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Research Article

Evaluation of a Polyvalent Vaccine Obtained From Divergent Low Pathogenic H5N2 Isolates of the Avian Influenza Virus in Mexico

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Abstract

In Mexico, the strategy used for controlling the Avian Influenza Virus (AIV) involves the use of immunizations through an inactivated emulsion vaccine (H5N2), which protects birds from the disease. It has been shown that the strain used in this vaccine is phylogenetically distant from the strains that are isolated in the field. Therefore, the goal of this study was to prepare and evaluate a polyvalent vaccine with genetically divergent isolates of the low-pathogenicity H5N2 avian influenza virus strains that are prevalent in Mexico. A polyvalent vaccine (Poly-AI) was prepared using five isolates that exhibited phylogenetic divergence from the low-pathogenicity avian influenza H5N2 virus strains found in Mexico. Chickens were immunized with Poly-AI and challenged 28 days post-vaccination with two Low Pathogenic Avian Influenza Virus (LPAIV) isolates contained in the vaccine and one High Pathogenic Influenza Virus (HPAIV). Serology was done at different times and clinical signs were recorded. This is the first study that documents the degree of pathogenicity differences between various isolates that exhibit genetic variation in the nation. The experimental Poly-AI vaccine eliminated the clinical signs of the disease, demonstrated 100% protection against the challenge with a highly pathogenic strain and decreased excretion when challenged with homologous and high virulence strains, which was detected by qRT-PCR.

Introduction

The Avian Influenza (AI) virus belongs to the influenza virus A genus of the *Orthomyxoviridae* family. Type A influenza viruses are important in veterinary medicine because they cause respiratory or systemic infections that are highly contagious in birds and potentially contagious in some mammals and humans [1]. The influenza A viruses are classified into subtypes based on two expressed proteins in the phospholipid membrane: Hemagglutinin (HA) and Neuraminidase (NA). Currently, in birds 16 HA and nine NA proteins have been identified, which can generate 144 possible combinations of subtypes [1-7], however in bats two more HA and NA have been demonstrated. Subtypes H5 and H7 are the most studied because they have shown the capacity to change from low to high pathogenicity in domestic poultry, which has been reported to the World Organization for Animal Health [8,9]. Methods to control the diseases caused by subtypes H5 and H7 include slaughtering infected birds, increasing biosecurity measures, and viral monitoring. In some countries, these measures also include the use of monovalent or polyvalent vaccines prepared with inactivated viruses because these vaccines have demonstrated their effectiveness in reducing or eliminating morbidity, mortality, and virus transmission, as well as reducing viral excretion in chickens [3,-5,9,10], peacocks [11,12] and ducks [12].

During 1994, Mexico had an avian influenza virus, the highly pathogenic H5N2, cause high mortality in flocks of different States. Isolates were made in different states and a scheme control for eradication of the virus was established in a vaccination program against the AI. Initially, the program was formulated to control the outbreak of the highly pathogenic virus and this was achieved in a few months, however, the low pathogenic H5N2 virus has been circulating since then [13,14]. The long-term massive vaccination of poultry in Mexico is a unique case. The presence of the Low-Pathogenic Avian Influenza Virus (LPAIV), subtype H5N2, has been present in Mexico since 1994, and it has been partly controlled using vaccinations. However, during this time, the virus has shown antigenic drift and genetic distance when compared to the virus, which was used to prepare the vaccine [15-18]. Because phylogenetic changes have been observed within the past year and in various specific regions of Mexico, it has been suggested that this virus can be controlled through the use of a polyvalent vaccine that includes isolates of phylogenetic divergence. To determine if

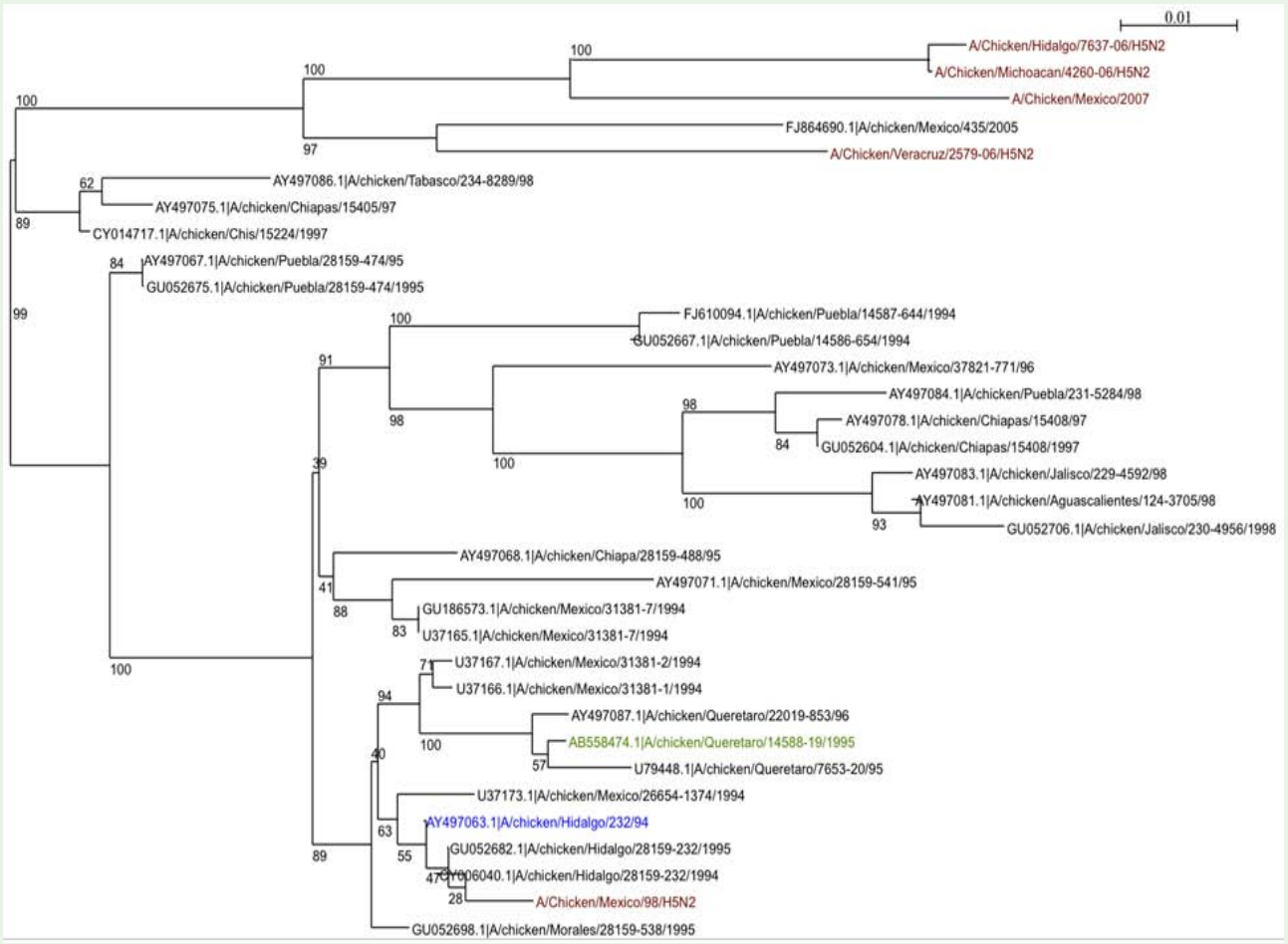

Figure 1: Phylogenetic tree based on the nucleotide sequence of the HA gene cleavage site sequence from Avian Influenza Virus (AIV) strains. The tree was constructed with maximum parsimony (Seaview 4) and 1000 replicates of bootstrap and rotated with the vaccine sequence (blue). The tree consisted of isolate sequences from different regions of the country isolated during the first outbreak from 1994 to 2007, including viruses provided by SENASICA-CPA (red) to prepare the vaccine and the HPAIV-Qro as challenge strain (green).

a polyvalent vaccine can protect against the clinical signs caused by different LPAIV H5N2 isolates in Mexico, this study prepared an adjuvant-free vaccine using five isolates that exhibited phylogenetic divergence [18,19].

Materials and Methods

Animals

Eighty-one Specific-Pathogen-Free (SPF), Leghorn, 10-day-old chicks (ALPES, Tehuacan, Puebla) were used in this study. The chicks were maintained in isolation units with level III biosecurity measures and a controlled environmental temperature in the Centro Nacional de Investigación Disciplinaria en Microbiología Animal (Mexico City). The birds received water and food ad libitum. This study was approved by the Institutional Animal Care and Use Committee (IACUC) of the Centro Nacional de Investigación Disciplinaria en Microbiología Animal (No. approval: 003-2014).

Virus

The LPAIV H5N2 subtype strains isolated in Mexico were used to prepare the experimental vaccines that were phylogenetically classified using HA gene sequencing (Figure 1). To prepare the vaccine, five genetically divergent isolates were selected from different years and shown in Table 1; additionally one HPAIV strain was used at the challenge the abbreviation for each virus are used throughout the manuscript [19]. The viruses were kindly donated by the Laboratory of the Mexico-United States Commission for the Prevention of Foot and Mouth Disease and Other Exotic Animal Diseases (CPA-SENASICA-SAGARPA).

The challenge trial used two low pathogenic strains: LPAIV-98 and LPAIV-Mich, corresponding to viruses that showed different

Table 1: Virus strains used in the polyvalent vaccine and challenge.

Strain	Year	Abbreviation	Title (HAU.)
A/Chicken/Mexico/98/ H5N2	1998	LPAIV -98	256
A/Chicken/Mexico/2007/H5N2	2007	LPAIV -2007	512
A/Chicken/Hidalgo/7637-06/H5N2	2006	LPAIV -Hgo	512
A/Chicken/Michoacan/4260-06/H5N2	2006	LPAIV -Mich	512
A/Chicken/Veracruz/2579-06/H5N2	2006	LPAIV -Ver	512
A/Chicken/Queretaro/7653-20/95	1995	HPAIV-Qro	512

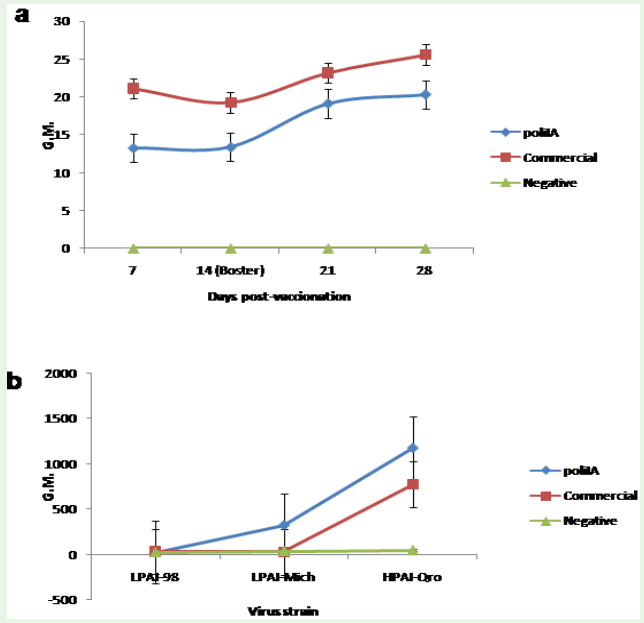

Figure 2: Titers of antibodies in vaccinated and non-vaccinated chickens. a) Antibodies promoted by non-vaccinated and vaccinated chickens with the inactivated avian influenza oil-emulsion monovalent commercial vaccine and Poly-AI before challenge, detected with the HI test b) Antibodies elicited after challenge against the influenza virus with each of the LPAIV strains and with the HPAIV-Qro. The challenge was conducted 28 days after the first vaccination. The negative control (non-vaccinated) group did not show evidence of any antibodies. Data are expressed as Geometric Means (GMs). (P = 0.046).

clinical signs in inoculated chickens (personal observation); similarly, a highly pathogenic influenza virus was used: HPAIV-Qro, which was isolated in Mexico during the 1995 outbreak and eradicated the same year.

Preparation of the polyvalent vaccine

To prepare the experimental vaccine (Poly-AI), the strains mentioned above were grown by passage in nine-day-old embryonating chicken eggs twice. Allantoic fluid was removed and diluted in MEM medium to a final titer of 106 for a 50% embryo infectious doses, and all viruses were mixed in equal proportion. Viruses were titrated with the hemagglutination assay, and the results are shown in Table 1. To evaluate the safety of the polyvalent vaccine [19], 15 chicken embryos were inoculated through the allantoids with 0.2 ml of the Poly-AI vaccine. Mortality over the following seven days was recorded daily; embryos that died in the first 24 h were eliminated. To demonstrate that the virus used for vaccine formulation were inactivated, the absence of hemagglutination activity was demonstrated in the allantoic liquid of embryos ten days after inoculation, using the hemagglutination test [20].

The absence of bacteria and fungi in the Poly-AI vaccine was verified through bacteriological culture in blood agar that was incubated at 37°C for seven days, following Mexican regulations [19].

Immunization

Twenty–seven 10-day-old SPF chicks were immunized with the non-adjuvanted Poly-AI vaccine, and another 27 chickens were vaccinated with an inactivated avian influenza oil-emulsions monovalent commercial vaccine (donated by PRONABIVE, México) for a comparison. Each group was vaccinated subcutaneously with 0.5 ml in the middle third of the dorsal region of the neck. Fourteen days post-vaccination, the chickens received a booster with the same dose and in the same manner. Another 27 10-day-old chicks were used as negative controls. Each group was kept in a separate cage in the biosecurity isolation units of INIFAP.

Detection of antibodies

The presence of antibodies against the avian influenza virus in the sera of vaccinated and non-vaccinated chickens was detected with the Hemagglutination Inhibition (HI) test using four Hemaglutinant Unit (UHA) of the antigen for avian influenza that was provided by the CPA. The presence of antibodies in the serum was evaluated at 0, 7, 14, 21, and 28 day's post- vaccination and 10 days after challenge. The results of the HI test were expressed as geometric means.

Challenge

Three groups of chickens immunized with the experimental, commercial and negative vaccine group were divided into three groups with nine animals each. This was done to challenge the animals with the two strains of low and high pathogenicity. Animals were maintained in separate cages in the isolation units of CENID-MA, INIFAP. To compare clinical signs among groups, the vaccinated and non-vaccinated chicken groups were challenged using two low pathogenic strains: LPAIV-98 and LPAIV-Mich. These isolates were chosen because they are phylogenetically divergent as shown in figure 1. Also, in a previous study developed very different signs (data not shown). Additionally, a Highly Pathogenic Avian Influenza Virus (HPAIV) strain A/Chicken/Queretaro/20/95 was used. Challenges were given through the intranasal route using 106 EID50. Animals that survived were humanely sacrificed using carbon dioxide, according to the guidelines of the Mexican Official Standard NOM-062-ZOO-1995 [21] for the humane euthanasia of domestic and wild animals.

Observation of clinical signs and lesions

Groups were evaluated daily for 10 days after challenge to observe the manifestation of clinical signs for groups challenged with the LPAIV strains, and mortality in chickens challenged with the HPAIV. These measurements were indicators used to evaluate the protection that the vaccine demonstrated against the challenged strains. General signs of avian influenza disease include the following: blepharoconjunctivitis, depression, epiphora, ruffled feathers, rales, nasal secretions, necrosis of the skin, edema of the comb, emerald green-colored feces, a decrease in activity, prostration, and death [22].

A positive sign (+) or a negative sign (-) was used to record if a clinical sign was present or absent, respectively.

Evaluation of viral excretion

To evaluate viral excretion, samples were collected using tracheal and cloacal swabs in MEM transport medium at 72, 120, 168, and 240 h after challenge, and they were filtered using 0.22-μm filters. Subsequently, RNA was extracted for qRT-PCR.

Real-time RT-PCR (qRT-PCR)

RNA was extracted from the filtered transport medium of each of the tracheal and cloacal samples using TRIzol LS (Invitrogen) for

Table 2: Clinical signs observed in non-vaccinated and vaccinated chickens with the inactivated avian influenza oil-emulsion monovalent commercial vaccine and Poly-AI vaccine; challenged with A/Chicken/Mexico/98/ H5N2.

Signs	Vaccine group	24 h	48 h	72h	96h	120h	144h	168h	192 h	216 h	240 h
Blepharoconjunctivitis	Unvaccinated control	+	+	++	+++	+++	+++	+++	++	++	-
	Vaccinated PolyAI	-	+	++	+	+	-	-	-	-	-
	Commercial vaccine	-	+	++	++	++	++	++	+	+	+
Depression	Unvaccinated control	-	-	++	+++	+++	++	++	++	++	+
	Vaccinated PolyAI	-	-	-	-	-	-	-	-	-	-
	Commercial vaccine	-	-	++	++	++	++	+	+	+	-
Epiphora	Unvaccinated control	-	-	+	+	+	+	+	+	+	-
	Vaccinated PolyAI	-	-	-	-	-	-	-	-	-	-
	Commercial vaccine	-	-	-	+	+	+	+	+	+	-
Ruffled feathers	Unvaccinated control	-	-	-	+	+	+	+	+	+	-
	Vaccinated PolyAI	-	-	-	-	-	-	-	-	-	-
	Commercial vaccine	-	-	-	-	+	+	+	+	-	-
Rales	Unvaccinated control	-	-	+	+	+	+	+	-	-	-
	Vaccinated PolyAI	-	-	-	-	-	-	-	-	-	-
	Commercial vaccine	-	-	-	-	-	-	-	-	-	-

-: non clinical sign presented; +: mild; ++: moderate; +++: severe; ++++; very severe

Table 3: Clinical signs observed in non-vaccinated and vaccinated chickens with the inactivated avian influenza oil-emulsion monovalent commercial and experimental vaccines; challenged with A/Chicken/Michoacan/4260-06/H5N2.

Signs	Vaccine group	24 h	48 h	72h	96h	120h	144h	168h	192h	216 h	240 h
Blepharoconjunctivitis	Unvaccinated control	+	+	+	++	++	++	++	+	+	+
	Vaccinated PolyAI	-	+	+	++	+	-	-	-	-	-
	Commercial vaccine	+	+	++	++	++	++	+	+	+	-
Depression	Unvaccinated control	-	-	+	++	++	+++	++	+	+	+
	Vaccinated PolyAI	-	-	-	-	-	-	-	-	-	-
	Commercial vaccine	-	-	+	+	+	+	-	-	-	-
Epiphora	Unvaccinated control	-	-	-	+	+	+	+	+	-	-
	Vaccinated PolyAI	-	-	-	-	-	-	-	-	-	-
	Commercial vaccine	-	-	+	+	+	+	+	+	-	-
Ruffled feathers	Unvaccinated control	-	-	-	+	+	+	+	+	-	-
	Vaccinated PolyAI	-	-	-	-	-	-	-	-	-	-
	Commercial vaccine	-	-	-	+	+	+	+	-	-	-
Rales	Unvaccinated control	-	-	-	-	-	-	-	-	-	-
	Vaccinated PolyAI	-	-	-	-	-	-	-	-	-	-
	Commercial vaccine	-	-	+	+	+	+	-	-	-	-

-: non clinical sign presented; +: mild; ++: moderate; +++: severe.

qRT-PCR [23]. Briefly, 250 μl of the sample and 750 μl of TRIzol LS were vortexed and incubated for 7 min. Next, 200 μl of chloroform were added; the mixture was centrifuged at 12,500 rpm, 4°C, for 15 min. Then, 500 μl of isopropanol were added to the supernatant, and the mixture was centrifuged at 10,000 rpm for 10 min. The pellet was washed with 1 ml of 70% ethanol and centrifuged for 10 min at 7,500 rpm. The pellet was then resuspended in 35 μl of RNAase-free water. RNA was frozen at -70°C until use. The qRT-PCR reaction was performed using the AgPath-ID One-step RT-PCR reagent kit (Ambion) with 1X reaction buffer master mix, 60 nM of the probe (InfA-M 5'-FAM-TCAGGCCCCCTCAAAGCCGA-BHQ1- 3'), 400 nM of each primer (InfM+ Fw 5'-AGATGAGTCTTCTAACCGAGGTCG-3'; InfM- Rv 5'-TGCAAAAACATCTTCAAGTCTCTG-3'), 1X enzyme mix, and RNAase-free water [23]. The PCR reaction was conducted in a LightCycler 480 (Roche) thermal cycler under the following conditions: 48°C for 30 min, 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min. The positive reaction control was established using a standard curve calculated with 1:10 serial dilutions of the LPAIV-Mich strain. Samples with a Ct lower than 31 and a fluorescence value of 0.2 were considered positive.

Statistical analysis

Statistically significant differences in antibody titers for vaccinated groups and quantitative virus shedding was compared between each vaccinated group and the non-vaccinated (control) group using the unpaired t test. Efficacy of the vaccine was determined based on increased survival, and the significant differences among groups were determinate using the Kruskal-Wallis variance analysis. Values of $p < 0.05$ were considered statistically significant.

Table 4: Presentation of clinical signs in chickens immunized with the inactivated avian influenza oil-emulsion monovalent commercial vaccine and experimental vaccine; challenged with A/Chicken/Queretaro/20/95 (HPAIV-Qro).

Signs	Vaccine group	24 h	48 h	72 h	96 h	120 h	144h	168h	192 h	216 h	240 h
Blepharoconjunctivitis	Unvaccinated control	+	++	+++	++++	++++	100% mortality				
	Vaccinated PolyAI	+	++	++	++	+++	++	+	+	-	-
	Commercial vaccine	+	++	++	+++	++++	++++	++++	++++	+++	+++
Depression	Unvaccinated control	-	++	+++	++++	++++	100% mortality				
	Vaccinated PolyAI	-	-	++	++	+++	++	+	-	-	-
	Commercial vaccine	-	+	++	++	+++	++++	++++	++++	+++	+++
Epiphora	Unvaccinated control	-	-	+	+	+	100% mortality				
	Vaccinated PolyAI	-	-	+	+	+	+	+	-	-	-
	Commercial vaccine	-	-	+	+	+	+	-	-	-	-
Ruffled feathers	Unvaccinated control	-	-	+	+	+	100% mortality				
	Vaccinated PolyAI	-	-	+	+	+	+	-	-	-	-
	Commercial vaccine	-	-	+	+	+	+	+	+	+	+
Fever	Unvaccinated control	-	-	+	+	+	100% mortality				
	Vaccinated PolyAI	-	-	+	+	+	-	-	-	-	-
	Commercial vaccine	-	-	+	+	+	+	-	-	-	-
Necrosis	Unvaccinated control	-	-	+	+	+	100% mortality				
	Vaccinated PolyAI	-	-	-	-	-	-	-	-	-	-
	Commercial vaccine	-	-	-	-	-	-	+	+	+	+
Oedema	Unvaccinated control	-	-	+	+	+	100% mortality				
	Vaccinated PolyAI	-	-	-	-	-	-	-	-	-	-
	Commercial vaccine	-	+	+	+	+	+	+	+	-	-
Emerald green faeces	Unvaccinated control	-	-	+	+	+	100% mortality				
	Vaccinated PolyAI	-	-	-	-	-	-	-	-	-	-
	Commercial vaccine	-	-	-	-	+	++	+	+	+	-
Decrease activity	Unvaccinated control	-	-	+	+	+	100% mortality				
	Vaccinated PolyAI	-	-	-	-	-	-	-	-	-	-
	Commercial vaccine	-	-	-	-	+	+	+	+	-	-
Prostration	Unvaccinated control	-	-	+	+	+	100% mortality				
	Vaccinated										
	Vaccinated PolyAI	-	-	-	-	-	-	-	-	-	-
	Commercial vaccine	-	-	-	-	-	+	+	+	+	-
Mortality	Unvaccinated control	-	33	80	100	100	100	100	100	100	100
	Vaccinated	-	-	-	-	-	-	-	-	-	-
	Vaccinated PolyAI										
	Commercial vaccine	-	-	-	-	-	-	-	-	-	-

-: non clinical sign presented; +: mild; ++: moderate; +++: severe; ++++; very severe

Table 5: Evaluation of viral excretion determined by qRT-PCR in chickens challenged with different avian flu strains. Data are expressed as EID50 equivalents. Different literals show a statistically significant difference (P>0.085) determined by the Kruskal-Wallis test.

Virus	Immunogen	72hr		120hr		168hr		240 hr	
		Trach	Cloacae	Trach	Cloacae	Trach	Cloacae	Trach	Cloacae
LPAI-98	PolyAI	1.31E+05^{a}	3.84E+06^{a}	8.26E+03^{a}	N	N	N	N	N
	Com	1.78E+08^{a}	5.17E+03^{b}	5.05E+01^{a}	1.18E+11^{a}	6.18E+05^{a}	2.64E+09^{a}	1.90E+12^{a}	2.89E+12^{a}
	Neg	6.61E+02^{a}	9.46E+03^{c}	8.55E+07^{a}	1.45E+02^{a}	5.17E+04^{b}	3.43E+04^{a}	2.07E+03^{a}	5.59E+06^{a}
LPAI-Mich	PolyIA	2.33E+08^{a}	2.29E+06^{a}	6.68E+09^{a}	1.12E+09^{a}	1.10E+07^{a}	N	N	N
	Com	1.23E+02^{b}	3.36E+01^{b}	2.83E+03^{a}	2.91E+11^{a}	2.18E+11^{b}	5.09E+12^{a}	5.26E+12^{a}	7.95E+12^{a}
	Neg	2.50E+02^{c}	1.55E+03^{c}	3.72E+04^{a}	4.19E+05^{a}	1.53E+02^{c}	3.00E+03^{b}	9.27E+04^{b}	2.48E+03^{a}
HPAI-Qro	PolyIA	7.15E+05^{a}	1.19E+05^{a}	6.56E+07^{a}	3.03E+01^{a}	N	N	N	N
	Com	3.43E+07^{b}	nd	6.60E+09^{a}	1.18E+11^{a}	N	N	N	N
	Neg	7.20E+03^{c}	2.66E+03^{b}	Mortality					

Traq = Trachea; Com = commercial vaccine; Neg = non-vaccinated birds; N = no detection for qRT-PCR.

Results

Preparation of polyvalent vaccine

The safety test did not indicate any embryo mortality, contamination, or hemagglutination. Negative results were also obtained for the sterility test in culture media.

Clinical signs

Birds that were immunized with the Poly-AI vaccine and challenged with only the LPAIV-98 strain showed signs of blepharoconjunctivitis at the inoculation site. Unvaccinated birds that were challenged with the same virus showed blepharoconjunctivitis, depression, epiphora, ruffled feathers, and rales (Table 2). Depression was assessed by the observation of decay in animals. Similarly, the immunized birds challenged with the LPAIV-Mich strain showed only blepharoconjunctivitis. The non-vaccinated birds that were challenged with this strain showed blepharoconjunctivitis, depression, epiphora, and ruffled feathers (Table 3).

The birds that were vaccinated and challenged with the highly pathogenic strain HPAIV-Qro showed blepharoconjunctivitis, depression, epiphora, and ruffled feathers. There was no mortality in any of the vaccinated birds; however, non-vaccinated chickens challenged with the HPAIV showed additional signs, such as necrosis of skin on the limbs, edema in the combs, emerald green feces, prostration, and activity reduction during the first five days, eventually all birds in this group died (Table 4).

Birds immunized with the commercial vaccine and challenged with LPAIV-98, showed blepharoconjunctivitis, depression, epiphora, and ruffled feathers similar to the negative controls (Table 2). Comparable results were observed in chickens challenged with LPAIV-Mich (Table 3). Furthermore, chickens challenged with HPAIV survived the challenge, although they showed exacerbated

signs compared with the birds vaccinated with Poly-AI, which were similar to the non-vaccinated chickens (Table 4).

Significant differences among groups were observed in mortality using the Kruskal-Wallis variance analysis (P=0.001).

Serology

Antibody titers prior to and after the challenge were measured using the HI test, and the results are shown in Figure 2. The results are presented as Geometric Means (GMT). The results showed that the Poly-AI vaccine without adjuvant induced antibodies to a GMT of 20.3 prior to challenge, while the commercial vaccine showed higher antibody titers (25.06). A t-test showed statistically significant differences between groups (P = 0.046).

Challenge

Chickens vaccinated and challenged with LPAIV strains showed decreased clinical signs, but no mortality (Table 2 and Table 3). In the group of birds immunized with the commercial vaccine, clinical signs were higher in intensity; there was no mortality (Table 4). Nevertheless, it is known that this increase is not connected to protection. As observed in Figure 2, the protection demonstrated by the Poly-AI and commercial vaccines reached 100%, compared with 100% mortality of the non-vaccinated birds challenged with the HPAIV.

Viral excretion measurements with qRT-PCR

Table 5 shows qRT-PCR results for viral shedding, expressed as an embryo infectious dose (EID50) equivalent. Viral shedding was primarily detected in the trachea, with less shedding in the cloaca. The result was expected, since the virus replicates mainly in upper respiratory tract, so it is one of the main sites of virus elimination or exit. Although, moreover, it is well known that the virus has a replication intestine and therefore is eliminated in feces, the use of the polyvalent vaccine was able to decrease said excretion. Viral shedding decreased in birds immunized with the experimental vaccine compared to the commercial, and this was observed in both LPAIV challenged birds. For the LPAIV-Mich strain excretion was observed for up to 168 h, while for the LPAIV-98 strain, observations in the trachea lasted for 120 h. For birds challenged with HPAIV, the experimental vaccine decreased viral excretion, although both groups (Poly-AI and commercial vaccine) showed excretion for up to 120 h.

Discussion

In Mexico, inactivated vaccines against the H5N2 avian influenza were applied in chickens and have demonstrated protection against morbidity, mortality, and reduced egg production. The vaccine used in Mexico (H5N2) is monovalent and contains the A/Chicken/Mexico/232/94/CPA strain as the master seed, as well as an adjuvant [24]. Observations conducted by the poultry sector have indicated that this vaccine, produced in 1994, is not providing adequate protection because signs of the disease have been seen even in vaccinated birds. Demands from the poultry industry have indicated a need for an updated vaccine. It was inferred that the reduced protection might be due to genome differences in the viruses encountered in the field, compared to the genome of the strain used for the vaccine. Analysis of the gene sequences identified genetic divergence among recent isolates and the vaccine (Figure 1). Therefore, it is important to prepare a polyvalent vaccine that includes the five main Mexican variants of the LPAIV H5N2 virus and to evaluate this polyvalent vaccine in target species. This study showed that the clinical signs produced in chickens challenged with both LPAIV (LPAIV-98 and LPAIV-Mich) are the same [25]. Nevertheless, the intensity of the signs induced by the various isolates of the LPAIV used in this experiment differed [14,25-27]. Remarkably, the experimental Poly-AI vaccine prevented most of the disease's clinical signs in animals challenged with the LPAIV, compared with the signs seen in birds vaccinated with the commercial vaccine. Moreover, when inoculating the control group, the LPAIV-98 strain was more pathogenic than the LPAIV-Mich strain, even though both strains were of low pathogenicity. This is the first study to demonstrate the degree of pathogenicity in a targeted species for the different isolates that exhibit genetic variation in Mexico. This indicates that there are strains of medium and low virulence. The signs reported in Tables 1 and 2 occurred in chickens challenged with the LPAIV virus [14,26]. The results also identified that chickens immunized with the experimental vaccine Poly-AI and challenged with the highly pathogenic HPAIV, showed only mild signs and avoided mortality.

Although antibody titers induced by the Poly-AI vaccine were low and the vaccine formulation did not contain an adjuvant, these titers were sufficient to protect 100% of the birds that were challenged, regardless of the pathogenicity of the challenge strain. Therefore, the use of an adjuvant was unnecessary because the antibody titers with the experimental vaccine not only protected against the challenge but also reduced the disease signs. This characteristic was observed with inactivated vaccines against the LPAIV, such as H5N1. The necessity of using a higher immune response threshold was suggested and would require the use of adjuvants, an increased antigen quantity, or an amino acid sequence similarity of at least 96.8%, which would induce antibodies with high affinity for the epitopes of the LPAIV [27-32]. However, an important detail is that the hemagglutinins (H5) of the LPAIV-2007, LPAIV-Hgo, LPAIV-Mich, and LPAIV-Ver isolates are 89 to 90% similar to that of the highly pathogenic strain, whereas the LPAIV-98 strain exhibits a similarity of 99%.

Viral shedding after challenging was evaluated with qRT-PCR, and this was the first time shedding was evaluated using this method in Mexico. The results indicated that the Poly-AI vaccine excreted a lower quantity of the virus compared to with the control (P>0.05). qRT-PCR detected up to 1012 EID50 equivalents (Table 5). The results showed that chickens and unvaccinated challenged LPAIV-Mich excrete less virus than those vaccinated with Poly-AI, this may be because these animals did not develop sufficient amount of antibodies to neutralize the virus. However, these antibodies were sufficient to confer protection. The results of antibody titles correlated with shedding after challenge with the LPAIV and HPAIV. Conversely, there was an inverse relationship between pre-challenge antibody titers and mortality and virus shed titers. These results are similar to previous studies that evaluated the shedding in other avian influenza vaccines [33]; in this study, Spackman, et al. investigated the efficacy of vaccines against the H7 Mexican strain that circulated in Mexico during 2012-2013.

Conclusion

The pentavalent vaccine produced and evaluated in this experiment demonstrates adequate protection against clinical signs

and mortality and lowers viral excretion compared with the control commercial vaccine. Although their sequences exhibit approximately 90% similarity, the isolates used for the challenge behaved differently. The experimental Poly-AI vaccine eliminated the clinical signs of the disease and led to 100% protection against challenge with a HPAIV-Qro strain. This is the first study to prepare and evaluate a pentavalent vaccine. Furthermore, it showed that the LPAIV virus produces different degrees of pathogenicity due to the various national isolates.

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