

## RESEARCH / INVESTIGACIÓN

# Safety and immunogenicity in piglets of two immunization schedules initiated at two or three weeks of age with Porvac<sup>®</sup>, a classical swine fever subunit marker vaccine

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**Abstract:** Classical swine fever is a highly contagious viral disease with a significant impact on food production worldwide. It currently represents one of the main limitations for the development of the pig industry in Cuba. Porvac<sup>®</sup> is a subunit marker vaccine that confers a very rapid onset of protection. Since there are different production systems in pig breeding, readjustments in the vaccination program are often required. This study compares the safety and efficacy in piglets of two vaccination schedules with Porvac<sup>®</sup> (0-2 weeks and 0-3 weeks), initiated at two or three weeks of age. Clinical monitoring was conducted, and a neutralization peroxidase-linked assay was used to measure the neutralization titers. All immunization regimens were safe and well-tolerated, without local or systemic adverse reactions in the vaccinated animals. Geometric mean neutralizing antibody titers higher than 1/1500 were detected in all groups during the six months of the trial. One month after the second immunization, piglets primed at two weeks of age, and boosted three weeks later, developed significantly higher neutralization titers (1/15644) compared to those vaccinated at a similar age but with a two-week interval between doses (1/5760). However, no significant differences in the titers were found three and six months after vaccination among the four regimens. In summary, all the variants studied are effective, but it is recommended to start vaccination at two weeks old, with the second dose at either two or three weeks later, depending on the production system and the purpose of the farm.

**Key words:** Classical swine fever, E2CD154, subunit vaccine, piglets, neutralizing antibodies.

## Introduction

Classical swine fever (CSF) is an infectious disease with the most significant economic impact on the swine industry in Cuba and many other countries. The disease is caused by the classical swine fever virus (CSFV), an enveloped, single-stranded RNA Pestivirus. Due to its devastating effects, CSF has been listed as a notifiable disease by the World Organization for Animal Health (OIE)<sup>1</sup>. In endemic areas, prophylactic vaccination is commonly used to limit the effects of the disease or as a first step within a general program to control and eradicate the virus. The modified live vaccines (MLV) are the most widely used in CSF endemic regions and have effectively controlled the disease in several countries such as Brazil, Argentina, and Uruguay.

On the other hand, Cuba is one CSF endemic country where the virus has been emerging for more than 20 years despite the use of an attenuated C strain MLV<sup>2-4</sup>. The lack of discrimination between infected and vaccinated animals, the requirement for a cold chain of distribution, and the interference of maternal-derived neutralizing antibodies (MDNA) are the main drawbacks of MLV, which have limited its use in disease-free regions. Several subunit vaccines have been developed to overcome these drawbacks, but they exhibit a late onset of protection as compared to MLV and provide insufficient protection against vertical transmission<sup>5-8</sup>.

Porvac<sup>®</sup> is a Cuban subunit marker vaccine against CSFV, which is based on a recombinant chimeric antigen 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, which is similar to the one described

for MLV<sup>9-11</sup>. Porvac<sup>®</sup> induces both high neutralizing antibodies (NAb) titers and cell-mediated immune response and can interfere with the transmission of the virus from pregnant sows to their offspring<sup>12</sup>.

Porvac<sup>®</sup> has been registered in Cuba and is being currently used in both, large state-owned pig production units and small private farms. Since there are different production systems in swine breeding, readjustments in the immunization schedules must make them compatible with those systems. The present study aimed to compare the safety and efficacy of two different immunization schedules with Porvac<sup>®</sup> (0-2 weeks and 0-3 weeks), starting at either two or three weeks after birth. These experiments must provide the scientific basis for a more flexible approach in terms of vaccination schedules for the different production systems.

## Methods

### Porvac<sup>®</sup> vaccine

The vaccine preparation was obtained from the stable HEK 293 cell line (ATCC CRL1573), which expressed the CSFV E2 antigen fused to the porcine CD154 protein. It was produced under Good Manufacturing Practices in a certified production area of the Center for Genetic Engineering of Camaguey, Cuba, and formulated as a water-in-oil formulation with Montanide ISA50V2 (SEPPIC, France) at a final concentration of 25 µg of E2-CD154 antigen/mL.

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## Experimental animals

The study included the litters from four unvaccinated CSFV-negative sows (Duroc x Yorkshire crossbred swine). The piglets were kept with their mothers until weaning, between 33 and 42 days, each group in a separate experimental room. Trials were carried out under appropriate high containment conditions following the animal welfare regulations and standards according to Good Clinical Practices<sup>13-15</sup>. The study was approved and supervised by the Committee for Care and Use of Laboratory Animals (CICUAL) of the Center for the Production of Laboratory Animals (CENPALAB).

The general clinical conditions of the animals were evaluated during the immunization period, especially the occurrence of adverse reactions at the injection site. The rectal temperature was measured immediately before the first vaccination and 72 hours after each vaccine administration. Blood samples were taken from the ophthalmic venous sinus in the absence of anticoagulant, previous to the first immunization (T0) and at one, three, and six months after the second immunization (T1, T3, and T6, respectively). Hematological and biochemical analyses were conducted at the analytical laboratory of CENPALAB. The levels of total protein, glucose, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, albumin, creatinine, uric acid, and urea were measured a Cobas Integra 400 PLUS Analyzer (Roche DiagnosticSystems).

## Vaccination

Four experimental groups (G2-2, G2-3, G3-2, and G3-3) were formed with complete litters comprising 9 and 12 piglets per group. Two groups included 2-week old piglets, and the other two were formed with 3-week old piglets on the day of prime vaccination. Each group was further subdivided to receive the vaccine with either two or three-week intervals between the priming and the booster. The intramuscular route inoculated a dose of 2 mL (50 µg of E2-CD154). The first immunization was performed on the right side of the neck and the second on the left side, using 18 x 1" needles, in agreement with the good veterinary clinical practices.

## CSFV-neutralizing antibodies detection

The serum samples were tested by Neutralization Peroxidase Linked Assay (NPLA) to determine the NAb titers against CSFV, following the methods described by OIE Manual<sup>16,17</sup>. National Center provided Margarita CSFV strain for Animal and Plant Health, Mayabeque, Cuba, and the anti E2 Mab CBS-SE2.3 conjugated to horseradish peroxidase by CIGB-Sancti Spiritus, Cuba.

## Data analysis

Data analysis was performed using package GraphPad Prism 6 (Prism 6 for Windows, Version 6.01, GraphPad Software, Inc., La Jolla, USA). The Kolmogorov-Smirnov test was used to evaluate normality, and the Levene test assessed the homogeneity of variances in the data. One-way ANOVA, followed by Tukey multiple comparison tests, was applied to compare the temperature values among groups. Kruskal-Wallis non-parametric test, followed by the Dunn test, was used to compare the geometric means (GM) of the antibody titers among the different groups. Statistical significance was considered when  $p < 0.05$ .

## Results

### Evaluation of the clinical signs during the 6 months follow-up

The animals exhibited good health conditions at the beginning of the trial. After vaccination, they showed no signs of inflammation, redness, or induration at the inoculation site. No systemic adverse effects or other alterations were observed, regardless of the age of the animals at vaccination and the vaccination schedule applied.

### Evaluation of the body temperature during the immunization period

The rectal temperature was monitored before the immunizations as a part of the assessment of the health status of the animals. Subsequent measures of the body temperature were done at 72 h after each immunization (Figure 1). Mean body temperature in all the groups remained within the physiological range, although some hyperthermia values were observed in G2-2 and G2-3 before vaccination and 72 h later. No significant differences were found between the immunization days and 72 h later; therefore, the high values of temperature registered for some individuals were not associated with vaccination. Animals remained healthy until the end of the trial.

### Evaluation of hematological and biochemical parameters

The hematological and biochemical parameters of the animals were evaluated before and three months after vaccination (tables 1 and 2). Although some variability was observed at the initial measurements at 15 or 21 days of age, no differences were found among the four groups three months after vaccination (ANOVA  $p < 0.05$ ). At that time all the values were within the normal ranges for the species.

### Immunogenicity of Porvac<sup>®</sup> in piglets

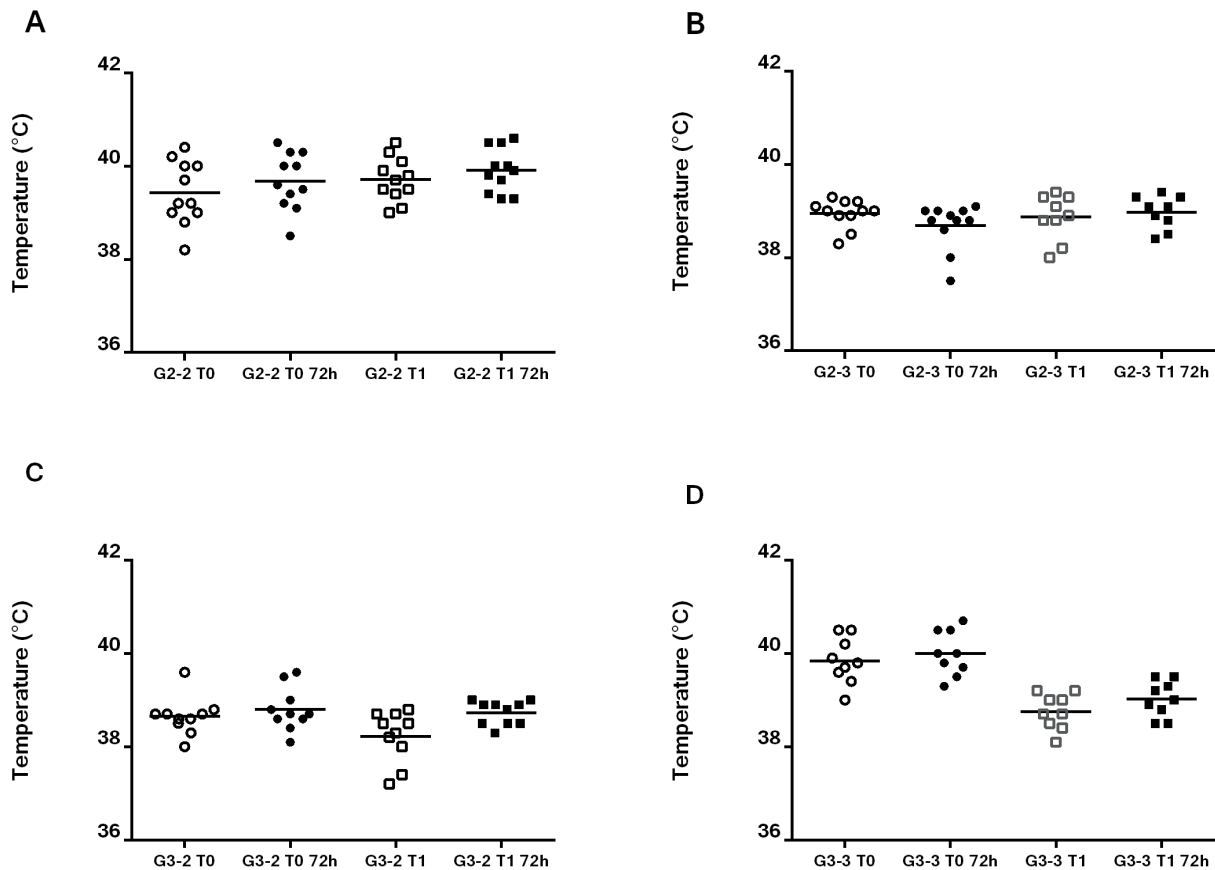
All piglets were CSFV seronegative at the beginning of the study, with NAb titers lower than 1/5. One month after receiving two immunizations, all animals developed NAb titers of 1/3200 or higher, regardless of the schedule applied (Figure 2). Statistical differences were documented among groups (Kruskal-Wallis,  $p < 0.05$ ). NAb titers were lower in group G2-2 (GM = 1/5552) as compared with groups G2-3 (GM = 1/12800) and G3-3 (GM = 1/8300) (Dunn test,  $p < 0.05$ ). No significant differences were found between the groups immunized at two and three weeks (G2.2 vs. G3.2 (GM = 1/11943) and G2.3 vs. G3.3).

In the evaluation conducted three months after the second immunization, all groups exhibited GM NAb titers above 1/9000, without significant differences among them (Kruskal-Wallis,  $p > 0.05$ ).

Six months after vaccination, the NAb titers had decreased as expected, although they remained equal to or higher than 1/400 in all animals. Finally, six randomly selected animals from the different schemes were followed up one year after vaccination. Interestingly, NAb titers of these pigs remained  $\geq 1/400$  at this time.

## Discussion

Vaccination is the most important activity within the CSF prevention program in endemic countries like Cuba since different pig farms use diverse production systems, the evaluation of different vaccination regimes with Porvac<sup>®</sup> is an important step to adjust vaccination to those production systems.



**Figure 1.** Evaluation of the rectal temperature before and after the vaccination.

In this study, we have evaluated the safety and immunogenicity of four immunization regimens with Porvac®. Two variables were explored: (1) age for the first administration and (2) time interval between the priming and booster doses.

The four regimens were safe and well-tolerated. Neither systemic nor local adverse reactions were observed nor temperature changes associated with vaccination. Those results confirm pre-registration studies in pigs of different ages with this vaccine<sup>10-12,18</sup>. They are also by those obtained in a pre-registration study with a CP7\_E2alf marker vaccine and animals of similar age<sup>19</sup>.

Concerning some elevated temperature values registered during the immunization period, the physiological temperature in pigs ranges between 38.7 and 39.7 °C, although this range could reach 40.2 °C in piglets during the first weeks after birth<sup>20</sup>. Our study was conducted between spring and summer with maximum values of ambient temperature above 30° C.

This environmental situation can lead to a condition called heat stress, which is characterized by modifying the physiological constants of the animals<sup>21</sup>. Therefore, the experimental manipulations in one of the most sensible pig's categories, and the heat stress could have influenced the temperature ranges observed.

The development of the innate immune system in pigs begins early during gestation. The newborn piglet already has a naive adaptive immune system with low blood levels of natural antibodies, which recognize the most common pathogens<sup>22,23</sup>. Piglets achieve full maturity in their immune system after four weeks of life, which guarantees an adequate immune response to vaccines<sup>24</sup>. Previous studies conducted with other vaccines have found that five-week-old piglets developed a superior NAb response compared to those immunized two weeks earlier<sup>24</sup>.

However, in the present study, even two-week-old naïve

	Time (months)	G2-2	G2-3	G3-2	G3-3
Leukocytes (10 <sup>9</sup> cells/L)	0	10.8 ± 1.6	11.3 ± 3.1	13.1 ± 3.1	9.0 ± 2.6
	3	16.0 ± 1.8	16.9 ± 2.7	13.9 ± 2.6	15.7 ± 1.6
Neutrophils (%)	0	19.4 ± 5.9	29.5 ± 7.6	38.4 ± 11.1	23.9 ± 5.3
	3	21.1 ± 9.4	30.3 ± 10.5	33.5 ± 8.7	31.1 ± 13.6
Lymphocytes (%)	0	80.7 ± 6.0	69.5 ± 7.6	60.9 ± 11.5	75.1 ± 6.2
	3	78.0 ± 9.8	68.9 ± 10.7	64.4 ± 8.1	68.0 ± 13.7
Monocytes (%)	0	0.55 ± 0.7	0.64 ± 0.7	0.44 ± 0.73	0.68 ± 13.7
	3	0.50 ± 0.8	0.43 ± 0.5	0.5 ± 0.53	0.3 ± 0.5
Eosinophils (%)	0	0.27 ± 0.5	0.36 ± 0.5	0.22 ± 0.44	0.7 ± 0.9
	3	0.38 ± 0.5	0.43 ± 0.5	1.63 ± 1.6	0.9 ± 0.9
Basophils (%)	0	0	0	0	0
	3	0	0	0	0

**Table 1.** Hematological parameters of the animals before and 3 months after vaccination.

Biochemical Parameters	Time (months)	G2-2	G2-3	G3-2	G3-3
Glucose (mmol/L)	0	8.9 ± 1.31	7.1 ± 0.8	7.3 ± 1.8	6.9 ± 1.5
	3	6.0 ± 0.5	5.0 ± 1.0	6.5 ± 1.9	5.2 ± 1.0
Albumin (g/L)	0	38.3 ± 2.6	34.3 ± 3.73	36.5 ± 5.7	32.8 ± 8.7
	3	38.3 ± 2.6	30.7 ± 1.4	38.3 ± 4.7	33.9 ± 4.4
Total protein g/L	0	50.6 ± 3.0	52.1 ± 3.1	50.9 ± 7.8	44.0 ± 10.7
	3	55.6 ± 6.6	55.1 ± 11.0	66.0 ± 5.0	55.9 ± 5.1
Urea (mmol/L)	0	2.4 ± 0.7	3.7 ± 1.44	4.2 ± 1.7	5.2 ± 6.0
	3	7.7 ± 1.4	7.7 ± 1.3	9.5 ± 0.8	6.0 ± 1.5
Creatinine (µmol/L)	0	79.0 ± 6.6	82.2 ± 13.8	91.1 ± 12.5	80.8 ± 30.3
	3	92.1 ± 11.9	103.4 ± 14.0	98.4 ± 20.5	89.9 ± 9.7
Uric acid (µmol/L)	0	4.0 ± 1.6	2.2 ± 1.32	1.7 ± 2.4	3.0 ± 5.6
	3	1.25 ± 1.4	0.63 ± 1.4	0.4 ± 0.5	0.4 ± 0.5
Aspartate aminotransferase (UI/L)	0	59.2 ± 6.7	34.8 ± 10.6	61.1 ± 27.5	50.4 ± 34.2
	3	38.5 ± 5.7	44.1 ± 10.2	66.0 ± 41.2	51.7 ± 17.0
Alkaline phosphatase (UI/L)	0	1013.6 ± 182	617.2 ± 236	290.9 ± 46.1	316.1 ± 139
	3	129.8 ± 26.8	129.6 ± 20.1	125.6 ± 18.4	109.7 ± 20.0
Alanine aminotransferase (UI/L)	0	34.5 ± 4.5	28.2 ± 7.1	41.0 ± 9.9	31.1 ± 12.3
	3	63.3 ± 11.7	55.5 ± 9.0	54.4 ± 4.1	63.8 ± 14.5

Table 2. Biochemical parameters of the animals before and 3 months after vaccination.

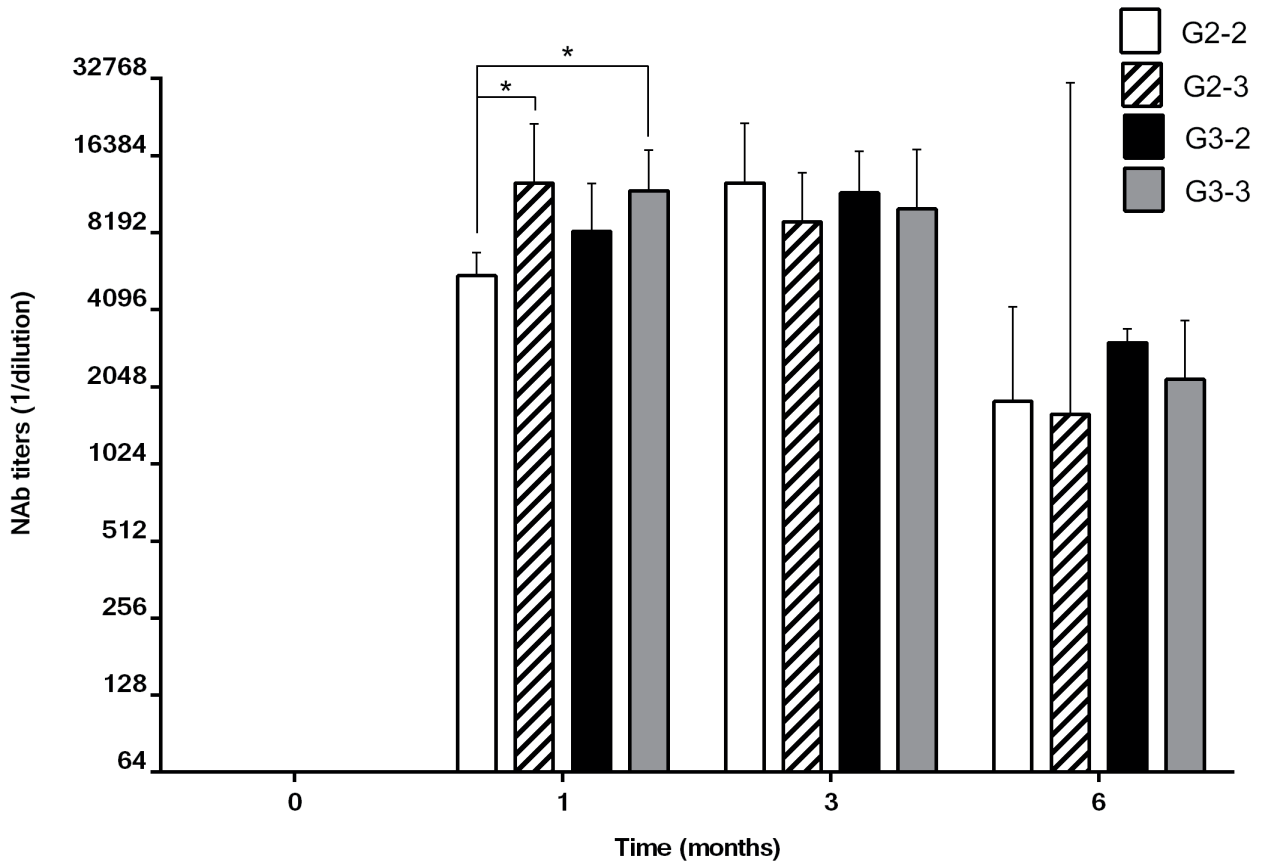


Figure 2. CSFV NAb titers in all experimental groups during the six months follow-up study.



piglets could induce a potent NAb response against CSFV after vaccination with Porvac<sup>®</sup>. Similar levels of NAb were measured in those animals, at any of the time points evaluated, compared with the group immunized at the age of three weeks.

Additionally, the findings suggest that a three-week interval between immunizations is more effective than two weeks to induce NAb, although these results were found only one month after vaccination. Those differences faded over time and were found neither at 3 nor at 6 months after vaccination.

Even so, it should be noted that, from a practical point of view, the differences above are irrelevant since the NAb titers elicited by the four regimes studied were manifold higher than the protective threshold of 1/50, previously defined by other investigators<sup>25,26</sup>.

The high immunogenicity of Porvac<sup>®</sup>, even at this very early age, is probably due to the action of the CD154 protein (CD40L), which functions as a molecular adjuvant. This molecule is directly involved in the activation and maturation of B lymphocytes, which occupy a central role in the immune response, functioning as precursors of antibody-secreting cells and effective antigen-presenting cells (APC). CD154 also interacts with professional APC such as dendritic cells, promoting their maturation and activation<sup>27,28</sup>.

Finally, those results confirm previous findings that Porvac<sup>®</sup> can promote a long-lasting response, which is welcome for breeding gilts and boars, although immune protection for 7–8 months is sufficient for growing and fattening pigs<sup>29</sup>. In the present study, when the vaccinated animals arrived at reproductive age still exhibited protective NAb levels against CSFV, demonstrating that a long-lasting humoral immune response can also be generated by immunizing two-week-old naïve piglets Porvac<sup>®</sup>. This agrees with the results of pre-registration controlled trials conducted by Suárez *et al.*, 2011<sup>11</sup>, where protective NAb titers were still detected at 9 months post-vaccination.

This study was conducted in naïve animals born to unvaccinated sows; therefore, they did not have MDNA at vaccination. It is well known that MDNA can interfere with active immunity, mainly described for MLVs<sup>24,30,31</sup>. However, previous studies have already demonstrated that pre-existing MDNA does not interfere with the immunogenicity of Porvac<sup>®</sup><sup>18</sup>. Therefore, it is very likely that the results shown here could be safely extrapolated to those piglets born to Porvac<sup>®</sup> vaccinated sows.

## Conclusions

A three-week interval between doses induced higher NAb titers than a 2-week interval in piglets vaccinated with 2 weeks of age. Those differences were only observed at 1 month, but not at 3 and 6 months after vaccination.

Regarding the time of the prime vaccination, no differences in the NAb response were found between 2 and 3 weeks of age.

The four immunization schedules evaluated in this study induced high titers of NAbs in naïve piglets, well above the protective threshold; therefore any of them could be used in pig production units, harmonized with their respective productive systems. As a general recommendation, all producers could apply the first dose of Porvac<sup>®</sup> at two weeks of age. The animals born in the genetic farms and reproductive centers would receive the second dose two weeks later. The rest of the farms, including medium and small-scale producers, may apply the second dose with either two or three week's interval depending on their production systems.

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