ARTICLE / INVESTIGACIÓN

Neutralizing antibodies as a correlate of protection against classical swine fever in Porvac® vaccinated pigs

Marisela Suárez-Pedroso[1]*, Yusmel Sordo-Puga[1], María Pilar Rodríguez-Moltó[1], Paula Naranjo-Valdés[2], Danny Pérez-Pérez[1], Iliana Sosa-Teste[3], Carlos Montero-Espinosa[1], Yohandy Fuentes-Rodríguez[1], Talía Sardina-González[1], Elaine Santana-Rodríguez[1], Milagros Vargas-Hernández[1], Ayme Oliva-Cárdenas[1], Nemecio González-Fernández[4], Eddy Bover-Fuentes[4], Carlos A. Duarte[1], Mario Pablo Estrada-García[1]

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[1] Departamento de Biotecnología Animal, Centro de Ingeniería Genética y Biotecnología, La Habana, Cuba.
[2] Unidad de Laboratorio Central para Salud Agropecuaria (ULCSA), La Habana, Cuba.
[3] Centro de Toxicología Experimental (CETEX), Centro Nacional para la Producción de Animales de Laboratorio (CENPALAB), Cuba.
[4] Departamento de Investigación Desarrollo, Centro de Ingeniería Genética y Biotecnología, Camagüey, Cuba.
Corresponding author: marisela.suarez@cigb.edu.cu

Abstract: Porvac is a classical swine fever (CSF) subunit vaccine. It is safe and induces a robust neutralizing antibody response, sterilizing immunity, and early protection, and it prevents vertical transmission in pregnant sows. The methodology to approve Porvac batches is a challenging experiment in pigs with a virulent CSF virus strain. However, there is an ethical reason to reduce, at minimum, the use of animals in these lethal experiments. The knowledge indicates that neutralizing antibody titers in the blood could be a good correlate of protection. The results of 22 challenge experiments involving 116 Porvac vaccinated and 38 unvaccinated animals were analyzed. All vaccinated animals remained free from CSF clinical signs and pathological lesions and were negative for viral isolation after the challenge. In contrast, all unvaccinated pigs developed clinical and pathological signs of the disease and had to be euthanized eight days post-challenge. All vaccinated pigs exhibited high neutralizing antibody titers, with a geometric mean value of 1: 5153. The lower titer registered was 1: 800. A complete correspondence between neutralizing antibody titers and protection was demonstrated. These results support substituting the viral challenge test for the neutralizing peroxidase-linked assay in the release of Porvac® batches.

Key words: Classical swine fever, virus, subunit vaccine, viral challenge, neutralizing antibodies.

Introduction

Classical Swine Fever (CSF) is a highly contagious and devastating viral disease with a severe socio-economic impact on the pig industry worldwide[1]. The etiological agent, CSF virus (CSFV), is icosahedral and enveloped positive-stranded RNA virus member of the Pestivirus genus of the *Flaviviridae family*[2].

In endemic areas, vaccination using modified live vaccines (MLV) is mainly directed to limit the effects of the disease or as a first step within a general program for the control and eradication of the virus[3]. However, MLV can't discriminate between infected and vaccinated animals, require a cold distribution chain, and maternal-derived neutralizing antibodies compromise its immunogenicity in the piglets[4]. These drawbacks have limited the application of MLV in disease-free regions. Several subunit vaccines have been developed to substitute MLV, but they show a late onset of protection compared to MLV and provide incomplete protection against vertical transmission[3,5-7].

Porvac® is a novel subunit marker vaccine against CSFV. It is based on a chimeric antigen (E2-CD154), comprising the E2 protein of CSFV and the molecular adjuvant CD154. This vaccine has been safe and capable of inducing an unusually rapid onset of protection against a challenge with a highly pathogenic CSFV strain, similar to the one described for MLV[8-10]. The animals immunized with Porvac® develop high neutralizing antibodies (NAb) titers and cell-mediated immune responses. Furthermore, the administration of Porvac® to pregnant sows interferes with the transplacental transmission of the virus[11].

Porvac® has been registered in Cuba since 2017 (Sanitary Registry 763) and has currently been used in large state-owned pig production units, genetic units, and small private farms. A potency test, consisting in the challenge of the vaccinated animals with 10^5 PLD_{50} of a highly virulent strain, has been established to release the batches of Porvac®. This test has been previously used in the batch release process for MLV[12,13]. However, it involves suffering for control animals, which would have to die to ensure vaccine quality. Furthermore, it requires the handling of large amounts of virus, and, therefore, special contention facilities are needed to avoid dissemination of the virus, increasing the costs of quality control.

As stated in the last edition of the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals[14], the efficacy of veterinary vaccines should be demonstrated through accurate statistical analysis of the host species. Nevertheless, challenge experiments in the final species may be unnecessary if data on the predictive value of serological tests are available. Wherever possible, applying procedures to replace, reduce and refine (the three R rule) the animals in-

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volved in such studies should be encouraged. The potency tests required for the approval of each vaccine batch must correlate with the vaccine's efficacy in the host species[15].

Previous work has established that neutralizing antibody (NAb) titers are a good correlate of protection against CSFV in swine. A NAb titer equal to or higher than 1:50 is considered protective[16-18]. In line with these experimental data, the chapter of the OIE Terrestrial Manual about protein subunit vaccines based on the CSFV E2 protein recommends that the induction of specific anti-E2 antibodies in vaccinated pigs can be used to confirm the potency of each batch once the title has been correlated with the results of the efficacy test[14].

Therefore, this study aimed to demonstrate the correspondence between the NAb titers in the serum of Porvac-vaccinated animals and protection from lethal viral challenges. The final goal is replacing the viral challenge test for the neutralizing peroxidase-linked assay in the routine release of Porvac® batches.

Materials and methods

Vaccine

Five batches of the Porvac® were evaluated (P51031, P61011-1, P61021-1, P71011-1, P81011-1). Each 2 mL dose contains 50 µg of E2-CD154 antigen (25 µg/mL) in Montanide™ ISA 50V2 oil adjuvant (SEPPIC, Paris). The vaccine was kept at 2-8 C until use. Vaccination and viral challenge were repeated every six months for some batches, which increased the number of animals evaluated in this analysis to a total of 116 vaccinated animals and 38 unvaccinated control animals.

Experimental animals

Nine weeks old Crossbred Duroc/Yorkshire swine (25-30 Kg) (CENPALAB, Cuba), belonging to an unvaccinated, CSF-free herd, were used. A total of 154 animals were used, 116 were vaccinated with Porvac®, and the other 38 were used as unvaccinated controls for the challenge. The animals were housed in separate experimental boxes, following the animal welfare regulations and standards established by the Animal Health Department of the Ministry of Agriculture in Cuba. Vaccination/challenge experiments were conducted under appropriate high containment facilities, following the animal welfare regulations and standards according to EU Directive 2010/63/EU and Good Clinical Practices[14]. The Ethical and Animal Welfare Committee of CENPALAB, Cuba, approved and supervised the protocols.

Vaccination and challenge schedules

A two-dose vaccination schedule was conducted, with 21 days interval between injections. A volume of 2 ml was administered intramuscularly in the neck. The animals were challenged between 8 and 10 days post-vaccination (dpv) with 10^5 PLD_{50} of the highly virulent CSFV "Margarita" strain, belonging to subgenotype 1.4[19,20], by intramuscular injection in the neck.

The clinical signs compatible with CSFV infection in the animals after viral challenge were scored from 0 to 18, according to Mittelholzer *et al.*[21], with some modifications. The changes consisted of eliminating three parameters (body tension, body shape, and breathing), and adding the rectal temperature. The pigs were visually inspected for at least 15 minutes per day before and during the feeding and cleaning of the boxes. A dairy registry was filled for each animal with all the relevant clinical signs (rectal temperature, dullness, anorexia, prostration, constipation, diarrhea, cutaneous hyperemia, posterior paralysis and paresis, conjunctivitis, unsteady gait, convulsions, lack of response to stimulus, and presence of cutaneous or mucosal lesions. For ethical reasons, control animals were euthanized eight days post-challenge (dpc) when they reached clinical scores higher than 10. Vaccinated animals were evaluated until 21 dpc. The macroscopic anatomopathological analysis was conducted immediately after the sacrifice. Samples from the spleen and tonsils were collected in some cases. Some of these samples were processed for virus detection through the viral isolation technique (VI).

The challenge test for Porvac® includes several acceptance criteria, which are listed below:

- All control animals must get the disease severely or die after the challenge.
- More than 80 % of the animals vaccinated with the full schedule must show clinical protection.
- No vaccinated animal must develop a fever (temperature above 40 ºC) for 3 or more consecutive days.
- After the viral challenge, vaccinated animals should be free from CSF-compatible macroscopic lesions.

Blood samples

Blood samples were taken in the presence or absence of an anticoagulant. Whole blood and serum samples were stored at -70 °C or -20 °C, respectively, until their use for virus isolation or antibody detection. The samples were taken on day 0 (before vaccination), day 21 (after one immunization), at 28-30 days (7-10 days after the second administration), and 7 and 21 days after the challenge.

Cell line

The PK-15 cell line (ATCC CCL-33TM) was used for the Neutralizing Peroxidase Linked Assay (NPLA)[22] and viral isolation[23]. A cell culture-adapted variant of the highly virulent CSFV strain Margarita, gently provided by the Centro Nacional de Sanidad Agropecuaria, Mayabeque Cuba, was used in NPLA.

CSFV NAb detection (NPLA test)

NAb titers in the serum were determined by the Neutralizing Peroxidase Linked Assay (NPLA)[22]. Titers were expressed as the reciprocal of the higher dilution of serum that neutralized 100 $TCID_{50}$ of Margarita strain in 50% of the culture replicates. The virus was detected by incubation with the anti-E2 Mab CBSSE2.3 (CIGB-SS, Cuba) conjugated to horseradish peroxidase, followed by 3-amino-9-ethyl carbazole substrate and hydrogen peroxide.

Viral isolation

For viral isolation, organs of vaccinated and control animals were taken at sacrifice, and blood samples at different post-challenge moments. One cubic centimeter of each organ was macerated in a mortar with 1 mL of DMEM medium. The macerated material was suspended in 4 mL of DMEM medium and allowed to stand for one hour at room temperature, then centrifuged for 15 min at 1200 g. The supernatant was collected and stored at -70 °C. Two successive passages of this supernatant were made in the PK15 cell line (ATTC CCL-33). After the last incubation, the cells were fixed with heat. The presence of CSFV antigens

in the cells was detected by incubation with the anti-E2 Mab CBSSE2.3 (CIGB-SS, Cuba) conjugated to horseradish peroxidase, followed by 3-amino-9-ethyl carbazole substrate and hydrogen peroxide.

Statistical analysis

The software GraphPad Prism v6.0 (GraphPad Software, Inc., La Jolla, USA) was used for statistical analysis. The geometric mean (GM) of the NAb titers and the confidence intervals of the GM were calculated for each vaccine batch and all vaccinated animals after one or two administrations of the vaccine. The Kolmogorov-Smirnov test and variance homogeneity determined normality by the Levene test. Mann-Whitney test was used to compare the NPLA titers between two experimental groups and the Kruskal-Wallis test, followed by the Dunn test, was applied to compare the GM of the NAb titers among several experimental groups.

Results and discussion

NAb response in Porvac® vaccinated animals

The 116 Porvac-vaccinated pigs seroconverted after the first administration of the vaccine (Figure 1). The GM of the NAb at day 21 after the first immunization was 1: 358.6, and the GM's lower and upper 95% confidence intervals (CI) were 302.3 and 425.4, respectively. The lowest NAb titer observed was 1:10 (in only one pig), and the highest was 1:1600.

A strong anamnestic response was observed after the second immunization (Figure 1). The GM of the NAb titers increased to 1: 5217, with CI of 4571 and 5954. Those NAb titers are significantly higher than those observed after day 21 after the first immunization (Mann-Whitney, p = 0.0001). The lowest individual NAb titer was 1:800, and the highest was 1:25600. Moreover, 78 % of the NAb titers were more increased than 1:3200.

The frequency distribution of the NAb titers at 21 and 28 dpv is presented in Figure 2. The marked differences between one and two doses of Porvac® are evident. From these results, and based on the published data that indicate that a NAb titer of 1:50 is sufficient to protect pigs from CSFV challenge[16-18], it is possible to predict that all animals should be protected after the challenge experiments.

Even after a single immunization, only one animal out of 116 showed NAb titers below the protective threshold. Therefore, it is very likely that the protective status of the animals can be achieved after only one dose in the 99.14% of vaccinated animals. This is an advantageous property for a vaccine that is intended to be applied in an endemic region where a rapid onset of immunity is needed. Other recombinant subunit vaccines have conferred only incomplete protection after one administration[18,24,25].

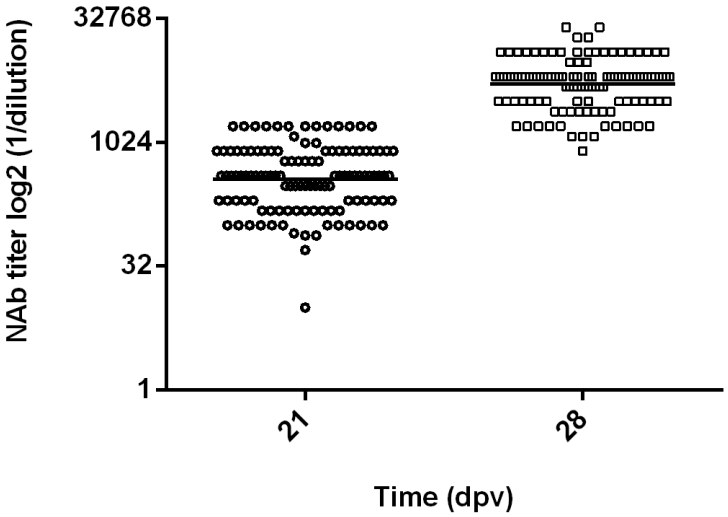

Figure 1. NAb titers in Porvac® vaccinated pigs. The X-axis scale is log2. Dpv: days post-vaccination. NAb was measured at 21 dpv (1 immunization) and 28 dpv (2 immunizations). n = 116. Horizontal lines represent the geometric mean of each group. Significant statistical differences were found between the two groups (Mann-Whitney, p = < 0.0001).

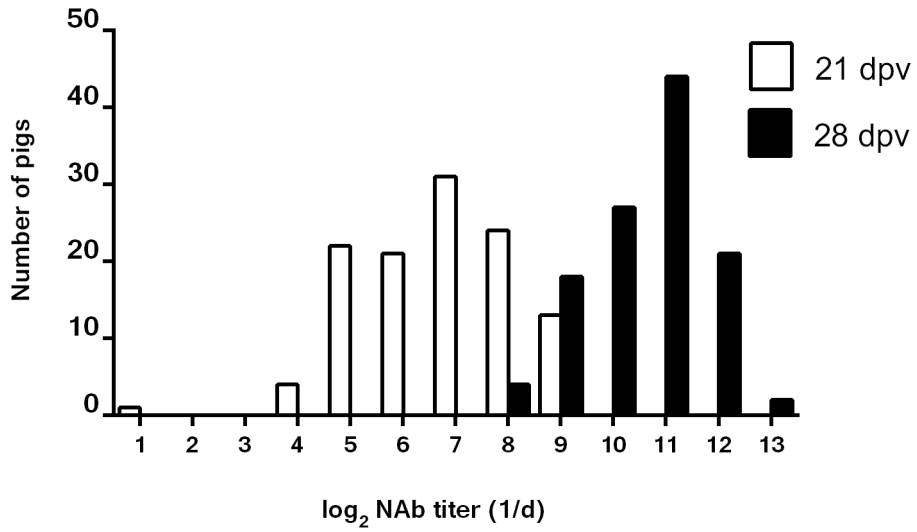

Figure 2. Frequency distribution of the NAb titers in pigs with 1 or 2 immunizations with Porvac®. Dpv: days post vaccination, 21 dpv (1 dose), 28 dpv (2 doses), n = 116.

The five batches of Porvac® studied induced similar levels of NAb

Figure 3 shows the NAb titers induced by the five batches of Porvac looked at 28 dpv. No statistically significant differences were observed among the five batches (Kruskal-Wallis, p = 0.4207). These results demonstrate the robustness and stability of the Porvac® production process.

All Porvac vaccinated pigs were protected against the CSFV challenge

All 22 independent challenge assays fulfilled the success criteria established for the test. All 116 vaccinated animals were protected from the viral challenge. They remain with good health status and appetite and without clinical signs of the disease. All pigs gain weight during the post-challenge period. Even the pig with the lowest NAb titer on the day of the challenge (1:800) was fully protected. The macroscopic examination of the main target organs conducted after the sacrifice at 21 dpc showed no pathological lesions in the vaccinated animals.

95 % of the vaccinated animals show typical temperature values after the challenge. The average temperature of the animals during the 21 dpc, did not exceed 39.6 ºC, within the physiological values for species[26] (Figure 4). Temperature values above 40 ºC were observed in 6 out of 116 animals in the study (5%) in punctual days after the challenge. However, the vaccines passed the test since the acceptance criterion established that temperature must be above 40 ºC for 3 consecutive days to be considered as relevant fever. Therefore, these transient temperature increases were supposed to be related to other punctual health issues in the animals.

Moreover, all samples from vaccinated animals analyzed in these studies were negative for viral isolation. These results correspond with o the animals' good health after the viral challenge and the examination of the target organs after the necropsy.

In contrast, control animals experience a rapid rise in the rectal temperature (Figure 4) and CSF-compatible clinical signs from 3 dpc. In this group, the temperature raised sustainably above 40 ºC up to values above 41.4 ºC. From six dpc, a marked deterioration in the animal health was observed (>10 points in the clinical score), therefore, all control animals were sacrificed at 8 dpc to avoid unnecessary suffering. The pigs suffered diarrhea, conjunctivitis, respiratory disorders, anorexia, ataxia, and prostration due to severe nervous symptoms. Figure 5 shows the time course of the clinical score in vaccinated and control animals after the viral challenge.

Moreover, CSF-compatible macroscopic lesions were also observed in the target organs. Histological examination of the immune system in unvaccinated animals exposed to the virus shows peripheral and local depression (lymphopenia in the spleen, mesenteric lymph nodes, and Peyer's patches in the ileum). An inflammatory reaction (diffuse and perivascular) in the central nervous system with the predominance of lymphocytes at the cortex level and meninges was observed in the control animals evaluated. These lesions corresponded with this group's clinical score and the disease's development.

Finally, and in agreement with the lesions found in the target organs, 100 % of blood samples and organs analyzed from control pigs were positive for viral isolation.

These findings confirm that the viral challenge was effective in all the independent experiments conducted and, therefore, the high NAb titers developed in Porvac® vaccinated pigs conferred full protection against a lethal CSFV challenge. NAb are capable of sequestering the virus outsi-

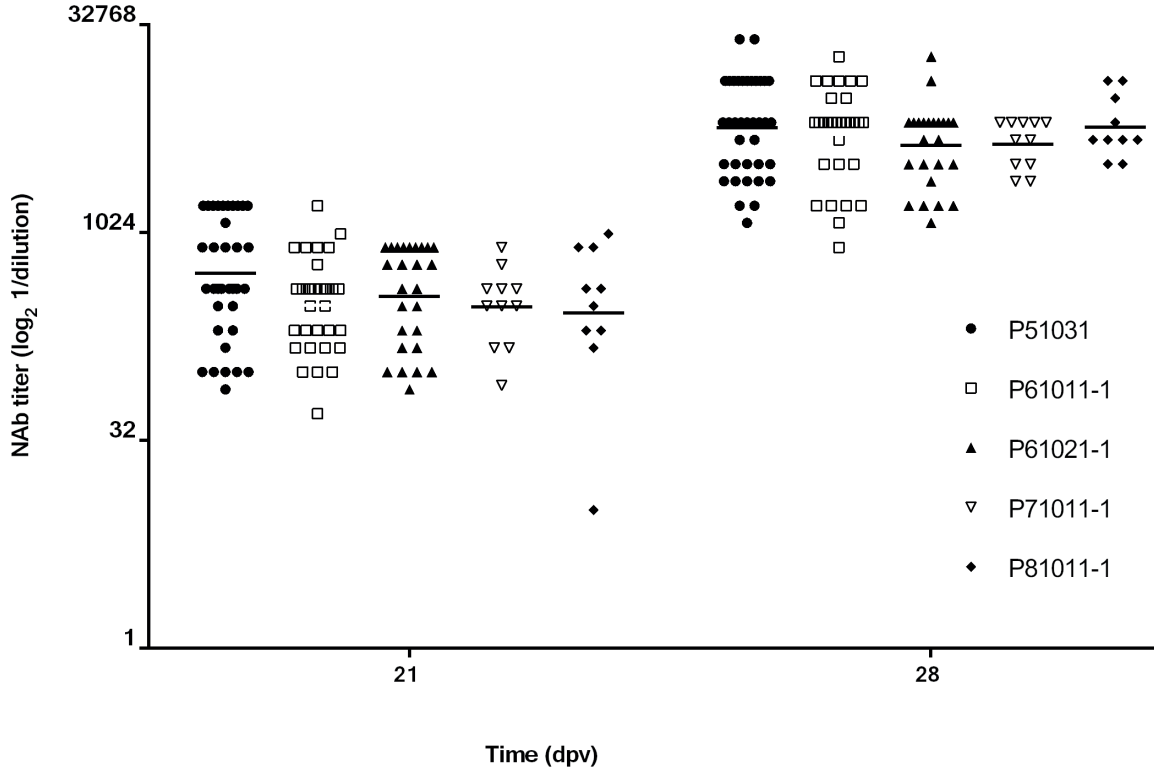

Figure 3. NAb titers induced by five production batches of Porvac. NAb titers were measured at 21 dpv (1 dose) and 28 dpv (2 doses). Horizontal lines represent the geometric mean of each group. Batches: P51031, n=37; P610111-1, n=32; P61021-1, n=26; P71011-1, n =11; P81011-1, n=10). No significant differences were found among the different batches after one or two immunizations (Kruskal-Wallis p>0.05).

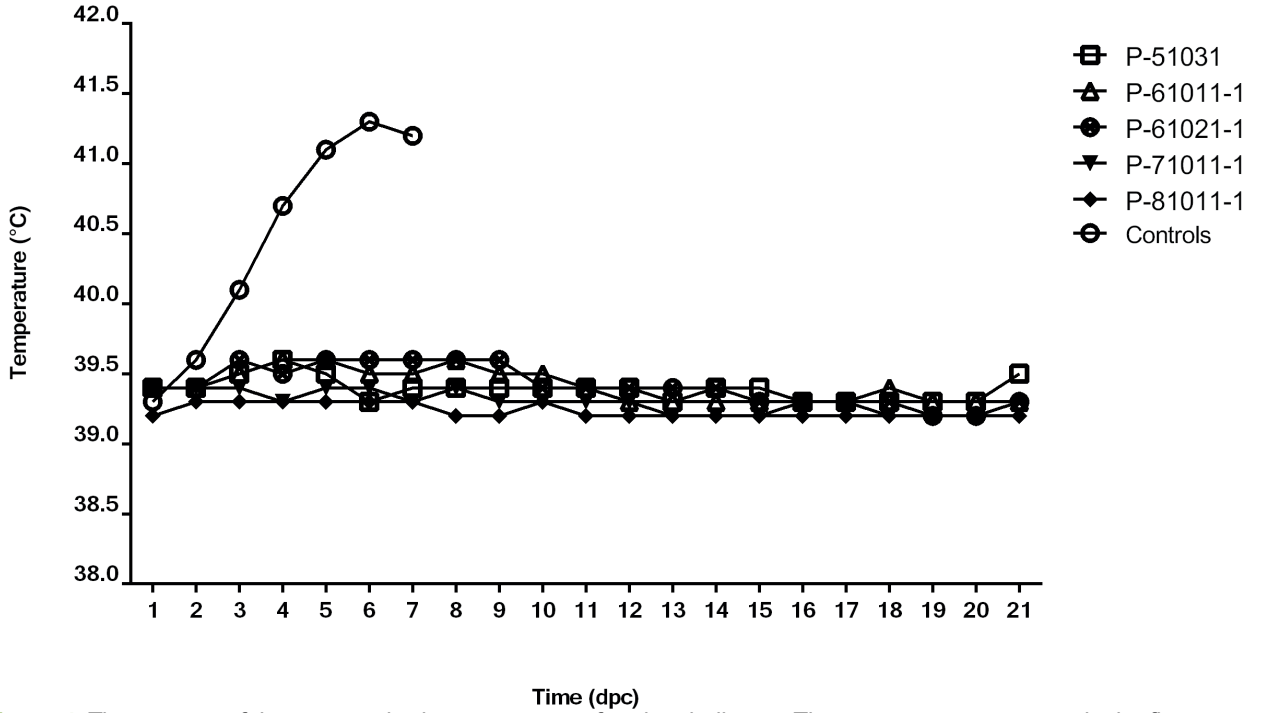

Figure 4. Time course of the average body temperature after the challenge. The average temperatures in the five groups of animals vaccinated with different batches of Porvac® and in control animals are represented. Vaccinated pigs were monitored during 21 dpc. All control pigs were euthanized at 8 dpc. dpc, days post challenge.

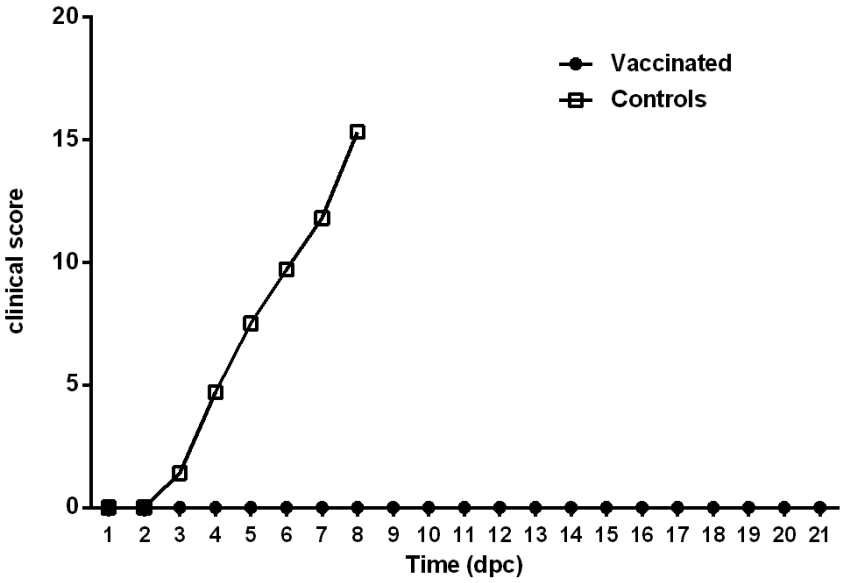

Figure 5. Time course of the clinical score after the challenge. The average clinical scores in vaccinated and control animals after the challenge are represented. Vaccinated pigs were monitored for 21 dpc. All control pigs were euthanized at 8 dpc. dpc, days post challenge.

de the target cells and arresting its multiplication.

Table 1 summarizes the results of the 22 challenge experiments conducted. There is an absolute correspondence between the presence of CSFV NAb in the serum of Porvac® vaccinated animals and protection against the viral challenge. Taking together, these results demonstrate for the first time that NAb elicited by immunization with Porvac® is a good correlate of protection against CSFV infection, as reported by other authors for MLV or other subunit vaccines in vaccinated/challenge animals.

Therefore, a potency test consisting of vaccinating pigs with Porvac® and measuring the NAb titers with the NPLA test can be proposed as a surrogate for the viral challenge experiment.

A NAb titer of 1:400 could be established as a cutoff value for approval of the production batches. This value is well above the 1:50 titer proposed by others as the cutoff for protection, implying a safety margin for a vaccine intended to be used in CSFV-endemic regions.

The following acceptance criteria would be considered for each production batch:

- All animals must develop NAb titers equal to or higher than 1: 400 after completing the vaccination schedule (21-day interval between the two doses)
- The geometric mean of the NAb titers of all vaccinated animals must be greater than 1: 1000.

By eliminating the viral challenge, the suffering of the animals will be reduced, as well as the risk of dissemination of the high-virulence strain used. The duration of the test and the costs will also be reduced.

Summary of the viral challenge potency tests					
Batch	Challenged animals	Protected animals	GM of NAb titers at 28 dpv	Lower 95% CI of GM	Upper 95% CI of GM
P51031	37	37	1: 5902	4523	7702
P61011-1	32	32	1: 5257	3964	6975
P61021-1	26	26	1: 4397	3341	5788
P71011-1	11	11	1: 4480	3410	4118
P81011-1	10	10	1: 5940	5886	8569
All vaccinated animals	116	116	1: 5217	4571	5954
Unvaccinated controls	38	0	< 1:5	-	-

NAb, neutralizing antibodies; dpv, days post-vaccination; GM, geometric mean; CI, confidence intervals of the geometric mean

Table 1. Summary of the challenge experiments with Porvac® vaccinated pigs.

Discussion

In Colombia, approximately 176,000 cultivated hectares benefit 52,000 families in 422 municipalities of 30 departments,

Monte Carlo Simulation Analysis (MCS)

After structuring costs, the most influential cost component was direct labor, representing 53% of the total cost. The cost of culture media was 12% of the total, IMC represented 5%, and operating expenses, including administrative expenses and infrastructure, were 30% (Figure 2).

Conclusions

In summary, a complete correspondence between the neutralizing antibody titer in the sera of Porvac© vaccinated pigs and protection from CSFV challenge have been demonstrated in a large set of experimental data. These results support substituting the viral challenge test for the neutralizing peroxidase-linked assay in the release of Porvac© batches. By eliminating the viral challenge, the suffering of the animals will be alleviated, as well as the risk of dissemination of the high-virulence strain used in those experiments. The duration of the test and the associated costs will also be reduced.

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Ethics

The Center for Genetic Engineering and Biotechnology ethics committee approved the study design.

Conflicts of Interest

The authors declare no conflicts of interest regarding the results presented in this article.

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