The Journal of Veterinary Medical Science

FULL PAPER

Virology

Immunogenicity of a recombinant hemagglutinin neuraminidase-*Porcine rubulavirus* produced by *Escherichia coli* of *Porcine rubulavirus* gives protective immunity of litter after challenge

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ABSTRACT. *Porcine rubulavirus* (PRV) is a contagious virus that affects the Mexican swine industry. This work aimed to evaluate the immunogenicity of an recombinant hemagglutinin neuraminidase-*Porcine rubulavirus* (rHN-PorPV) candidate vaccine on pregnant sows, and the protective efficacy afforded to their 7-day-old suckling piglets against PRV lethal challenge. Three sows were immunized with *r*HN-PorPV formulated with immune-stimulating complex (ISCOMs) and two sows with *r*HN-PorPV protein alone as well as a mock-immunized pregnant sow (negative control). Quantitative ELISA detected a high concentration of anti-*r*HN-PorPV Immunoglobulin G (IgG) antibodies in sow sera after the second dose of vaccine administered on day 14 until farrowing, showing viral-neutralizing and cross-neutralization activity against different variants of PRV. Sera samples from piglets of immunized sows (with or without adjuvant), showed high concentrations of IgG antibodies. As expected, piglets from the negative control sow (n=5), exhibited severe signs of disease and 100% of mortality after PRV challenge study. Conversely, 75% and 87.5% of the piglets born from the *r*HN-PorPV and the *r*HN-PorPV-ISCOMs-immunized sows (n=8), survived, respectively, showing milder PRV clinical signs. Our data indicate that *r*HN-PorPV candidate vaccine produced in *Escherichia coli* induces efficient humoral response in pregnant sows and that the maternally derived immunity provides high protection to suckling piglets against PRV lethal challenge.

KEYWORDS: colostrum, hemagglutinin-neuraminidase, immune-stimulating complex, *Porcine rubulavirus*, recombinant vaccine

J. Vet. Med. Sci.
84(12): 1595–1604, 2022
doi: 10.1292/jvms.22-0207

Received: 8 August 2022
Accepted: 11 October 2022
Advanced Epub:
21 October 2022

Porcine rubulavirus (PRV) belongs to the family *Paramyxoviridae*, subfamily *Rubulavirinae* and was recently renamed *Porcine orthorubulavirus*-La Piedad Michoacan Mexico virus [28]. PRV is the causative agent of "Blue Eye Disease" (BED), a disease first reported in the early 1980s in central Mexico around the town La Piedad in the State of Michoacán, affecting piglets associated with disorders of the central nervous system (CNS) and corneal opacity with morbidity of about 20% and mortality up to 50% [27, 36, 37]. Since the report of the initial outbreaks, the economic impact after PRV infection is related to low fertility, increased percentage of stillbirth pigs (up to 19%) and mummies (up to 36%), and decreased number of total piglets born [15, 20]. Currently, PRV infection is endemic in the central and western-central parts of Mexico, whereas the disease remains unreported in other countries [15, 20, 37].

PRV infection follows diverse epidemiological patterns, being most severe in newborn piglets that usually die within 2–7 days

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of the appearance of clinical signs; including fever, rough hair coat, progressive neurological disturbance, pneumonia, and uni- or bilateral corneal opacity. The disease is much less severe and with lower mortality rates in older pigs, showing clinical signs mainly associated with reproductive disorders [18, 23, 27, 36, 37]. However, neurological signs in fattening and adult pigs associated with mutations in the PRV hemagglutinin-neuraminidase (HN) gene have been reported [33]. Pigs are the only animals known to be affected clinically by PRV under natural conditions.

Like other *Rubulaviruses*, the PRV envelope contains two transmembrane fusion glycoproteins (F) and the mentioned HN [38, 39]. PRV infection induces strong and life long-lasting antibody responses againtst the immunodominant HN protein and also against two additional PRV proteins: M and NP [12, 19, 36]. PRV mRNA persists in pigs that have recovered from a natural infection and possible reactivation of the virus have been reported [11].

Aiming to contribute to PRV control, during the past years, efforts have been spent to develop vaccines for PRV. Presently, in México, there are two licensed vaccines to control PRV infection, based on live attenuated and inactivated classical vaccines, respectively. Despite their demonstrated effectiveness in preventing disease, sometimes-sporadic outbreaks occur in vaccinated farms, opening some biosafety concerns [5]. Work performed in our laboratory has shown the feasibility to produce two recombinant variants of the PRV HN proteins as candidate antigens, one of them *r*HN-PorPV becoming an alternative economic and scalable *Escherichia coli* platform to produce large amounts of safe and effective recombinant subunit vaccines in a short timeframe [7, 14]. Thus, three-dimensional modeling showed that the main conformational and functional domains of the *r*HN-PorPV protein were preserved, results further confirmed with functional assays demonstrating that this protein maintained antigenicity and immunogenicity characteristics similar to native HN-PRV protein [14]. Aiming to extend these studies, here we aim to evaluate the safety, immunogenicity, and protective efficacy of *r*HN-PorPV candidate vaccine either alone or formulated in immune-stimulating complex (ISCOMs), designed to induce long-lasting biologically active antibodies. ISCOMs® are antigen-releasing systems with a 40-nm micellar assembly made up of cholesterol and phospholipids. They combine amphipathic antigens with adjuvants (such as Quillaja saponin) and antigen-presenting cells (APC) targeting delivery devices, in order to transport and distribute antigens towards the cytosolic and endosomal pathway and thus stimulate the immune response [1, 2, 21].

Due to the fact that newborns are the main targets affected by PRV, here we experimentally immunized pregnant sows to evaluate the immunity induced and the passive protection afforded by the maternal immunity to suckling piglets via the colostrum. Two different clinical trials were performed, the first one using a lethal challenge of a highly virulent PRV strain. A second trial was perform using a low virulent PRV strain (PAC3) characterized by causing a mild respiratory disease in piglets [29], concomitantly circulating in endemic PRV areas [34].

MATERIALS AND METHODS

Production and purification of rHN-PorPV

The HN protein antigen open reading frame obtained from the reference strain PRV (GenBank accession number KC928078), was the original source for the *E. coli* expressed recombinant *rHN-PorV* protein and the *rHN-PorV* was purified by the GE AKTA Prime Liquid Chromatography System (GE Healthcare, Chicago, IL, USA) and quantified, before the immunization of pregnant female sows by standard protocols [14]. Briefly, the *r*HN-PorPV clone was grown in 250 mL of LB medium supplemented with kanamycin (50 µg/mL) and expressed in *E. coli* KRX competent cells (Promega, Madison, WI, USA). The culture was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) (Merck KGaA, Darmstadt, Germany) at a final concentration of 0.5 mM and 0.01% of L-rhamnose followed by incubation for 5 hr at 37°C and purified by Ni-NTA resin (GE Healthcare, Chicago, IL, USA). The protein concentration was determined by the Bradford assay and analyzed by SDS-PAGE and Western blot (WB) by standard protocols [14].

Animals and experimental design

To evaluate the immunogenicity of *r*HN-PorPV candidate vaccine (with or without adjuvant), six clinically healthy Landrace-hybrid pregnant sows, without clinical history and serological negative examinations of BED, were immunized in the second third of gestation (8 weeks). Three experimental groups were performed; one group of three pregnant sows immunized by the *r*HN-PorPV protein formulated in AbISCO-100 (ISCOMs) adjuvant (Isconova, Uppsala, Sweden) (*r*HN-PorPV-ISCOMs-vaccine) (1:1) [1]. A second group included two pregnant sows immunized by *r*HN-PorPV protein alone plus phosphate buffered saline (PBS) (*r*HN-PorPV-non-adjuvant vaccine), and the last group was one mock-immunized pregnant sow (control group) administered with ISCOMs plus PBS i.e. no antigen. The immunizing dose (ID) was established at 350 µg/sow, the immunization and bleeding scheme was as follows; the pregnant sows were intramuscularly (i.m.) immunized into the lateral neck region with a final volume of 3 mL. The sows were boosted with the same dosage two weeks later. During the experimental phase, the temperature in the immunized sows and control group was monitored every day and blood samples were collected from the jugular vein into sterile Vacutainer tubes (Becton Dickinson, Rutherford, NJ, USA) at 7, 14, 21, 30 days, post-farrowing; the suckling piglets were sampled at 0, 7, 14 and 30 days of age. Serum samples from all animals were separated and stored at −20°C until use, to evaluate the antibody response.

In a second part of the study, two different experimental challenge studies were performed using on seven-days-old suckling piglets. Briefly, suckling piglets from sows with different immunization treatments, were randomly selected to perform two different PRV challenges, as schematically shown in Table 1. For the study, piglets were challenged by intranasal instillation with $10^{-2.69}$ LD_{50}/mL (lethal dose which causes the death of 50%), of the wild-type lethal viral strain (PRV/Mx/2/Jalisco/2009, GenBank accession number KT037090.1), obtained from National Microbiology Research Centre (CENID-SAI), INIFAP. The starting point for the calculation of 50% endpoint dilution (LD_{50} titers), was previously calculated using Reed and Muench method (LD_{50} $10^{-2.69}$ LD_{50}/mL) using

Table 1. Suckling piglet challenge treatment scheme

Trial of immunized sow	Sucking piglets 7 days old post-colostrum ingest		
	Experimental group	Number of Piglets (n)	Challenge strain type Low virulence (1×10^6TCID$_{50}$/mL) High virulence ($1 \times 10^{2.69}$ LD$_{50}$/mL/dose)
*r*HN-PorPV-ISCOM-vaccine	A	16	Low virulence
	B	8	High virulence
*r*HN-PorPV protein plus PBS vaccine	C	8	Low virulence
	D	8	High virulence
Mock-immunized (control group)	E	5	Low virulence
	F	5	High virulence
Total	5	50	

TCID$_{50}$, median tissue culture infectious dose; LD$_{50}$, lethal dose which causes the death of 50%; ISCOM, immune-stimulating complex; PBS, phosphate buffered saline.

seven-days-old SPF piglets by serial 10-fold dilutions [26]. Post-challenge, the piglets were evaluated daily by a veterinarian and the criteria for the clinical score were assigned from 0 (healthy) to 4 (dead or moribund) for each piglet based on parameters as previously described [22], such as fever and rough hair coat [1], mild respiratory sign [2], incoordination and walking in circles [3], progressive neurological disturbance and death [4]. At 30 days post-challenge (dpc), all piglets were euthanized by CO_2 asphyxiation for determining the viral titres found in mid-brain tissue by real-time quantitative polymerase chain reaction (qPCR). The animals were handled at the house facility at National Microbiology Research Centre (CENID-SAI), Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP)-Mexico City and they were fed with a commercial diet twice daily, having free access to water.

For the second experiment, the PRV/PAC3/1992 (GenBank accession number EF413173) low-virulence strain [18] was used, provided by the Departamento de Microbiología e Inmunología, Facultad de Medicina Veterinaria y Zootecnia, UNAM, Mexico. The viral titer was determined by the calculation of 50% endpoint by serial dilution, the titer of virus stock was 1×10^6TCID$_{50}$/mL (median tissue culture infectious dose).

Real-time quantitative polymerase chain reaction (qPCR)

The qPCR assay was performed on the midbrain from suckling piglets to estimate the PRV viral load, specific primers to the P-gene were designed as described by Cuevas-Romero *et al*., 2013 [10]. Briefly, 50 ng of total RNA in a volume of 2 µL was used as templates for the SuperScript II Reverse Transcriptase, followed by qPCR amplification (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The thermal cycling protocol was as follows: 42°C for 5 min, 60°C for 20 min, for reverse transcription and 2 min at 95°C for initial activation step, followed by 48 cycles of two steps consisting of 15 sec at 95°C for denaturation, 60 sec at 58°C for annealing, and extension. Real-time detection of the PCR products was performed on a Rotor Gene Real-Time (QIAGEN, Hilden, Germany) and the copy numbers were calculated using the P-gene plasmid as a standard template [6]. All samples and controls were run in duplicate. A no-template control of nuclease-free water was included in each run.

Quantitative ELISA for antibody detection

A homemade, quantitative antigen-specific ELISA capture system targeting the HN glycoproteins of the PRV was performed. Briefly, MaxiSorpTM 96-well microplates (Nalge Nunc International Corp., Rochester, NY, USA) were coated with the recombinant antigen (200 ng/well) in 50 mM carbonate-bicarbonate buffer (pH=9.6, 0.5 M Na_2CO_3, 0.5 M $NaHCO_3$) and incubated overnight at 4°C. Subsequently, plates were blocked (50 mM Tris, 0.14 M NaCl, 1% Albumin bovine serum, pH 8.0), and incubated for 60 min at room temperature with the proper dilution of the corresponding samples diluted in PBS-Tween 0.3%. Serum and colostrum samples from immunized sows and suckling piglets were used (typically: serum at 1/150 and colostrum at 1/5) to detect specific IgG antibodies to the *r*HN-PorPV protein.

The plates were washed and anti-isotype-specific goat anti-pig IgG-HRP conjugated (Bethyl Laboratories Inc., Montgomery, TX, USA) was added and incubated for 60 min at RT. After washing, the chromogenic reaction was developed using 3,3′,5,5′-tetramethylbenzidine substrate solution (Sigma-Aldrich, St. Louis, MO, USA) for 3 min. The reaction was stopped using 1 M H_2SO_4 followed by reading the absorbance of each well at 450 nm. All plates included a standard curve using a reference serum provided in the kit (Bethyl Laboratories Inc., Montgomery, TX, USA) to quantify the antibody concentration as described by Salinas-Zacarias *et al*., 2020 [32].

Hemagglutination inhibition test (HI)

For hemagglutination-inhibiting test (HI) on colostrum, samples from immunized sows (with or without adjuvant) were evaluated according to standard procedures [29]. Briefly, colostrum samples were diluted (1:7) and centrifuged at 2,000 rpm for 10 min. Sera collected were heat-inactivated and adsorbed to remove nonspecific inhibitors of hemagglutination. HI assay was performed in 96 well plates using chicken erythrocytes (0.8%). A titre of the HI antibodies was expressed as the maximum dilution in which the serum inhibited the hemagglutinating activity of the virus. A positive response was considered >to 1:8. The titres were transformed into $\log_2$ values.

Plaque forming units (PFU) and cross-neutralizing antibody assays

Virus-neutralizing antibodies were detected in serum samples from immunized sows (with or without adjuvant) by using the plaque reduction neutralization test in porcine kidney (PK-15) cell monolayers, prepared in 12-well polystyrene culture plates according to the procedure described by Cerriteño-Sánchez *et al.,* 2016 [7]. Briefly, serum samples were heat-inactivated at 56°C for 30 min. Serial serum dilutions (1:8 to 1:64) were prepared in Gibco Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific), without serum and each one was mixed with the reference virus strain PRV 1984 (previously titrated 70 PFU). The mixture was incubated at 4°C for 1 hr, subsequently; PK-15 cell monolayers were infected with 400 µL of the mixture and incubated at 37°C, 5% CO_2 for 1 hr. The supernatants were removed from the wells and overlaid with 0.35% agarose in DMEM (containing 2.5% of bovine fetal serum). Plates were incubated for plaque formation for 6 days and stained with 0.2% crystal violet solution. The formed plaques were counted, and neutralization activity was assessed by comparing the plaque numbers obtained from PRV infected cells and treated with negative control serum. Additionally, cross-viral neutralizing assay (VN) was carried out using immunized sows' sera (with or without adjuvant) against three different PRV viral strains reported in the GenBank (KP 229773-PAC1/1990; EF 413172-PAC2/1990 and EF 413173-PAC3/1992). Briefly, three different 96-well tissue culture plates were prepared with duplicate serially 2-fold serial dilutions of 50 µL pig serum samples (complement inactivated) and incubated (5% CO_2, 37°C) for 1 hr with 300 TCID50 of different viral strain (PAC 1; PAC 2; PAC 3 respectively) in DMEM. Following incubation, PK15 cells were infected with 100 µL of the virus-serum mixture and incubated for 72 hr in 5% CO_2, 37°C. After incubation, when the virus control wells exhibited advanced virus-induced cytopathic effect (CPE), the neutralizing capacity of individual serum samples was assessed by determining the presence or absence of CPE. VN titres was expressed as the lowest dilution fold capable of completely preventing virus-induced CPE in 100% of the wells [24]. Control positive hyperimmune pig sera and non-immunized negative sera were run in all the assays. Titres were measured as plaque forming units (PFU).

Ethics approval and consent to participate

All procedures were in accordance with the Mexican legislation (NOM-062-ZOO-1999; SAGARPA), based on the Guide for the Care and Use of Laboratory Animals, NRC. The experiment was previously approved under a permit from the IACUC (Institutional Animal Care and Use Committee), CENID-MA, INIFAP.

Statistical analysis

Statistical analyses of the results were performed using one-way ANOVA to compare immunized groups with control group for each day post-immunization. Differences at $P<0.05$ were considered statistically significant with 95% confidence interval, (*$P<0.05$; **$P<0.005$; ***$P<0.0005$) and graphs were constructed using SigmaPlot version 12.5 (Systat Software Inc., San Jose, CA, USA). All summary data are presented as means ± standard error of mean (SEM).

RESULTS

rHN-PorPV protein is safe for the animals and induces specific antibody responses in pregnant sow's

The candidate vaccine was formulated with expressed *r*HN-PorPV protein (61.7 kDa) consistent with the molecular weight of the PRV-HN protein using 350 µg of *r*HN-PorPV protein without or plus ISCOMs in a final volume of 3 mL/dose (1:1). Following immunization in the second third of gestation at days 0 (8 weeks), all vaccinated pregnant sows remained clinically healthy. No febrile reactions (Fig. 1), nor clinical signs of illness were noted up to one-day post-farrowing. No apparent macroscopically changes and no harmful inflammatory response were observed in the application zone in any of the immunized sows.

Analysis of the humoral immune responses in serum samples of immunized pregnant sows (with or without adjuvant) showed specific serum antibodies that increased after the second dose given on day 14 and lasting to the delivery (day ~ 35), measured by quantitative ELISA (Fig. 2A). These antibodies recognized the *r*HN-PorPV purified protein by Western Blot analysis at 18- and 28-days post-immunization (Fig. 2B). The concentration of specific IgG antibodies in *r*HN-PorPV-ISCOMs-immunized sows was significantly higher at day 30 post-immunization (13.88 µg/mL) than found after immunization with non-adjuvant vaccine (2.19 µg/mL) and as expected, mock-immunized pig remained seronegative to the delivery (Fig. 2A). In colostrum, the average concentration of IgG-antibodies of *r*HN-PorPV-ISCOMs-immunized pregnant sows was 27,619 ng/mL at delivery and a statistically significant difference ($P<0.05$) was observed, when compared with that induced by *r*HN-PorPV protein alone (4,184 ng/mL) (Fig. 3), which decreased considerably at 36 hr postpartum to values close to zero. No significant differences in the IgG immunoglobulin concentrations

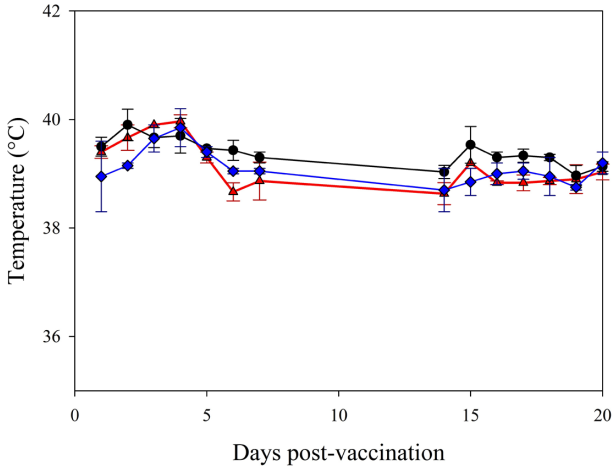

Fig. 1. Temperature of pregnant immunized sows with *r*HN-PorPV recombinant protein (with or without ISCOMs adjuvant); (–♦–) immunized sows by *r*HN-PorPV alone, (–▲–) *r*HN-PorPV-ISCOMs-immunized sow, (–●–) Control group (mock-immunized pregnant sow).

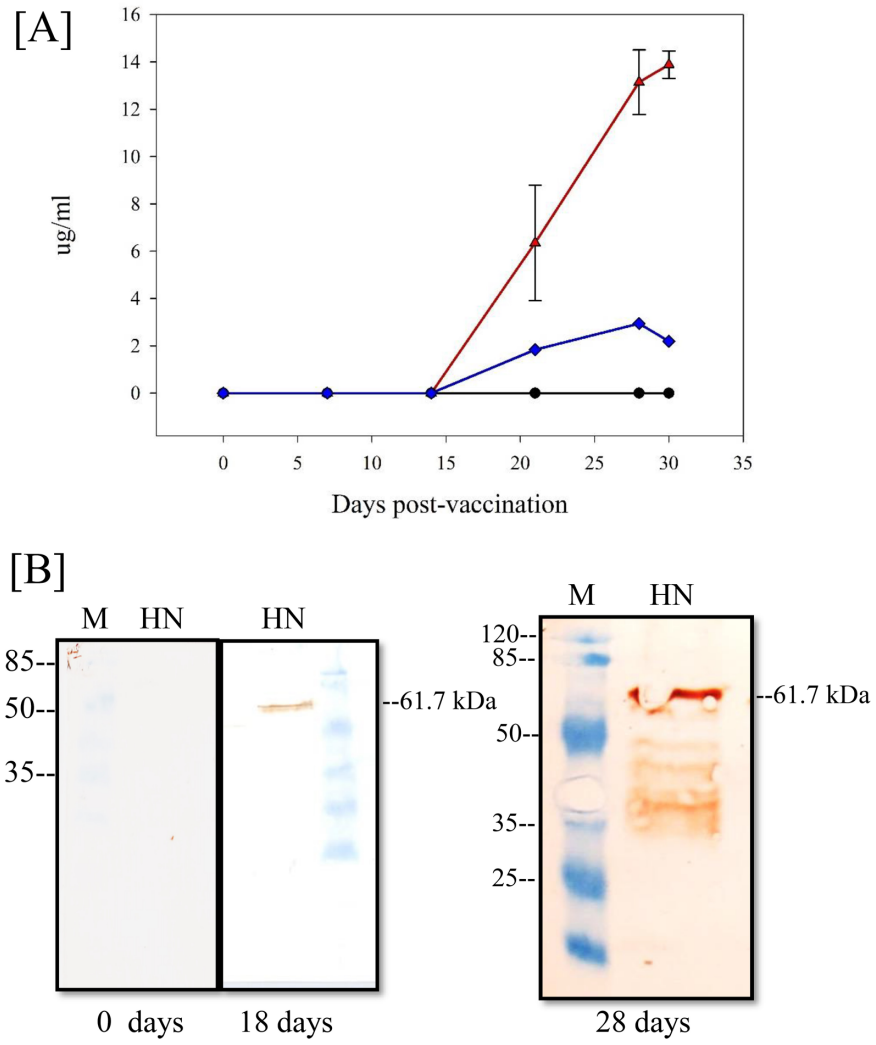

Fig. 2. Specific humoral immune responses induced by *r*HN-PorPV recombinant protein (without or with ISCOMs adjuvant) in serum from pregnant sows immunized at days 0 and boosted at day 14 measured by quantitative ELISA and WB. [**A**] Specific IgG anti-*r*HN-PorPV protein detected by quantitative ELISA suing sera from immunized pregnant sows with either: (–♦–) by *r*HN-PorPV alone, (–▲–) *r*HN-PorPV-ISCOMs-immunized pregnant sows; or (–●–) PBS. [**B**] Recognized *r*HN-PorPV protein by using a serum from *r*HN-PorPV-ISCOM-immunized pregnant sow at different times after immunization by Western Blot.

was observed between colostrum versus serum from immunized pregnant sows (with or without adjuvant) at the day of farrowing (Fig. 3). In addition, the presence of hemagglutination-inhibiting antibodies for PRV in colostrum samples were detected until 36 hr post-farrowing with a significant increase ($P<0.05$) rate of 4 to 5 $\log_2$ averages at 0 and 12 hr, but not at 36 hr post-farrowing. No HI activity in colostrum samples from the mock-immunized control sow was observed (data not shown).

The neutralization activity of anti-*r*HN-PorPV serum antibodies determined by counting the reduction of the number of PFU compared with a positive control at the same dilution (polyclonal serum) in a virus-neutralization assay presented a decrease of PFU observed at 1/8 to 1/64 dilutions (Fig. 4A). Positive and negative controls corroborated the fidelity of the assay. The percentage of inhibition was 75% and 87% at 1/16 and 1/8 and sera dilutions, respectively, compared with the positive control without serum dilution (Fig. 4B). The anti-*r*HN-PorPV-IgG antibodies tested at 21 days after sow's immunization showed cross-virus neutralization titers of 1:256 against three different PRV viral isolates measured by a virus-neutralizing assay. The cytopathic effect on PK15 cells was observed in the negative control sera from mock-immunized sow after 3 days of incubation with each viral isolate (Table 2).

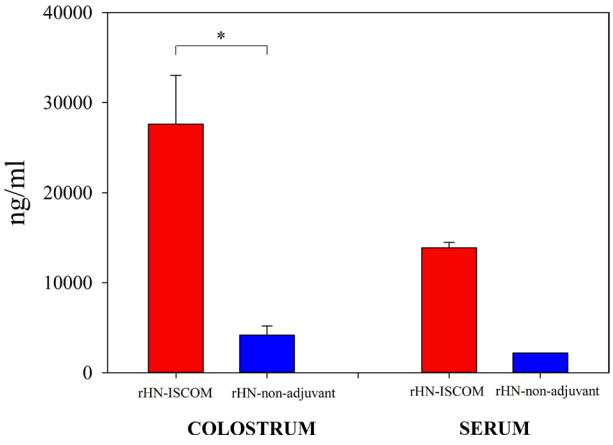

Fig. 3. Anti IgG-*r*HN-PorPV antibodies average concentration in colostrum and serum samples of immunized pregnant sows (with and without adjuvant) at 0 and 36 hr by ELISA test using samples from immunized pregnant sows with: *r*HN-PorPV alone or *r*HN-PorPV-ISCOMs. Asterisks indicate statistical significance between groups (*$P<0.05$).

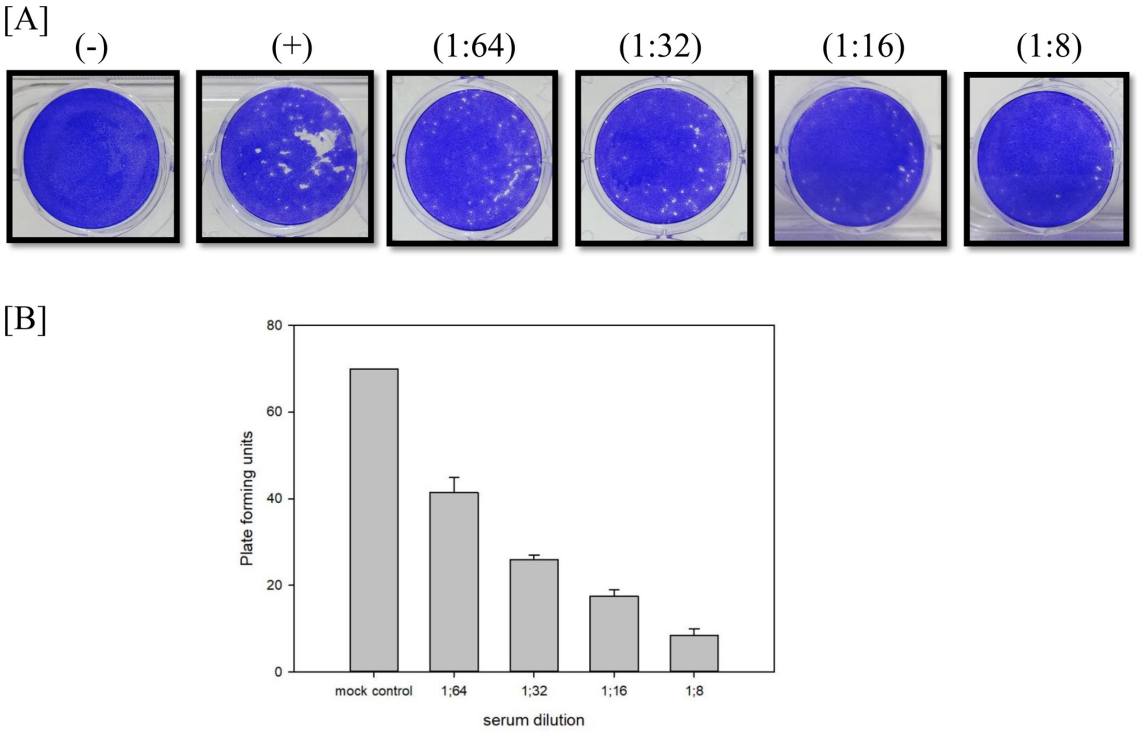

Fig. 4. Reduction of plate forming units (PFU) by *r*HN-PorPV antibodies from *r*HN-PorPV-ISCOMs-immunized pregnant sows; [**A**] PFU in PK-15 cell culture; (−) negative control without virus, (+) positive control with PRV reference virus. 1:8 to 1:64 serum dilutions, [**B**] Number of PFU at different serum dilutions.

Table 2. Serological cross-reactivity of serum samples tested at 21 days after sow's immunization against viral isolates

Strain/GenBank accession number	Neurovirulence in suckling pigs	VN
PAC-2/EF413172	<10%	1:256
PAC-4/EF413174	>30%	1:256
PAC 7/EF413176.1	10%	1:256

VN: virus neutralization titers, expressed as the reciprocal of the last serum dilution that completely inhibited the production of cytopathic effect (cpe) (>1:2 are considered positive).

rHN-PorPV immunization protect suckling piglets against lethal PRV challenge

As expected for the virus and dose used, suckling piglets (n=5) from the control group (mock-immunized mother sow) showed a 100% of mortality (Fig. 5A). In clear contrast, At 13 dpc, 7 out of 8 piglets (87.5%) of *r*HN-PorPV-ISCOMs-immunized sow survived and a survived rate of 75% (6 out of 8) was observed for piglets from immunized sows with the *r*HN-PorPV protein alone. The average clinical score for the suckling piglets of *r*HN-PorPV immunized sows (with or without adjuvant) shoed a score of 1, with increase of temperature during the first 5 days after challenge, next, the animals recovered the normal temperature from day 8 and 9 respectively (Fig. 5B). Only one piglet of the *r*HN-PorPV-ISCOMs-immunized sow, showed a very high clinical score at 9 dpc, increase of respiratory signs and progressive neurological disturbance with death at 12 dpc. Similarly, two piglets from *r*HN-PorPV immunized sows, showed progressive neurological disturbance, the clinical signs starting on 8 dpc with death at 11 dpc, one of them showing uni-lateral corneal opacity- Interestingly one different piglet without clinical signs showed uni-lateral corneal opacity. Compared with the immunized piglets; piglets from the control group showed average scores peaking on days 6 and 8 dpc, with all animals showing progressive neurological disturbance and death between 9 to 10 dpc. The surviving piglets of recombinant *r*HN-PorPV immunized sows recovered and were healthy without sequelae of the disease from 16 dpc until the end of the experiment (30 dpc) (results are summarized in Fig. 5B).

Clinical observation after challenge by a low virulence viral strain

At the time of the challenge, the antibody detection in sera collected from suckling piglets (7 days old), which had previously ingested colostrum of immunized sows (with or without adjuvant), showed higher concentrations of specific IgG anti-*r*HN-PorPV of 4,543 ng/mL and 2,887 ng/mL respectively, (Fig. 6, day 0). After experimental infection with a low virulence viral strain ($1 \times 10^6 TCID_{50}$/mL/dose), significant differences were observed in the IgG antibody concentration at 0, 7 and 14 days post-challenge between suckling piglets of the *r*HN-PorPV-ISCOMs-immunized sow and control group ($P<0.005$). On the other hand, significant differences were observed in the IgG antibody concentration only at 0 days post-challenge between suckling piglets of immunized sows with the *r*HN-PorPV protein alone and control group ($P<0.005$) but, no differences were observed at 30 days after the challenge in all groups (Fig. 6). The average temperature was increased at 4 dpc in all infected groups, indicative of the first replication stage by

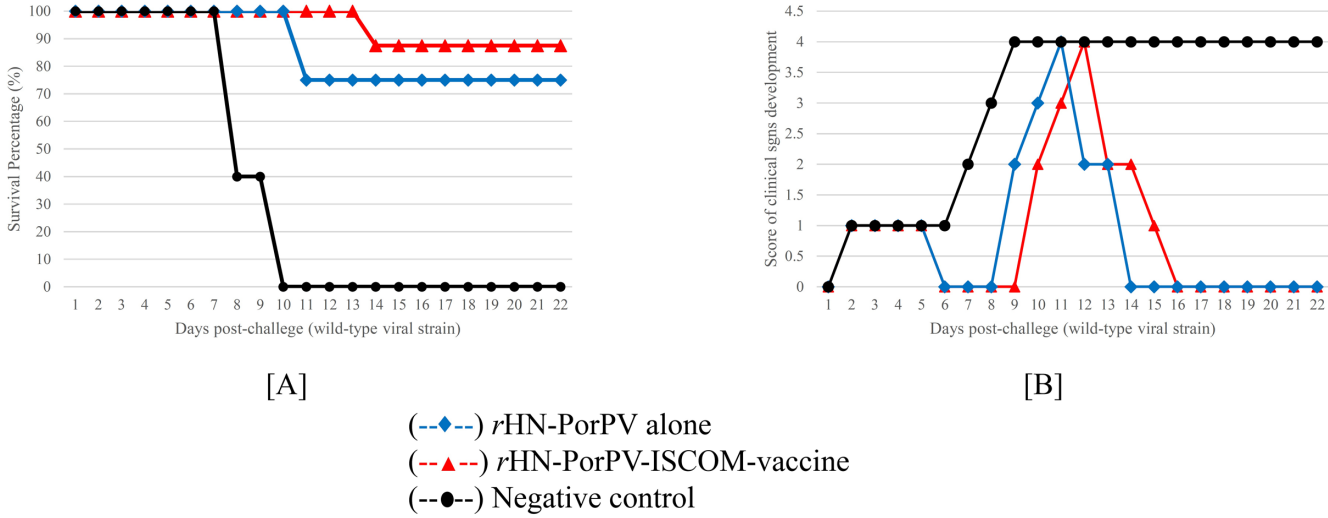

Fig. 5. Evaluation of clinical signs score and survival rate of suckling piglets of immunized sows after challenge by wild-type lethal viral strain. [**A**] Survival percentage along days after a challenge of suckling piglets of immunized sows with: (–♦–) suckling piglets of immunized pregnant sows by *r*HN-PorPV alone, (–▲–) suckling piglets of *r*HN-PorPV-ISCOMs-immunized pregnant sows, (–●–) negative control suckling piglets (mock-immunized pregnant sows). [**B**] Score of clinical signs development in piglets of immunized sows after challenged. Clinical score was assigned as 0 (healthy); fever and rough coat [1]; mild respiratory signs [2]; incoordination and walking in circles [3]; progressive neurological disturbance and death [4].

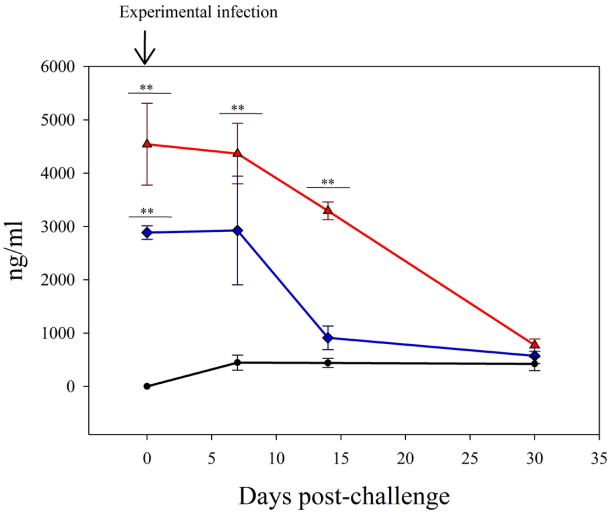

Fig. 6. Anti-*r*HN-PorPV IgG antibody concentration in sera collected from suckling piglets 7 days old (day 0) after ingesting colostrum of immunized pregnant sows (with and without adjuvant). The arrow indicates the challenge time by low-virulence-viral strain (PorPV/PAC-3) and antibody detection after experimental infection; (–♦–) suckling piglets of immunized pregnant sows by *r*HN-PorPV alone, (–▲–) suckling piglets of *r*HN-PorPV-ISCOMs-immunized pregnant sows, (–●–) negative control suckling piglets (mock-immunized pregnant sows). Asterisks indicate statistical significance between suckling piglets of the *r*HN-PorPV-immunized sows and control group, respectively (**P<0.005).

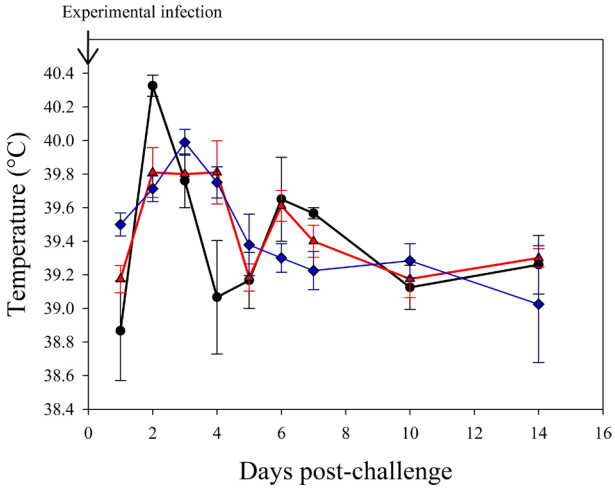

Fig. 7. Temperature detection after low virulence challenge of suckling piglets from immunized pregnant sows with: (–♦–) suckling piglets of immunized pregnant sows by *r*HN-PorPV alone, (–▲–) suckling piglets of *r*HN-PorPV-ISCOMs-immunized pregnant sows, (–●–) negative control suckling piglets (mock-immunized pregnant sows). Arrow indicates the beginning of viral infection.

the viral infection (Fig. 7). The exposure to a low-virulence viral strain produced no-clinical signs of illness in any suckling piglet of *r*HN-PorPV-ISCOMs-vaccine (n=16) and *r*HN-PorPV protein alone (n=8), showing average clinical scores close to zero. However, in suckling piglets (n=5) from the control group (mock-immunized sow), 3 out of 5 piglets showed mild respiratory signs by seven days post-challenge. All suckling piglets were recovered and healthy by 14 dpc until the end of the experiment (30 dpc). Viral infection

was confirmed in five euthanized-suckling piglets by qPCR of each group; no statistical difference in virus load was shown in midbrain samples collected at necropsy. The average of Ct value found in their brains was 24.73 (*r*HN-PorPV-ISCOMs) and 24.356 (*r*HN-PorPV-protein alone) and 24.75 in the control group; corresponding approximately to 9.50×10^4 and 6.32×10^3 copy numbers, respectively, according to the standard curve by qRT-PCR assay [10] (Table 3).

Table 3. Results of real-time PCR assay in midbrain samples using the P gene plasmid as reference positive template

Experimental infected suckling piglet		Ct value
Suckling piglets of rHN-PorPV-ISCOM-vaccine immunized sow	1	19.23
	2	24.27
	3	32.16
	4	23.26
	5	23.28
	Average	24.73
Suckling piglets of rHN-PorPV-protein alone immunized sow	1	21.32
	2	22.57
	3	24.56
	4	30.14
	5	23.19
	Average	24.35
Suckling piglet of control mock-immunized sow	1	25.85
	2	23.04
	3	20.71
	4	29.41
	5	22.13
	Average	24.50

Ct, cycle threshold; ISCOM, immune-stimulating complex.

DISCUSSION

PRV infection in pigs has only been detected in Mexico and is considered one of the important diseases affecting the Mexican swine industry. The last seroepidemiology study (2011) a significant serological prevalence was found ranging from 9% to 23.7% in four of the largest pig-producing states in Mexico (Guanajuato, Jalisco, Michoacán, and the Estado de Mexico) [15].

Our study has demonstrated the potency of the *r*HN-PorPV protein as an immunogenic vaccine alone and when combined with adjuvants (ISCOMs-Matrix®) to elicit a strong protective immune response in pregnant sows and maternally derived immunity. There was a high survival rate among piglets of *r*HN-PorPV-immunized sows (87.5% and 75% respectively) after the intranasal PRV challenge, with a 100% mortality in piglets of the control group (mock-immunized sow). These survival rates suggest the potential of the *r*HN-PorPV as a subunit candidate vaccine in preventing piglet mortality. Traditionally, viral vaccines for pigs are either based on attenuated-live virus strains or inactivated viral antigens. With the advent of genomic sequencing and molecular engineering, novel vaccine strategies and tools, including subunit and nucleic acid vaccines, became available and are being increasingly used in pigs [25].

According to our results, the *r*HN-PorPV candidate vaccine, no apparent macroscopically changes and no harmful inflammatory response was noted after i.m. immunization in the application zone indicative of the safety, including no febrile reactions in any of the immunized sows up to one-day post-farrowing. In addition, no clinical signs of illness were noted in vaccinated sows, despite that the dose of the *r*HN-PorPV-candidate vaccine (350 µg) was two times higher than the dose used in younger pigs (150 µg) [2]. In our study, the potential importance of successful absorption of colostrum was essential for disease prevention because the newborn piglets are highly susceptible [1]. The presence of hemagglutination-inhibiting antibodies to PRV in colostrum samples was indicative to have effective binding ability to recognize the virus. In this respect, immunoglobulins form an important component of the immunological activity found in milk and colostrum and it is the first immune protection of the newborn piglet [4]. Nevertheless, the amount of maternal immunity in the post-suckling pig depends on the concentration of immune and other bioactive products in colostrum, the amount of colostrum ingested, and the timing of gut closure [30]. Serum samples from immunized sows also showed the potential of IgG antibodies to inhibit viral replication by seroneutralization activity, it is agree with does reported by Cerriteño *et al.*, 2016 [7], with the same assay obtained >80% inhibition with anti-HN mice antibodies. Recently, Siañez-Estrada *et al.,* 2020 [35], predicted several linear B-cell and T-cell MHC class I epitopes into HN protein where two conserved significant epitopes; ATRSETDYY and AAYTTTTCF might be important for neutralizing the viral infection. In addition, maternal neutralizing antibodies not only bind to target protein of the virus, but also cross-neutralizing reactivity against other PRV variants was observed, suggesting that antibodies against the *r*HN-PorPV protein may provide broad neutralizing capability against multiple variants of the virus, despite genetic and antigenic differences of the PRV isolates [33]. However, Escobar-López, *et al*., 2012 suggested that the use of a monovalent vaccine would not generate complete protection against the different antigenic variants of PRV [15]. Nevertheless, our studies on molecular characterization of different PRV isolates, where some variants of PRV preserve the HN sequence for a long time and small variations in amino acid sequences suggests a high level of conservation of the HN protein and perhaps low antigenic variation [11, 13]. In fact, the antigenic prediction of the *r*HN-PorPV protein compared with published sequences of the HN of PRV showed the conservation of important residues in the active sites independently of the genetic mutation observed to occur through the years [3, 14, 33]. In addition, evaluation of a commercial PRV vaccine in piglets of 5 and 6 weeks of age by viral challenge using three different isolates with different genetic homology (100%, 99.7% and 97.6%, respectively) has been documented, which showed high efficacy and protection, regardless of the genetic variation of the strain used in the challenge [5].

In addition, the incorporation of adjuvants to elicit a strong protective immune response is often essential as the use of ISCOMs-based vaccines revealed an antigen-specific immune response characterized by long-lasting antibody production [2, 16] and several genes, genetic pathways and biological processes had been identified that are likely to shape the early immune response [1]. However, since subunit vaccines can be administered as crude purified proteins, the included antigens alone are not sufficient to induce adequate long-term immunity; as was reported in the PCV2 vaccination and E2 vaccine for classical swine fever [25, 31]. Nevertheless, it is not in agreement with our results, because the *r*HN-PorPV recombinant protein alone used in the vaccine formulation as essential antigen had potential effects in the survival rate of piglets after challenge (75%). In suckling piglets, serum antibodies remained high for up

to two weeks after the challenge, suggesting that protection of these piglets may last longer against a low and high lethal dose of the PRV viral strain used in viral challenge. As expected, the IgG antibody concentration of the negative control piglets group was higher at 30 days post-challenge, suggesting that transient passive immunity will not last long after weaning, as has been reported [8, 17]. The results confirmed a protective role of specific maternal anti-*r*HN-PorPV-IgG antibodies at challenge exposure (7 days old) by using a wild-type lethal viral strain showed a high rate of protection of suckling piglets against PRV infection (87.5% or 75%, respectively).

With this background, we suggest that the *r*HN-PorPV formulated in ISCOMs-Matrix® adjuvant, or protein alone is a good candidate to propose a vaccine for PRV, where the immunogenicity of recombinant antigen could not be affected by the use of *E. coli* as an expression platform as have been reported by using formulations of different recombinant proteins produced in *E. coli* [3, 9]. Virus recombinant proteins are promising tools for the development of vaccines, due to the absence of genetic material, which may induce adverse reactions and transmit the pathogenic virus [22, 25]. Consequently, the *r*HN-PorPV protein produced in *E. coli*, has the necessary elements to induce an efficient humoral response in pregnant sows and maternally derived immunity, important step to protect newborn piglets while they are highly susceptible; it is promising, giving the first advances in the development of a new generation vaccine to PRV. These results demonstrated alternatives for the use of traditional vaccines and provided important information for further development of subunit vaccines by using formulations of recombinant proteins produced in *E. coli.*

CONFLICTS OF INTEREST. None of the authors have any conflict of interests.

ACKNOWLEDGMENTS. This study was supported partiality by SAGARPA-CONACYT-12159, SEP-CONACYT 288942, CENID-Salud Animal e Inocuidad INIFAP (México) and IPVS México 2014 AC. We would like to thank Dr. Bror Morein and Dr. Eliseo Hernández Baungarten for technical assistance and Dr. Fernando Rodríguez (IRTA-CReSA) for his critical reviewing.

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