ORIGINAL ARTICLE

Safety and immunogenicity in piglets of the vaccine candidate E2-CD154, a subunit vaccine against classical swine fever. Results from phase III clinical trial

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Seguridad e inmunogenicidad en lechones con el candidato vacunal E2-CD154, una vacuna de subunidades contra la peste porcina clásica. Resultados del ensayo clínico de fase III

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ABSTRACT: E2-CD154 is a subunit vaccine candidate that has been proven to be safe and to protect piglets from classical swine fever (CSF). In this study, those previous findings were confirmed and extended to a larger number of animals in a phase III clinical trial conducted on two production farms in Pinar del Río province. All animals in both farms were vaccinated with two doses of E2-CD154 on days 0 and 21. The study extended up to 60 weeks. The vaccine was well tolerated in piglets between 15 and 28 days of life, with neither local nor systemic side effects documented. Immunized pregnant sows were capable of transmitting high levels of maternally-derived neutralizing antibodies (MDNAs) to their offspring (Unit A, geometric mean titer = 1:1295, minimum value 1:100 and Unit B geometric mean titer = 1:474, minimum value 1:150), well above the protection threshold (1:50). These high MDNA titers in the piglets did not interfere with the immunogenicity of the candidate. All vaccinated piglets evaluated at random (more than 10% of 2804 vaccinated) developed protective neutralizing antibody titers higher than 1:400 at the four time points analyzed (nine, 21, 41, and 44 weeks) in both farms. The results of this study confirm the safety, immunogenicity and robustness of this vaccine candidate in this sensitive pig category in the field.

Keywords: Subunit vaccine, classical swine fever, maternally-derived neutralizing antibodies (MDNAs), production farm, neutralizing antibodies, phase III clinical trial.

RESUMEN: El candidato vacunal E2-CD154 es una vacuna de subunidad cuya seguridad y capacidad protectora en crías frente a la peste porcina clásica ha sido demostrada en estudios previos. Esos hallazgos se confirman en el presente estudio de fase III realizado en dos unidades de producción porcina en la provincia de Pinar del Rio con un número mayor de animales. Todos los animales en ambas granjas recibieron dos dosis de E2-CD154 los días 0 y 21. El estudio se extendió por 60 semanas, la vacuna fue bien tolerada en las crías vacunadas entre los 15 y 28 días de edad, no se documentaron efectos adversos locales o sistémicos. Las cerdas gestantes inmunizadas fueron capaces de trasmitir altos títulos de anticuerpos a sus crías (Unidad A, media geométrica = 1:1295, valor mínimo 1:100 y Unidad B, media geométrica = 1:474, valor mínimo 1:150), muy por encima del umbral de protección (1:50). Estos elevados títulos de anticuerpos neutralizantes derivados de la madre no interfirieron con la inmunogenicidad del candidato vacunal en estudio. Todas las crías vacunadas estudiadas al azar (más del 10% de las 2804) desarrollaron títulos de anticuerpos neutralizantes mayores de 1:400 en los cuatro muestreos realizados (nueve, 21, 41, y 44 semanas) en ambas granjas. Los resultados de este estudio confirman la seguridad, inmunogenicidad y robustez del candidato vacunal en esta sensible categoría en condiciones de campo.

Palabras clave: Vacuna de subunidad, peste porcina clásica, anticuerpos neutralizantes derivados de la madre, unidad de producción, anticuerpos neutralizantes, estudio clínico fase III.

INTRODUCTION

Classical swine fever (CSF) is one of the more severe diseases of pigs, responsible for significant economic losses in endemic countries (1). The etiological agent of CSF is the CSF virus (CSFV), an enveloped single-stranded RNA pestivirus. Due to the elevated pathogenicity and morbidity, CSF declaration has been established as mandatory by the World Organization for Animal Health (WOAH, former OIE) and the principal policies used to control the disease are stamping out and preventive vaccination (2). Stamping out leads

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to the sacrifice of many uninfected swine, which has a very negative economic impact, and it is highly problematic from an ethical point of view (3, 4).

On the other hand, Modified Live Vaccines (MLVs) confer an effective and rapid onset of protection against CSFV, therefore they have been used in many countries as one of the main tools to control the transmission of CSFV (5). Yet, these MLVs do not permit the differentiation between naturally infected and vaccinated animals, and are extremely sensitive to temperature changes; therefore, they require a strict cold chain of distribution, which often fails in underdeveloped countries (6). A third limitation of MLVs is the risk of reversion of the virulence of vaccine strain, an issue that has been described in several countries (7-10). Due to this associated risk, the European Union prohibited the use of MLVs.

To avoid those issues connected to MLVs, our group has developed a CSFV subunit vaccine candidate based on the chimeric recombinant protein E2-CD154 formulated with Montanide™ ISA50V2. The CD154 protein functions as a molecular adjuvant to potentiate both the humoral and cellular branches of the immune response against the E2 viral glycoprotein. E2-CD154 conferred unusually rapid protection in swine against a highly virulent CSFV strain (11, 12). This onset of protection is comparable to the one provided by MLVs. Additionally, E2-CD154 prevented trans-placental infection in six pregnant sows (13).

In other studies conducted in controlled installations, E2-CD154 vaccinated sows were capable of transmitting high titers of maternally-derived neutralizing antibodies (MDNAs) to their offspring, and those MDNAs were protective against a lethal viral challenge (14-16). Moreover, it has been demonstrated that MDNAs do not interfere with the immune response of piglets to the vaccine (14).

However, the evaluation of the safety and immunogenicity of vaccine candidates in the field is a crucial step for the approval of the vaccine registry. The manipulation of large numbers of animals in remote locations is a challenging test for any vaccine candidate. The present study aims to present partial results from a phase III clinical trial with E2-CD154, manufactured under the Good Manufacturing Practice (GMP) facilities. The study was conducted on two large production farms according to VICH regulations, (17). The presence of MDNAs in the blood of piglets born from vaccinated sows, and the safety and immunogenicity of the vaccine candidate in piglets will be examined.

MATERIALS AND METHODS

Clinical sites

The study was conducted in two pig farms with closed production cycles (from newborn piglets to finishers). The first farm selected (Unit A) is located in the north of Los Palacios municipality, “Pinar del Río” province, and had a total of 2510 pigs. The second farm (Unit B) is located in the south of the same municipality and had 1232 pigs. Both units had no record of CSFV outbreaks during the three previous years, had good biosafety conditions, and pigs are grouped by age and weight in an all-in, all-out system. Both units also kept good tracking and documentation of their animals and the production and biological indicators.

Animals

All animals were Duroc x Yorkshire crossbreed swine. The pigs were grouped in rooms according to the animal welfare regulations and standards established in the Manual of Technical Procedures for Pig Farming (18). Pigs' rooms were cleaned daily.

Vaccine candidate

The generation of a transformed HEK 293 cell line (ATCC CRL1573) stably secreting the E2-CD154 antigen has been described elsewhere (11). The two vaccine lots used in this study were manufactured under GMP conditions at the production unit CIGB of Camaguey, Cuba. The stably transformed HEK 293 cells were cultured in a 10 L fermentor (BIOSTAT B Plus, Göttingen, Germany). The culture supernatant was filtered by a 0.2 µm capsule (Sartorius, Göttingen, Germany) and concentrated by a tangential ultrafiltration technology using a 100 kDa PESU cassette (Sartorius, Göttingen, Germany). Next, samples were dialyzed and re-filtered with a 0.2 µm capsule (Sartorius, Göttingen, Germany). The SDS-PAGE purity degree estimated for the E2-CD154 chimeric subunit protein was ≥80%. The E2-CD154 chimeric protein was emulsified with Montanide™ ISA 50V2 (SEPPIC-Castres, France). All vaccine vials were kept at 2-8 ˚C until the moment of application.

Vaccination

All animals in the farms, from 15 days piglets to the breeding stock, were vaccinated at time 0 of the study in a single day with the vaccine candidate by the intramuscular route in the neck. The booster was given 21 days later. At time 0 of the study, weaning and fattening pigs had been previously vaccinated with an MLV (Labiofam, Cuba). All piglets were sampled before vaccination to evaluate the initial level of maternally-derived neutralizing antibodies (MDNAs). The breeding stock was revaccinated after 6 months. All piglets born in the units during the length of the study (60 weeks) were vaccinated between 15 and 28 days of life. The presence of adverse effects at the inoculation site was monitored by clinical observation (visual and palpation of the inoculation site).

Sample collection

Animals fasted from 12-18 h before blood extraction. Blood samples were taken through ophthalmic venous sinus punctures. For serological analysis, blood was collected in sterile tubes without anti-coagulant. Tubes were incubated for 2 h at room temperature and kept overnight at 2-8 °C. They were then centrifuged for 10 min at 5000 x g, and sera were preserved at -20 °C until their use. For hematological analysis, blood was collected in 1.5 mL vials containing EDTA and kept at 4°C until used.

Serum samples from non-vaccinated piglets were taken at weeks 0, 26, and 41 of the study, to monitor the levels of maternally-derived neutralizing antibodies. The number of samples analyzed corresponded to the 11 %, 22 % and 14 % respectively of the total number of piglets in "Unit A", and the 12 %, 22 %, and 17 % in "Unit B".

Serum samples from vaccinated weaners were taken at weeks 0, nine, 26, 41, and 44. The number of samples collected represents 16 %, 25 %, 28 %, 20%, and 15% of the total number of animals in this section in "Unit A", and the 25 %, 23 %, 16 %, 31%, and 100% of the total in "Unit B". Weaners at day 0 had been vaccinated with the MLV. In the rest of the weeks, the samples were taken from weaners vaccinated at 15-23 days of age with E2-CD154.

Hematological analysis

The following cells were counted in the optical microscope (Olympus, Japan): thrombocytes, total leucocytes (TL), neutrophils (N), lymphocytes (L), eosinophils (E), and monocytes (M). Hematocrit (HTC) and hemoglobin (HB) levels were determined in the microhematocrit centrifuge.

Biochemical analysis

The following parameters were determined in an Automatic Analyzer Cobas Integra 400 PLUS (Roche Diagnostic Systems): alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), albumin (ALB), creatinine (CREA), total bilirubin (BIL-T), direct bilirubin (BIL-D), glucose (GLUC), cholesterol (CHOL), triglycerides (TG), Urea, uric acid (AU), gamma glutamyl transferase (GGT), cholinesterase (CHE), calcium (Ca), and phosphorus (Pho).

Neutralizing antibody titer determination

Serum samples were screened for their capacity to neutralize "Margarita" CSFV strain using the neutralizing peroxidase-linked antibody (NPLA) assay as described elsewhere (19). The labeled antibody used was the anti E2 monoclonal antibody CBSSE2.3 generated at the Center for Genetic Engineering and Biotechnology of Sancti Spíritus, Cuba, conjugated to horseradish peroxidase (SIGMA, St. Louis, Missouri, USA), followed by amino-9- ethylcarbazole substrate and hydrogen peroxide (SIGMA, St. Louis, Missouri, USA). Titers were expressed as the inverse of the higher dilution of serum that neutralized 100 $TCID_{50}$ of "Margarita" strain in the 50% of replicates

Statistical analysis

The normal distribution of the data was assessed by D'Agostino-Pearson tests. Kruskal-Wallis and Dunn's Multiple Comparison tests were applied to compare NAb titers among the groups. Chi-square test was used to compare mortality rates over years. Statistical significance was considered when $p < 0.05$. The statistical package GraphPad Prism 6 was used for all of the analysis (Prism 6 for Windows, Version 6.07, GraphPad Software, Inc., La Jolla, CA, USA).

Ethics statement

The experiments were conducted following the animal welfare regulations and the standards of the European Union (Directive 2010/63/EU). The Ethic Committee for Animal Breeding and Care of CENPALAB (Mayabeque, Cuba) approved and supervised the protocols.

RESULTS AND DISCUSSION

Maternally-derived neutralizing antibodies in the offspring of E2-CD154® vaccinated sows

The presence of MDNAs in the serum of non-vaccinated piglets was assessed at different time points of the study. On both farms, week 0 piglets born to MLV-vaccinated sows had significantly lower MDNA titers than piglets studied at weeks 26 and 41, which were born to sows vaccinated with the E2-CD154 antigen (Kruskal Wallis/Dunn tests, $p<0.01$). There were no statistical differences between farms at weeks 0 and 21, but MDNA titers at week 41 were higher in "Unit A" than in "Unit B".

Only half of the offspring of MLV vaccinated sows exhibit protective NAb titers higher than 1: 50, which is generally accepted as the threshold of protection (20-22). In contrast, all piglets born from vaccinated sows have NAb titers higher than 1:100 and the geometric mean of NAb titers of the group is higher than 1:400, above the protective threshold.

The MDNA titers measured in this study were lower than those described in previous controlled studies in 15 days old piglets born to E2CD154 vaccinated sows. In that study, MDNA titers remained higher than 1:1000 until day 63 (14). However, other reports described very similar MDNA titers with geometric means of 1:5000 at 15 days of age in piglets born from sows vaccinated with E2-CD154 or other E2 subunit vaccines (16, 23). In one of those studies, piglets born

from E2-CD154 vaccinated sows, challenged at day 63 with NAb titers of only 1:100, were protected from a lethal viral challenge (16).

Those findings confirmed previous results from Phase I and II studies conducted under controlled conditions. Large-scale vaccination of pregnant sows with the candidate vaccine E2-CD154 on a production farm induced elevated NAb titers that were passively transferred to the offspring during lactation, and those NAbs kept the piglets protected against CSFV infection during the first weeks of life and until vaccination.

Safety of vaccine candidate E2-CD154 in piglets

Local or systemic adverse effects

No local or systemic adverse effects were documented in the 2804 piglets vaccinated in both farms (1899 in the "Unit A" and 905 in "Unit B"). These results confirm and extend the safety studies of vaccine candidate E2-CD154 with a large number of piglets and in two production units. Additionally, the presence of a self-antigen (CD154) in the formulation did not cause adverse reactions of any kind in the short term. Long-term follow-up of vaccinated breeders is necessary to assess the potential adverse effects of this vaccine in the long term.

Although some level of mild local adverse reactions has been reported with subunit vaccines (21, 24), the new generation of subunit vaccines has a better safety profile than MLV (25). The use of MLV is contraindicated during pregnancy, as it poses a high risk of miscarriage or stillbirth to fetuses (26).

Mortality

The administration of the vaccine candidate started in March 2016. No CSF outbreaks were reported in the two units during the study. No significant differences were found in the mortality rate among the three years studied in piglets (Fig 2A) or weaners (Fig 2B) (Chi-square >0.05). Those results indicate that vaccination with this candidate did not affect mortality in the two units within these categories.

Hematological and biochemical parameters

All hematological parameters measured in vaccinated piglets were within the reference range for the species (table 1).

Table 2 shows the average values and standard deviation of the biochemical variables measured in the blood of vaccinated piglets. It is known that many factors such as diet, age, analytical method, climate, microbiological conditions, and altitude, among others, strongly influence these parameters. However, all the values remained within the normal ranges described for this species by different authors. This indicates that this vaccine candidate does not affect the main biochemical health indicators in the piglets.

Immunogenicity of E2-CD154 in piglets under production conditions

At day 0 of the study, weaning pigs had been vaccinated with MLVs. Those groups of animals in both farms exhibited very low NAb titers. The geometric mean of those titers was lower than the theoretical protection threshold of 1:50. In contrast, all animals immunized with E2-CD154 vaccine candidate at the different time points of the study developed a potent NAb response with the geometric means higher than 1:1000 and more than twenty-fold higher than the protection threshold.

There were some fluctuations in the NAb titers on the different sampling days. In “Unit B”, NAb titers at 41 weeks were significantly lower than at nine weeks,

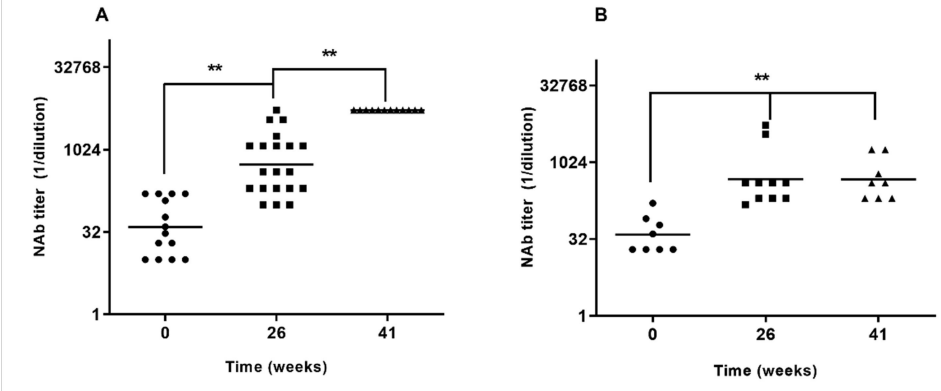

Figure. 1. Maternally-derived neutralizing antibody titers in non-vaccinated piglets at different time points of the study. A: “Unit A”, B: “Unit B”. Piglets at week 0 were born from MLV vaccinated sows; piglets at weeks 26 and 41 were born from E2-CD154 antigen vaccinated sows. ** Kruskal Wallis test and Dunn's Multiple Comparison tests, *p<0.01*. / *Títulos de anticuerpos neutralizantes de origen materno en lechones no vacunados en diferentes momentos del estudio. A: "Unidad A", B: "Unidad B". Los lechones de la semana 0 nacieron de cerdas vacunadas con MLV; los lechones de las semanas 26 y 41 nacieron de cerdas vacunadas con antígeno E2-CD154. ** Prueba de Kruskal Wallis y pruebas de comparación múltiple de Dunn, p<0,01.*

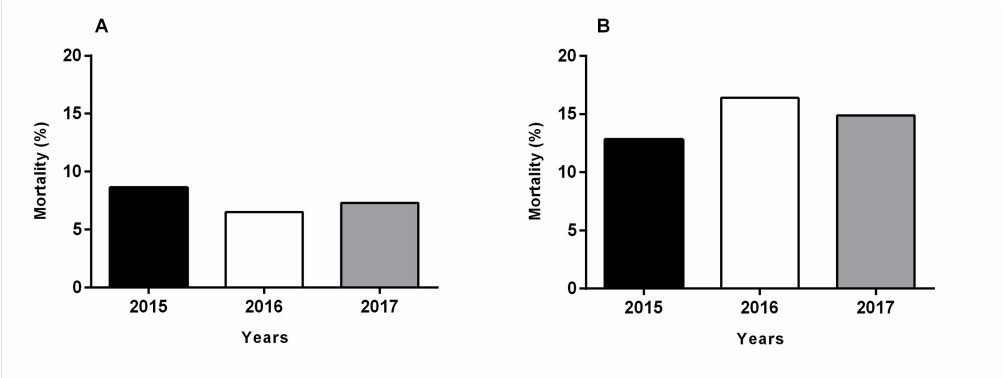

Figure 2. Mortality rate in the three years of the study. A: Mortality in piglets; B: Mortality in weaners. Mortality is expressed as the percent of deaths of the total number of animals in each category in both farms. No statistical differences among the three years studied were found (Chi-square > 0.05). / *Tasa de mortalidad en los tres años del estudio. A: mortalidad en lechones; B: mortalidad en destetados. La mortalidad se expresa como el porcentaje de muertes sobre el total de animales de cada categoría en ambas explotaciones. No se encontraron diferencias estadísticas entre los tres años estudiados (Chi-cuadrado > 0,05).*

Table 1. Hematological parameters in vaccinated piglets**.** / *Parámetros hematológicos en lechones vacunados*.

Parameter	E2-CD154 vaccinated piglets n=16	Referential values (27)
Hemoglobin (g/dl)	11.09 ± 2.3	10.0-16.0
Hematocrit (%)	33.94 ± 6.4	32.0-50.0
Thrombocytes (x10^9/L)	309.81 ± 57.6	320-520
Leukocytes (x10^9/L)	13.20 ± 2.2	11.0-22.0
Neutrophils (x10^9/L)	3.83 ± 1.8	3.1-10.5
Lymphocytes (x10^9/L)	9.10 ± 1.9	4.3-13.0
Monocytes (x10^9/L)	0.81 ± 1.1	0.2-2.2
Eosinophils (x10^9/L)	1.25 ± 1.2	0.05-2.4

Table 2. Biochemical parameters in vaccinated piglets. / *Parámetros bioquímicos en lechones vacunados*.

Parameter	Non-Vaccinated piglets n=12	Vaccinated piglets n=28	Referential values
Creatinine (µmol/L)	81.5 ± 52.9	88.3 ± 41,2	36.0 - 240 (22, 23)
Glucose (mmol/dL)	5.3 ± 0.3	4.4 ± 0.2*	3.5 -7.5 (22, 24)
ASAT (IU/L)	72.1 ± 47	55.4 ± 3.9*,	10-84 (22, 24)
ALAT(IU/L)	31.83 ± 4.6	40.79 ± 16	10-45 (24, 25)
Albumin (g/L)	29.2 ± 7.6	38.14 ± 4.3*	19-39 g/L (26)
Total bilirubin (mg/dL)	5.8 ± 3.4	1.67 ± 0.8*	0-10 (22)
Direct bilirubin (µmol/L)	2.6 ± 1.3	1.03 ± 0.5*	0-5.1 (22)
GGT (units/L)	5.08 ± 2.5	17.52 ± 8.7**	10-60 (22)
Cholesterol (mmol/L)	3.24 ± 1	2.82 ± 0.8	3.05-3.10 (22, 26)
Urea (mmol/L)	3.84 ± 1.3	5.37 ± 1.7**	3 -8.5 (22)
Triglycerides (mmol/L)	1.10 ± 0.6	1.04 ± 0.5	0.26-0.64 (26)
Calcium (mmol/L)	2.21 ± 0.2	2.50 ± 0.2	1.78-2.90 (22)
Phosphorous (mmol/L)	3.02 ± 0.4	3.20 ± 0.5	1.30-3.55 (22)

Values represent the arithmetic mean ± standard deviation from 28 piglets randomly selected from both farms at different times. *$p<0.05$; ** $p<0.01$; ASAT: aspartate aminotransferase; ALAT: alanine aminotransferase; GGT: gamma glutamyl transferase.

but the differences between NAb titers at 41 weeks and the rest of the time points were not statistically significant. On the other hand, in "Unit A", NAb titers at week 41 were lower than the rest of the time points. However, in both cases, NAb titers were more than 20 times higher than the theoretical protective threshold of 1:50.

Another important fact is that the piglets received the first dose of vaccine between 15 and 28 days of life, when circulating MDA levels were high, as shown in the previous section. Nevertheless, all piglets were able to develop a strong NAb response against E2-CD154.This lack of interference of MDA on the immunogenicity of the E2-CD154 antigen corroborates previous findings from controlled studies and confirms the advantage of subunit vaccines over MLV in this particular subject. Administration of MLV before six weeks of age is the most likely cause of vaccine

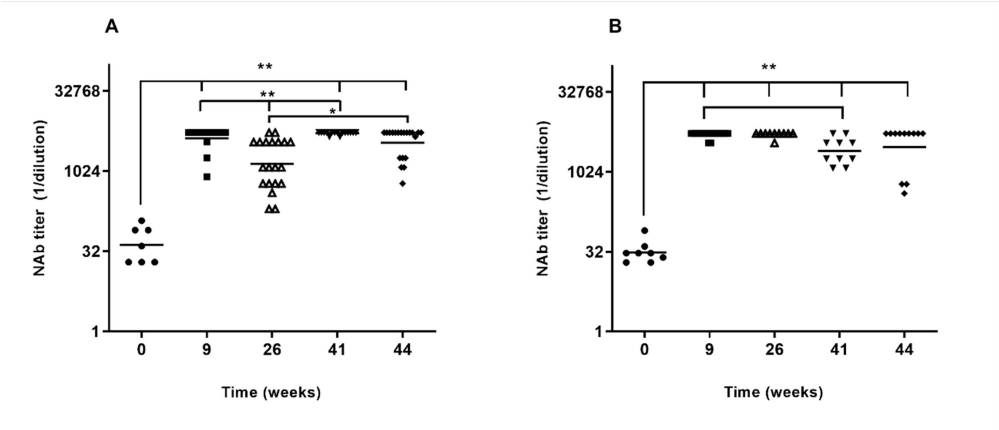

Figure. 3. Neutralizing antibody titers in vaccinated weaning pigs. A: "Unit A", B: "Unit B". Pigs at week 0 were vaccinated with MLVs; pigs at weeks nine, 26, 41, and 44 were vaccinated with E2-CD154. Kruskal-Wallis test with Dunn's Multiple Comparison tests. ***p<0.01*. * *p<0.05*. / *Títulos de anticuerpos neutralizantes en cerdos destetados vacunados. A: "Unidad A", B: "Unidad B". Los cerdos de la semana 0 fueron vacunados con MLV; los cerdos de las semanas nine, 26, 41 y 44 fueron vacunados con E2-CD154. Prueba de Kruskal-Wallis con prueba de comparación múltiple de Dunn. **p<0.01. * p<0.05.*

failure in endemic areas (32). Consequently, experts recommend waiting until NAb titers are below 1:32 to administer these vaccines (23). In contrast, subunit vaccines can be successfully administered as early as 15 days of age on a production farm because MDAs do not interfere with the immune response. This early vaccination is a great practical advantage for pig farmers.

In summary, the results of this phase III trial in piglets confirmed the safety profile and immunogenicity of E2-CD154 subunit vaccine candidate in this very sensitive category and support the extension of this subunit vaccine to other swine production farms in the country.

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