F) kit (New England Biolabs, Ipswich, MA, USA) according to the manufactured instructions. Briefly, the glycoproteins were denatured with 1X of Glycoprotein Denaturing Buffer at 100 °C for 10 min. Further, the mixture of 1X of GlycoBuffer, 1% NP40, water and PNGase F were added to the reaction followed by incubation of 37 °C for 1 h. The reaction was heat inactivated at 75 °C for 15 min. Samples were loaded in the SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and the molecular weight of AI HA analyzed by western blot using antisera against H7N3 [A/turkey/OR/1971 (H7N3)].

2.9. Western blot assay

Sample homogenates were prepared in Laemmli buffer v/v (62.5 mM Tris–HCl pH 6.8; 5% β-mercaptoethanol; 2%SDS; 20% glycerol; 0.01% bromophenol blue) (Bio-Rad, Hercules, CA, USA) and denatured 5 min at 95 °C. Samples were then separate on

8–16% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio Rad) with 1X Tris/Glycine/SDS buffer (Bio-Rad) and transferred to nitrocellulose membrane using liquid electrophoresis systems Criterion™ Vertical Electrophoresis Cell (Bio-Rad). The nitrocellulose membrane was blocked with 5% nonfat dry milk in Tris-buffered saline with Tween 20 (TBST) buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20) during 1 h at room temperature, and then incubated overnight at 4 °C with chicken antisera against H7N3 (A/turkey/OR/1971) at a dilution of 1:2000. The membrane was washed with TBST and incubated with an alkaline phosphatase (AP) labelled goat anti-chicken IgY (H + L) (Thermo Fisher Scientific, Rockford, IL, USA) diluted 1:10,000. The western blot was visualized with the substrate BCIP/NBT (Sigma-Aldrich) according the manufacturers protocol. The molecular size of the HA pre- and post-digestion with PNGase F was analyzed with the marker Precision Plus Protein Dual Xtra Standard (Biorad).

2.10. Statistical analysis

Statistical analyses were performed using Prism 7 (GraphPad software, San Diego, CA, USA). The survival rate data was analyzed using the Mantel-Cox Log-Rank test. Fisher's exact test was used to analyze statistical significance of virus shedding. Statistical differences in mean viral titers and antibody levels between groups were analyzed using Mann–Whitney test and Tukey one-way ANOVA. A P-value of <0.05 was considered to be significant.

3. Results

3.1. Morbidity and mortality in specific pathogen free (SPF) White Leghorn chickens challenged with HPAIV H7N3

One-day-old chickens were vaccinated once and challenged three weeks later with H7N3 HPAIV, Jalisco/37905/2015 (H7N3). After challenge, all the sham-vaccinated control birds developed acute severe clinical disease and died between 2 and 4 days post-challenge (dpc) (mean death time [MDT] = 3.20 days) (Table 1, Fig. 1). Most of the sham-vaccinated birds were found dead (11/20) but others (9/20) showed clinical signs like respiratory distress, listlessness, lethargy, and prostration. The latter were euthanized for humane reasons. Similar clinical results were observed for all rFPV-H7/2155 vaccinated birds (MDT = 3.15 days) (Table 1, Fig. 1); i.e. birds were not protected against challenge, and consequently had severe clinical signs (7/20) or died (13/20) within 4 dpc. By contrast, all rFPV-H7/3002 vaccinated birds had complete protection from clinical signs and death (Table 1, Fig. 1).

3.2. Evaluation of virus shedding

Virus shedding was evaluated by quantification of AIV RNA in OP and CL swabs from chickens at 2 and 4 dpc using qRRT-PCR [21]. Viral RNA was detected at 2 dpc in OP and CL swabs from all birds in the sham group, with mean titer of 4.9 and 6.4 $\log_{10}$ EID_{50}/ml, respectively. Similar results were observed for birds in the rFPV-H7/2155 group, with 4.8 $\log_{10}$ EID_{50}/ml (OP) and 5.9 $\log_{10}$ EID_{50}/ml (CL) titers (Table 1, Fig. 2). At 4 dpc, only one bird survived in the sham group with a viral titer in the OP and CL samples, 5.8 and 7.7 $\log_{10}$ EID_{50}/ml, respectively. In the rFPV-H7/2155 vaccine group, only three birds were alive at 4 dpc and shed virus both orally, mean titer of 4.4 $\log_{10}$ EID_{50}/ml, and cloacally, 6.2 $\log_{10}$ EID_{50}/ml (Table 1, Fig. 2).

In contrast, rFPV-H7/3002 vaccinated birds had either no or very low viral shedding titers. The mean virus titer shed in the CL samples was 2.7 (15/20) and 1.9 (12/20) $\log_{10}$ EID_{50}/ml at 2 and 4 dpc, respectively. Interestingly, in the rFPV-H7/3002-vaccinated group only 7/20 birds were positive at 2dpc and 5/20 were positive at 4 dpc for OP with a mean titer of 1.5 $\log_{10}$ EID_{50}/ml on both days (Table 1, Fig. 2).

Overall, rFPV-H7/3002 vaccinated birds had statistically significant lower OP and CL titers than sham and rFPV-H7/2155 vaccinated groups, and significantly fewer birds shedding virus from the respiratory tract ($p < 0.05$) (Fig. 2).

3.3. Antibody levels in immunized birds pre- and post-challenge with HPAIV H7N3

Sera collected from all chickens pre- (0 dpc) and the survivors at termination (14 dpc) were processed for HI assay to measure humoral immune response to the challenge virus antigen and vaccine seed strain (Table 1, Fig. 3). Sera from sham-vaccinated chickens were negative for anti-AIV antibodies on the day of challenge.

Table 1
Summary data from one-day-old WL chickens vaccinated with one of two constructs of recombinant fowlpox virus vaccine, rFPV-H7/3002 and rFPV-H7/2155, and subsequently challenged with Jalisco/37905/2015 H7N3 HPAIV.

Group	Vaccine[a]	N° of birds	Morbidity[b]	Mortality[b]	MDT[c]	Oral shedding[d]				Cloacal shedding[d]				HI serology[e]		
														Vaccine strain as antigen	Jalisco/37905/2015 as antigen	
						2 dpc		4 dpc		2 dpc		4 dpc		Pre-challenge	Pre-challenge	Post-challenge
1	rFPV-H7/3002	20	0/20	0/20	na	07/20	(1.5)	05/20	(1.5)	15/20	(2.7)	12/20	(1.9)	nd *	15/20 (3.0)	20/20 (5.0)
2	rFPV-H7/2155	20	20/20	20/20	3.2	20/20	(4.8)	3/3	(4.4)	20/20	(5.9)	3/3	(6.2)	20/20 (6.6)	3/20 (2.2)	nd
3	Sham	20	20/20	20/20	3.2	20/20	(4.9)	1/1	(5.8)	20/20	(6.4)	1/1	(7.7)	nd	00/20 (2.0)	nd

[a] Constructs of recombinant fowlpox virus vaccine containing an H7 LPAIV gene insert. Group 1, rFPV-H7/3002 vaccine, contain the HA from the Mexican strain A/chicken/Guanajuato/07437-15/2015 (H7N3), and group 2, rFPV-H7/2155 vaccine, the HA from North American strain A/turkey/VA/SEP-66/2002 (H7N2). Important, the HA gene had the cleavage site changed back to LPAIV.

[b] Morbidity = no. with clinical disease/total; mortality = no. survivors/total.

[c] MDT, mean death time, *#dead birds × dpc/total dead birds (expressed as dpc, days post-challenge).

[d] The numbers represent no. virus positive/total in group followed by mean virus shed titer. Results were reported as $\log_{10}$ EID_{50} titer/ml and the lower limit of detection was 1.1 $\log_{10}$ EID_{50}/ml; therefore qRRT-PCR negative samples were treated as $\leq$1.1 $\log_{10}$ EID_{50}/ml. For the purposes of statistical calculations, all the negative samples were assigned the value of 1.0 $\log_{10}$ EID_{50}/ml. Different superscript lowercase denotes statistical significance of number of birds shedding between vaccine and corresponding sham by Fisher Exact ($p < 0.05$). Different superscript uppercase denotes statistical significance of shedding titers between vaccine and corresponding sham by Mann-Whitney test and ANOVA ($p < 0.05$). §, number of positive birds was too low for statistical purposes.

[e] The numbers represent no. serology positive/total in group (geometric mean titers (GMT-$\log_2$) only includes positive birds) followed by mean HI titers against vaccine virus or challenge virus. Samples with titers below 3 $\log_2$ GMT were considered negative, and then assigned as 2 $\log_2$ GMT for statistical purpose. nd = not determined. nd * = The challenge virus and vaccine rFPV-H7/3002 are nearly identical, then only challenge virus was used as HI antigen in this vaccinated group.

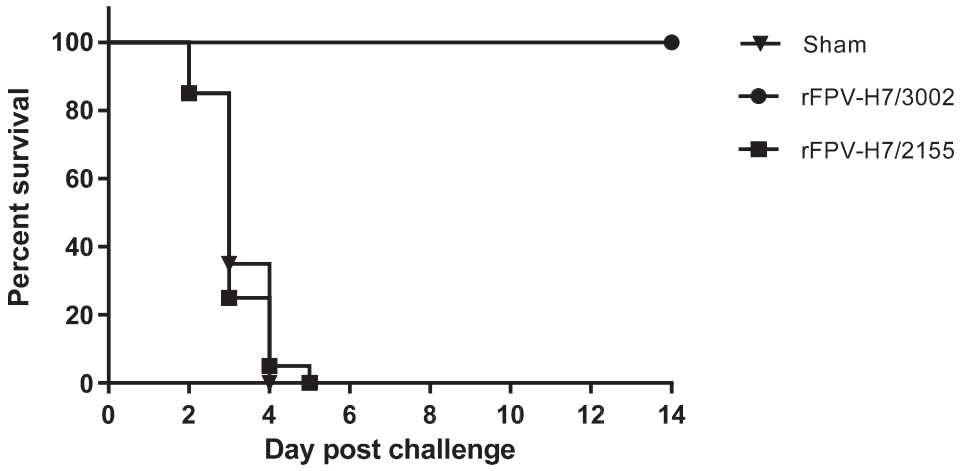


Fig. 1. Survival curve of vaccinated chickens challenged with Jalisco/37905/2015 (H7N3) HPAIV.

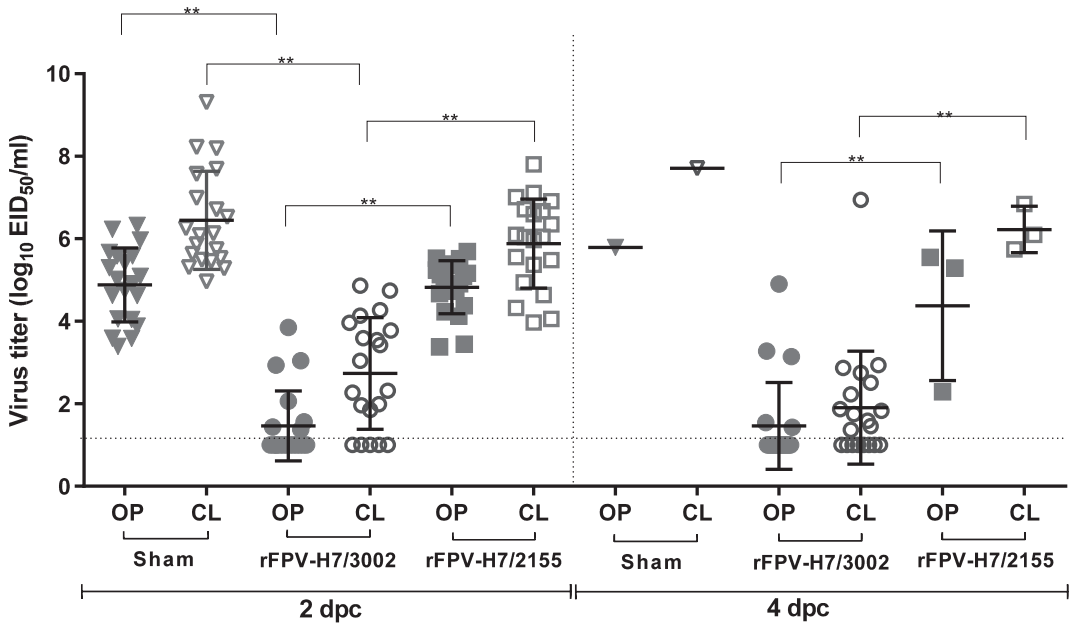


Fig. 2. Scatter plot of oropharyngeal (OP) and cloacal (CL) shedding detected by qRRT-PCR after challenge with the Jalisco/37905/2015 (H7N3) HPAIV. Viral titers in swabs from vaccinated birds, rFPV-H7/3002, rFPV-H7/2155, and Sham, at 2 and 4 days post challenge (dpc) are expressed as $\log_{10}$ EID_{50}/ml. The limit of detection was 1.1 $\log_{10}$ EID_{50}/ml and negative samples were treated as 1.0 $\log_{10}$ EID_{50}/ml. Statistical significance between mean titers was determined with ANOVA using the Tukey's Multiple Comparison Test ($p < 0.05$). The $p < 0.005$ are indicated by "**".

The pre-challenge serum from rFPV-H7/2155 vaccinated group had low levels of antibody ($\log_2$ GMT = 2.2) in a few birds (3/20) when using the challenge virus as the antigen (Table 1, Fig. 3a). However, when using the vaccine seed as antigen (A/turkey/VA/SEP-66/2002), all birds (20/20) had high titers of HI antibody ($\log_2$ GMT = 6.6) (Table 1, Fig. 3b), and when using Jalisco/12283/2012 (13/20), a high number of birds (13/20) had specific antibodies (Fig. 3c), confirming results previously published [5]. Therefore, the r-FPV-H7/2155 vaccinated-birds have specific antibodies that significantly ($p < 0.05$) recognize the virus from the first outbreak in 2012, as Jalisco/12283/2012, than Jalisco/37905/2015 (Fig. 3c).

In contrast, the rFPV-H7/3002 vaccinated birds had HI antibodies in the pre- (15/20, $\log_2$ GMT = 3) and post- (20/20, $\log_2$ GMT = 5) challenge serum when using the challenge virus (Jalisco/37905/2015) as HI antigen (Fig. 3a), and when using Jalisco/12283/2012 (6/20), a few number of birds (6/20) had specific antibodies (Fig. 3c).

3.4. Molecular analysis of H7 avian influenza virus

To understand the role of HA in the protection of chickens against challenge with H7N3 HPAIV, we compared the nucleotide identity for complete HA, specific amino acid sequences, and protein structure between HA of vaccine viruses and challenge strain.

The ML phylogenetic tree indicates the initial Mexican H7N3 HPAIV strain from June 2012 emerged from a North American H7 LPAIV lineage. Since then, it evolved through genetic drift to create the three contemporary genetic subgroups (I, II, and III) during 2015 and 2016 (Fig. 4a). We observed that the HA gene of the challenge virus (Jalisco/37905/2015) and the H7 strain in the rFPV-H7/2155 (A/turkey/Virginia/SEP-66/2002) had lower nucleotide identity (86.5%). The Jalisco/37905/2015 and the HA of rFPV-H7/3002 vaccine [H7 from 2015 strain (A/chicken/Mexico/2015)] not only had a higher identity (99.2%) but also cluster together in subgroup II.

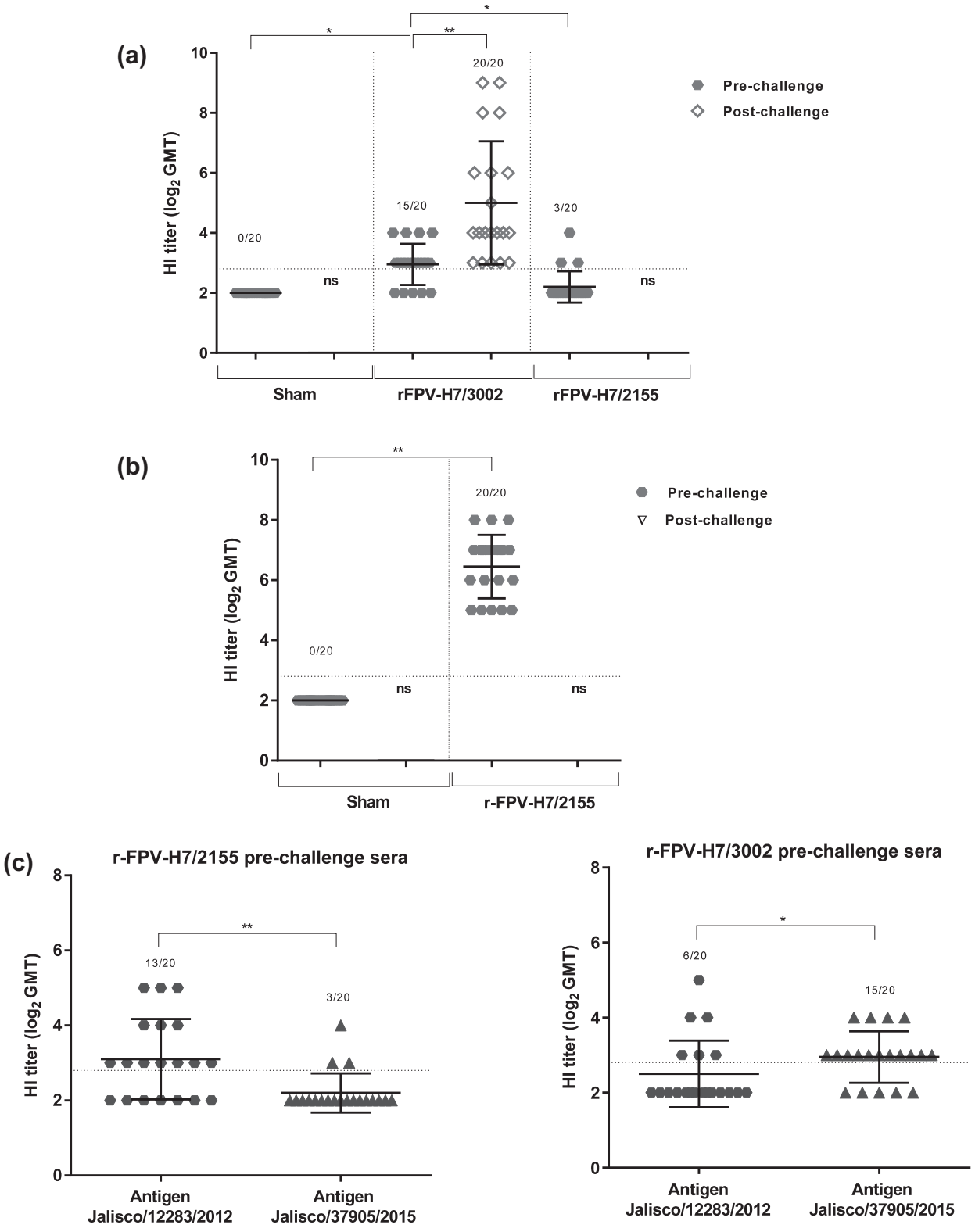


Fig. 3. Scatter plot of HI titers for vaccinated birds in groups, rFPV-H7/3002, rFPV-H7/2155, and Sham pre- and post-challenge. The HI titers were analyzed using the challenge virus (A), or vaccine strain as antigen (B), or Jalisco/12283/2012 as antigen (C). Titers were expressed as geometric mean titers (GMT-$\log_2$), and samples with titers below 3 $\log_2$ GMT were considered negative. Dotted horizontal lines indicate limit of detection. Statistical significance between mean titers was determined with ANOVA using the Tukey's multiple comparison and Mann–Whitney tests or *t*-test ($p < 0.05$). The $p < 0.05$ and $p < 0.005$ are indicated by "* "and "**", respectively. ns = birds did not survive.

Amino acid sequences were aligned to evaluate possible mutations in the HA of the challenge virus (Jalisco/37905/2015), vaccines (rFPV-H7/2155 and rFPV-H7/3002), and the initial Mexican H7N3 HPAIV outbreak strain (Jalisco/12283/2012). The Jalisco/12283/2012 strain was chosen for comparison because a previous study, the rFPV-H7/2155 vaccine provided 100%

protection from mortality against this lethal challenge strain [5]. The percentage of amino acid similarities among the HA sequences ranged from 98.6 to 87.3%. The HA gene in the early strains (vaccine rFPV-H7/2155 and Jalisco/12283/2012) showed few amino acid differences. However, when we compared these strains to HA of the new vaccine (rFPV-H7/3002) and the Jalisco/37905/2015, there was progressive accumulation of mutations from the early to recent strains (Fig. 4b). The majority of the amino acid substitutions are localized in the globular head of HA. Furthermore, these changes occurred in regions close or inside the antigenic sites (Fig. 4b). Some of these amino acid substitutions resulted in the formation of potential new predicted glycosylation sites.

Thus, we examined *in silica* the differences in the number of potential of N-glycosylation sites in the HA sequence (Fig. 4c). Positions 30, 46, 249, and 501 had potential glycosylation sites in Jalisco/12283/2012 virus, in which position 249 was the only one localized in the globular head. However, between late 2012 and early 2016, H7N3 Mexican strains obtained additional glycosylation sites in the globular head of the HA protein at the positions 141, 151 and 182: 1) subgroup I – 141 and 151, 2)

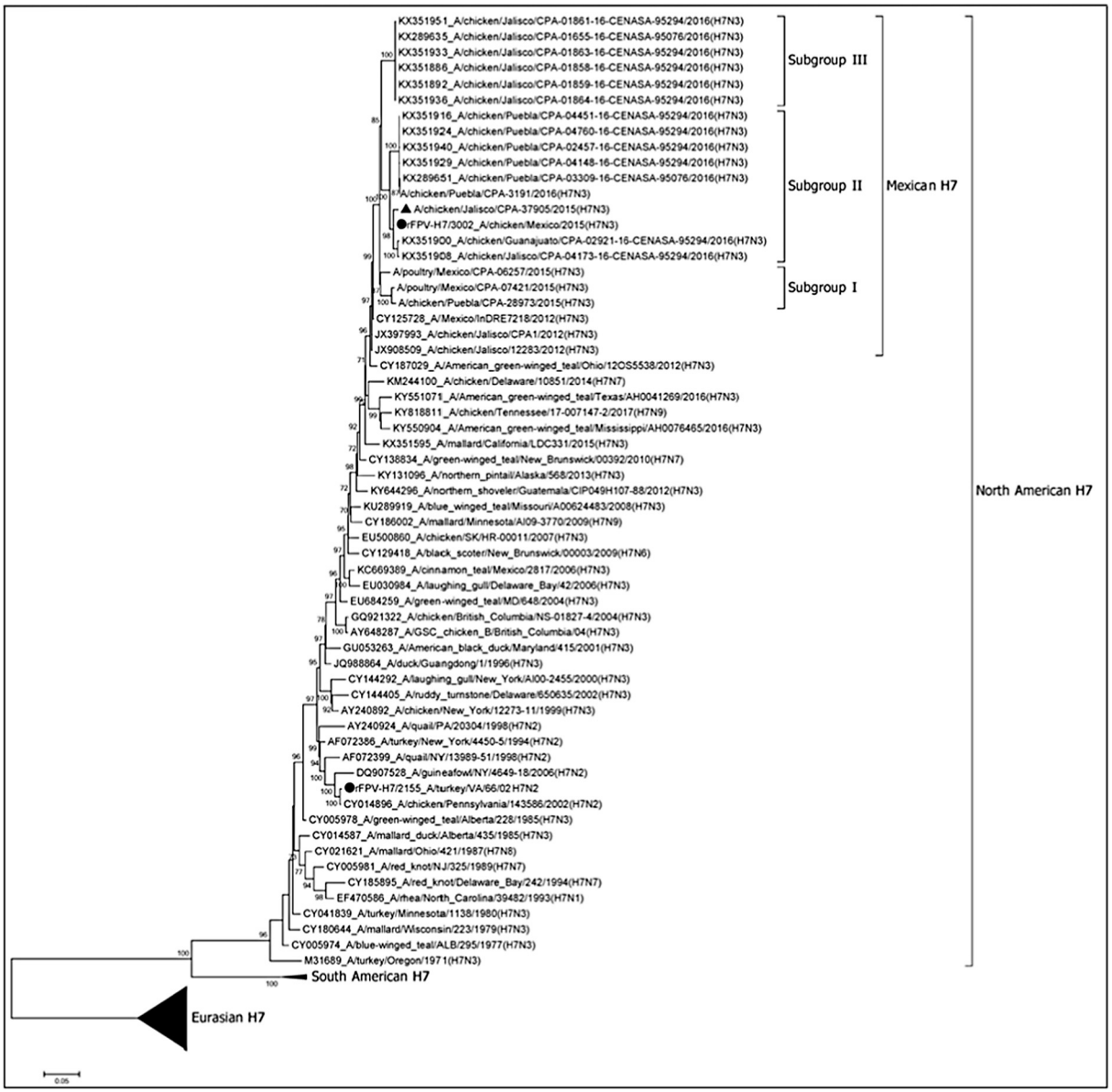


Fig. 4. Molecular analysis of H7 avian influenza virus hemagglutinin. (A) Phylogenetic tree created by the Maximum-likelihood algorithm for the HA gene of H7 avian influenza viruses. Available full-length HA gene sequence identified from North America, South America, Asia, and Europe (n = 2135) from the Influenza Virus Resource database were used. The vaccine strains and challenge virus used in this study are highlighted as black circles (•) and triangle (▲), respectively. Bootstrap values >80% are shown at the branch nodes. (B) Amino acids residues of AIV H7 HA were aligned in Lasergene MegAlign (DNAStar). Antigenic sites of H3 HA (A, B, C, D, and E) are shown in the boxes. Only the amino acids different from those in the challenge virus (Jalisco/37905/2015) sequence are shown. The asterisks represent the position of predicted N-glycosylation sites are shown in blue (position 30, 46, 249, and 501) and red (position 141, 151, and 182). The N-glycosylation sites shown in blue were observed in all samples analyzed, and red are exclusive to the Jalisco/37905/2015 and vaccine rFPV-H7/3002. (C) Ribbon diagram and surface of H7 HA. Only monomers are shown. Predicted antigenic sites A (cyan), B (orange), C (magenta), D (green), and E (yellow) are labeled. Locations of predicted N-glycosylation sites are represented as spheres labeled with red (position 141, 151, and 182) and blue color (position 30, 46, 249, and 501). Antigenic sites A and E are highlighted. D) Western blot comparison of Jalisco/12283/2012 and Jalisco/37905/2015HA treated with (+) and without (−) PNGase F. The HA was detected using chicken antiserum against H7N3 (A/turkey/OR/1971). The number of predicted N-glycans with and without PNGase treatment was shown as "HA N-Glyc". Results are representative of two independent replicates. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

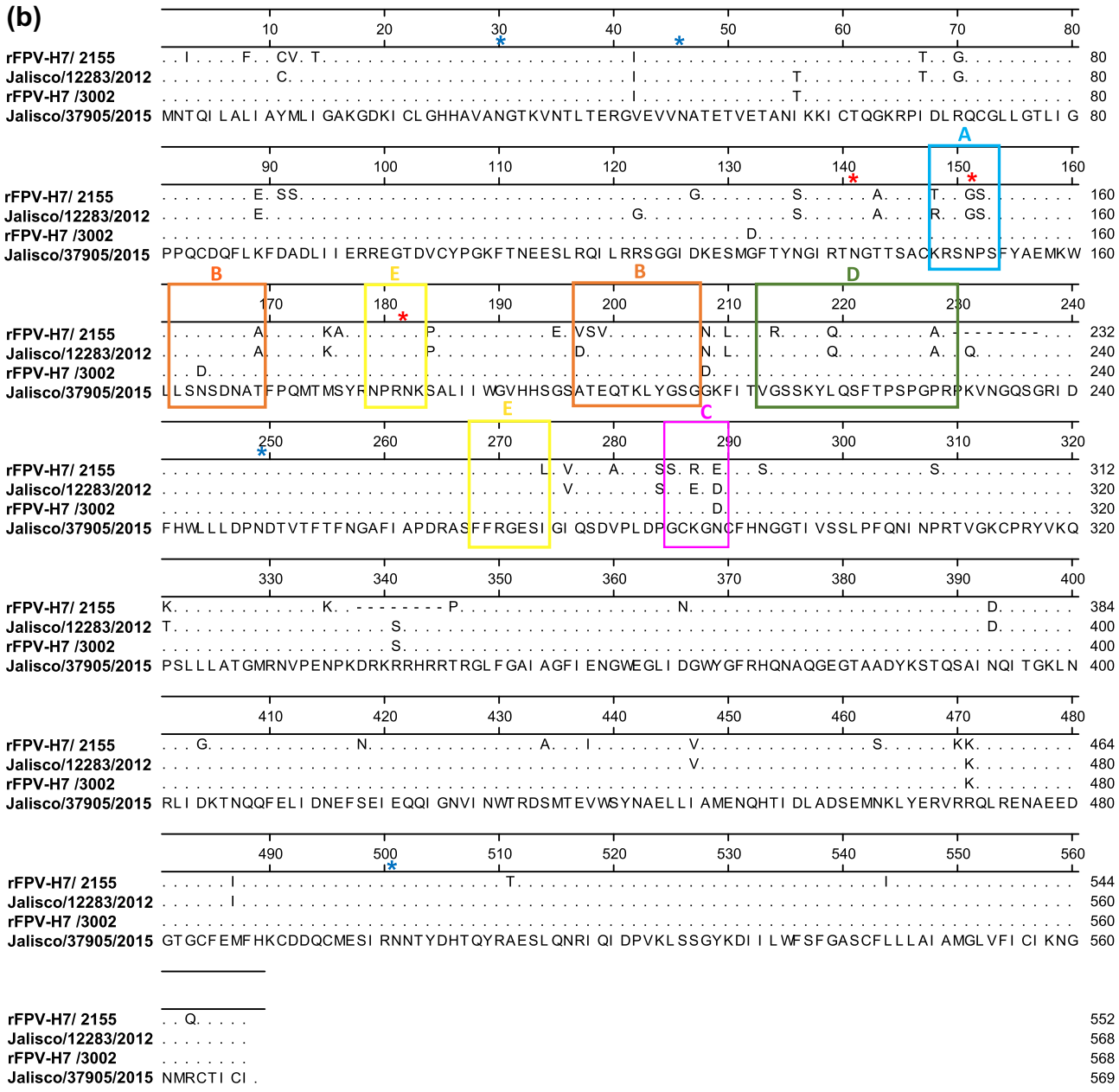


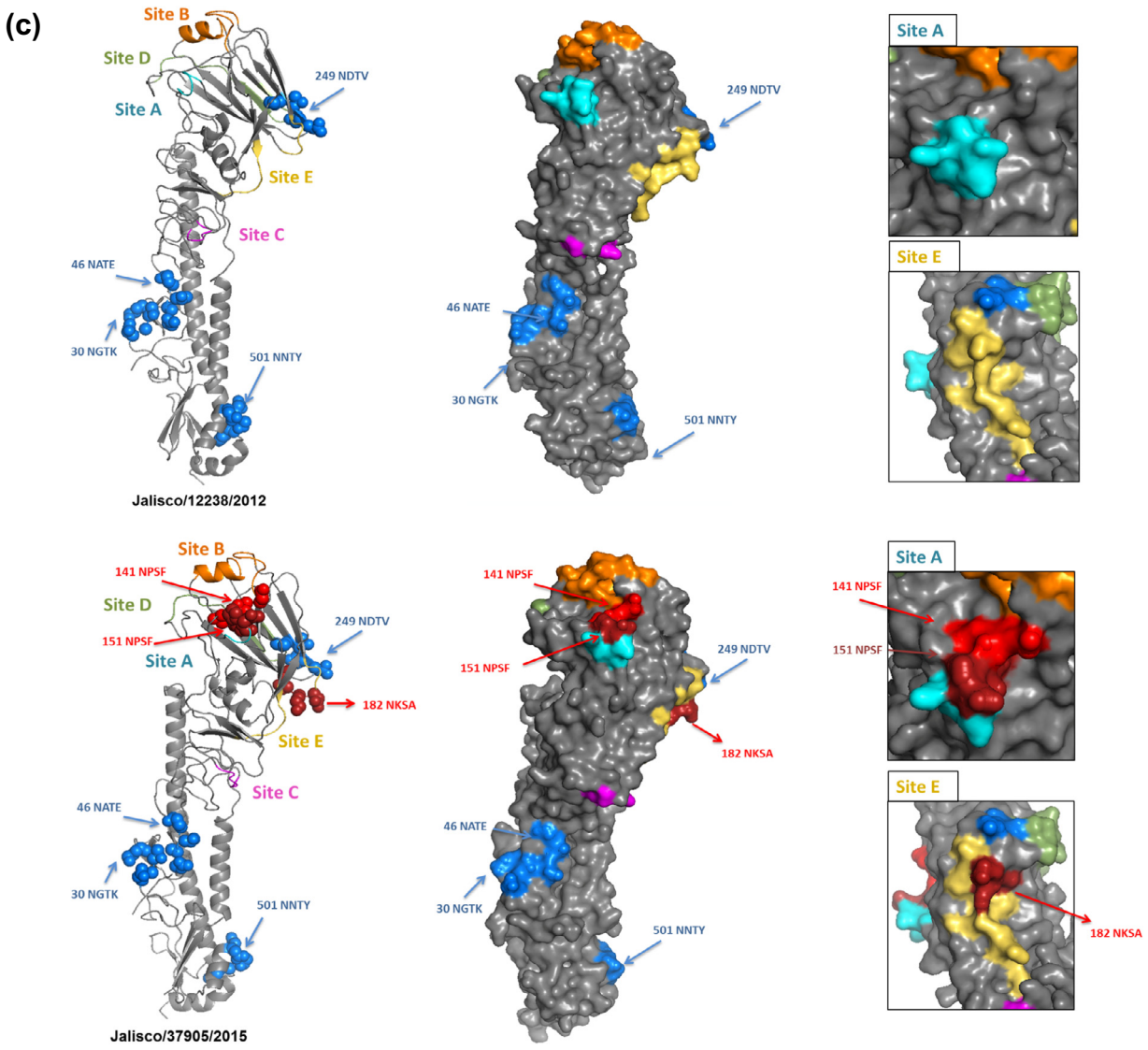
Fig. 4 (*continued*)

subgroup II – 141, 151, and 182, and 3) subgroup III: 141 (Fig. 4a). Specifically, the challenge virus (Jalisco/37905/2015) (Fig. 4b and c), which belongs to subgroup II, has additional potential N-glycosylation sites at the positions: 141, 151 and 182. Moreover, two of these sites, positions 151 and 182, were found within antigenic sites A and E, respectively. Protein alignment and prediction of N-glycosylation sites determine the structure of H7 HA from these strains affected the protein structure and possibly immune recognition in the antigenic sites (Fig. 4c).

To verify the *in silico* prediction of potential N-glycosylation sites in the Mexican H7 HA, we used pre-and post-digestion with PNGase F followed by SDS-PAGE. Western blot analysis demonstrated the HA of Jalisco/37905/2015 had a higher molecular weight than HA of Jalisco/12283/2012 (Fig. 4d). Moreover, PNGAse F treatment, which removes the N-link glycans, resulted in a decrease of the molecular weight of all HA to a similar size (Fig. 4d). Therefore, these results demonstrated that potential N-glycosylation sites were present in different quantities in the HA proteins of the Mexican viruses from 2012 (Jalisco/12283/2012) and 2015 (Jalisco/37905/2015) outbreaks, as predicted *in silico*.

4. Discussion

Both H5 and H7 HPAIV are the most frequent causes of severe disease outbreaks in poultry [2,34]. In developing countries, the use of vaccines is an important tool to control HPAI in combination with depopulation of infected poultry, strict epidemiological surveillance, and enhanced biosecurity measures [35]. However, continuous genetic evolution of H5 and H7 AIVs, as accumulation of nucleotides and amino acids substitutions in the HA, has led to significant antigenic diversity of these AIVs in poultry in many parts of the world [36–39]. Such diversity has led to antigenic drift allowing AIVs to evade the herd immunity established from



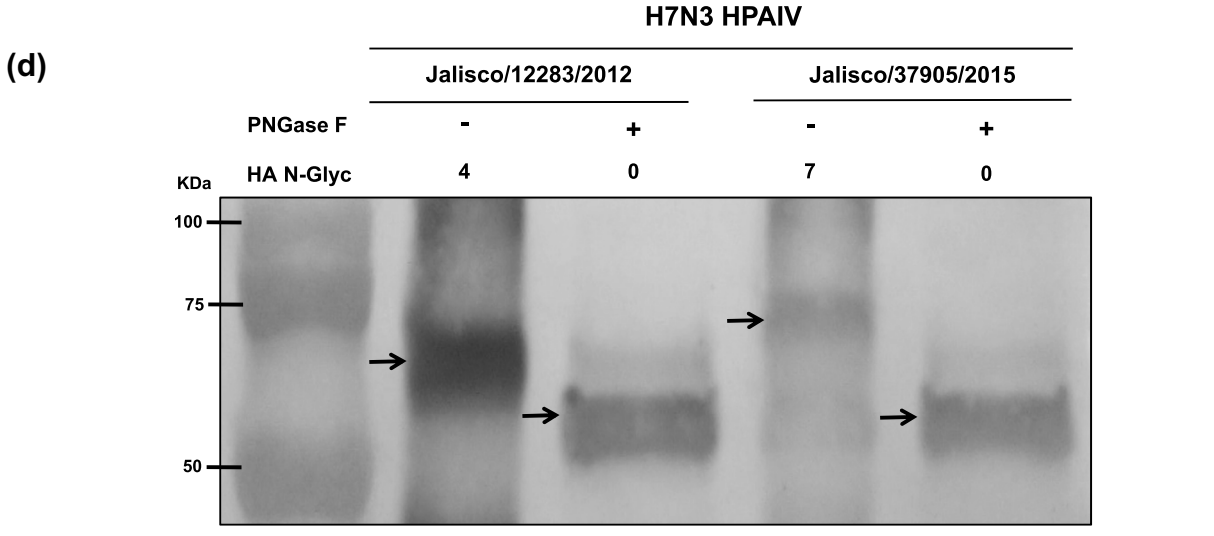


Fig. 4 (*continued*)

previous influenza infection or vaccination [9,35,40]. Therefore, AI vaccines must be periodically re-evaluated to determine the effectiveness against emergent variant circulating virus strains and should be replaced with updated vaccine seed strains if no longer protective. Outbreaks of H7N3 HPAI have been reported in vaccinated flocks in Mexico since 2016, suggesting significant antigenic

changes in the field viruses and a need to examine vaccine seed strains for needed updates [8].

In the current study, immunization of one-day-old SPF White Leghorn chickens with two different constructs of recombinant fowlpox virus vaccine (rFPV-H7/3002 and rFPV-H7/2155), containing a 2002 and 2015 LPAI H7 HA insert, respectively, were used to evaluate the protection against an H7N3 HPAI virus isolated in Mexico during 2015 (Jalisco/37905/2015). Interestingly, the closely related rFPV-H7/3002 vaccine conferred protection without morbidity or mortality together with no or low virus shedding from the respiratory and gastrointestinal tracts. A different scenario was observed for birds vaccinated with the old vaccine, rFPV-H7/2155, which previously conferred complete protection resulting in no clinical signs or death against the 2012 original outbreak virus (Jalisco/122083/2012) [5]. Our results showed that rFPV-H7/2155 and sham vaccinated birds after challenge with Jalisco/37905/2015 had 100% mortality within 4 dpc.

Previous studies have demonstrated that systemic humoral immunity induced by AI vaccines, especially HA subtype specific, contributed to protection against influenza [1,9]. Most of the rFPV-H7/3002-vaccinated birds had pre- and post-challenge HI antibodies titers against the challenge virus, which conferred protection. Even though the rFPV-H7/2155 vaccine induced a strong immune response against the homologous vaccine antigen, these HI antibodies were not sufficiently specific to confer any protection against the challenge virus. Antigenic differences between the old vaccine strain (rFPV-H7/2155) and the current challenge strain resulted in the recent H7N3 HPAI virus being poorly recognized by antibodies from the rFPV-H7/2155 vaccine. This difference was probably caused by antigenic drift in field isolates of H7N3 virus. In addition to a correlation between HA antibodies response and protection against influenza, the cellular immune response also can contribute to protection after vaccination [1,35]. In our study, the influence of cell-mediated immunity (CMI) perhaps contributed to the post-challenge protection of a few rFPV-H7/3002-vaccinated birds (5/20) which lacked pre-challenge HI antibodies, but were protected from morbidity and mortality. Future studies are needed to better understand the mechanisms and benefits that CMI could offer to vaccine effectiveness in poultry.

To better understand the difference observed in vaccine protection, nucleotide and amino acid sequences of the HA were further analyzed to identify possible antigenic changes in the evolving H7 HPAIV viruses in Mexico. The HA phylogenetic tree suggested that Jalisco/12283/2012 emerged from a North American H7 LPAIV lineage and evolved to create multiple subgroups as early as 2015, which includes the challenge virus used in this study, Jalisco/37905/2015. The amino acid sequence analysis showed that the challenge virus, Jalisco/37985/2015, and the original outbreak virus, Jalisco/12283/2012, have 94.72% of HA identity. Similarity of the whole HA sequence is not predictive of protection because a few specific changes in amino acid sequence or tertiary structure of HA protein can result in a lack of protection. In this case, the difference in the HA identity was the result of amino acid substitutions, especially in the globular head of HA, in regions close or inside the antigenic sites. It is well known that changes at the antigenic sites affect the specificity of neutralizing antibodies, but the trigger for when antigenic changes requires a vaccine change is not defined [1]. Overall, the amino acid substitutions at position 143 (alanine to threonine), 151 (glycine to asparagine), 152 (serine to proline), and 184 (proline to serine) from early to recent H7 AIV (Fig. 4b) suggest the formation of additional potential glycosylation sites according to *in silico* analyses.

Interestingly, we observed that H7 Mexican AIV strains have obtained potential additional glycosylation sites between June 2012, early 2015, and 2016. *In silico* analysis showed that 2 to 3 additional N-glycosylation sites were present on the globular head of the H7 HA. Recent advances in the interaction between AIV glycan and components of innate and adaptive immunity have been providing insights regarding the ability of N-glycans to target specific cells and consequently stimulate cytokines production [13,16,41]. The effect of glycosylation on the HA tertiary structure and possible immune recognition has been documented [13–15,42–44]. The progressive accumulation of N-glycan on the HA can physically mask access to antigenic sites allowing escape from antibody-mediated response produced against previous field strains and vaccine-induced immunity [13–15,44]. At the same time, additional glycosylation sites can interfere with the receptor binding properties, which consequently contributes to virus replication and fitness [43]. Furthermore, previous studies have shown that changes in HA glycosylation in AIV can also affect the CMI, helping AIV to escape T cell recognition [45,46], and inhibit the approach of clones bearing certain T cell receptors to the glycopeptide-MHC complex [47]. Dendritic cells mediate the link between innate and adaptive immune response [48]. A recent *in vitro* study showed that influenza virus HA with different N-glycan patterns could activate dendritic cells [48]. Although a few studies suggested that N-glycan could play a role in the regulation of CMI, more experiments are still necessary to fully understand how it can be modulated.

We found that the Jalisco/37905/2015 has 3 potential additional N-glycosylation sites on the globular head of HA at the amino acid position 141 (NGTT), 151 (NPSF), and 182 (NKSA), which were absent in previous strains, such as Jalisco/12283/2012. Western blot analysis results demonstrated the higher molecular weight of the Jalisco/37905/2015 (challenge virus) compared to the Jalisco/12238/2012 HA. Further, the glycosylation in the position 151 and 182 are localized on the globular head of HA in the antigenic sites A and E, respectively. Mutations in the antigenic sites have altered recognition of neutralizing antibodies and contributed to escape mutant generation [13,17]. A previous report highlighted the importance of antigenic site A in HA of H7 AIV for cross-reactive antibody response [49]. Even though residue 141 is not in the antigenic sites, N-glycosylation in this position also affects the neutralization by site A antibodies due to its location in the protein structure [50]. Next to it, N-glycosylation at position 142 was shown to be important to the HA and sialic acid interaction [18], playing a role in virus infectivity and host immune response observed in different AIV strains [15,18,51]. Thus, these modifications in the globular head of the H7 HA from Jalisco 2012 to 2015 viruses has potential to play a role in the changes observed in serological response in the rFPV-H7/2155-vaccinated birds, which antibodies produced poorly recognize the Jalisco/37905/2015. Future studies using reverse genetics should be done to better understand the mechanism of resistance to neutralization conferred specific amino acid modifications and increases in glycosylation sites in the H7 HA from Mexico strains.

Overall, our data showed that vaccine containing the HA closely related to recent outbreak H7 strains in Mexico, rFPV-H7/3002, completely protected chickens from morbidity and mortality following challenge with the 2015 Mexican H7 HPAIV strain, Jalisco/37905/2015. The vaccine significantly reduced the number of birds shedding virus and the virus shed titers from the respiratory and gastrointestinal tracts. A reduction in virus shedding is an important outcome of vaccination, which reduces environmental contamination and subsequent virus transmission from infected vaccinated flock to uninfected flock [22]. Interesting, prior to June of 2018, there was no licensed rFPV-H7 AIV in Mexico, although the rFPV-H5 AIV has been successfully used in the last two decades [1]. Additionally, rFPV vaccine choice, as opposed to the inactivated vaccine alone, has advantages as: (a) rapid insertion of any influenza HA gene due to constant antigenic shift and drift among influenza viruses [1,52,53], (b) stimulation of humoral and cellular

immunity when given parenterally [1], (c) priming the humoral immune response to enhance a boost by inactivated vaccines [1], and (d) mass application and early immunity with hatchery application [1,54]. This vaccine was licensed in Mexico on 8 June 2018 and such licensure was based upon these efficacy studies and will assist in reducing outbreaks of H7N3 HPAI.

H7N3 HPAIV outbreaks in Mexico have been reported to OIE since 2012 even though biosecurity measures and vaccination were already established. Molecular analysis showed that Mexican H7N3 strains have continued to rapidly evolve and recent strains have multiple amino acid changes and potential additional of N-glycosylation on the antigenic sites of the HA. It is therefore essential to continue strict epidemiological surveillance to identify emerging variant viruses of H7 AIVs circulating in the field. Epidemiological surveillance significantly contributes to the development of strategies for disease prevention and control, as well as more suitable vaccine strains for improving vaccination effectiveness. Altogether, this study confirms the importance of updating vaccines seeds strains for long-term effective control of H7 HPAIV.

Declarations of interest

None.

Acknowledgments

We thank Diane Smith, Scott Lee, Suzanne DeBlois, and Klaudia Chrzastek for excellent technical assistance. In addition, we acknowledge Roger Brock and James Doster for animal care assistance. We also thank MVZ Joaquín B.D. Álvarez and Mario Solís Hernández, Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria [SENASICA], Mexico that provide the challenge virus strain used in this study.

This research was supported by Agricutural Research Service project 6040-32000-066-00D and Boehringer Ingelheim Animal Health, Inc., trust agreement 58-6040-7-002.

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