**Authors: Aymé Oliva-Cárdenas, et. al.**

*Published on IVT Network (www.ivtnetwork.com)*

**JVT Volume 28, Issue 1 – February 2022**

**AUTHORS**

Aymé Oliva-Cárdenas Milagros Vargas-Hernández, Yamila Martínez-Cuéllar, Yusmel Sordo-Puga, María Pilar Rodríguez-Moltó, Carlos A. Duarte, Mary Karla Méndez-Orta, Elaine Santana-Rodríguez, Talia Sardina-González, Alianne Fundora-Llera, Danny Pérez-Pérez, Marisela F. Suárez-Pedroso.

# AN ALTERNATIVE POTENCY TEST IN MICE FOR PORVAC®, A SUBUNIT VACCINE AGAINST CLASSICAL SWINE FEVER.

## ABSTRACT

Classical swine fever (CSF) is the main sanitary problem of pig farming in endemic regions. Porvac® is a subunit vaccine against CSF with promissory results in clinical trials. Porvac® lots are released with a lethal viral challenge test in pigs. An alternative potency test that measures neutralizing antibodies (NAb) titers in mice was established. The dose-response curve was standardized in the range from 125 ng to 8 ng. The effective dose to induce a NAb titer ≥1:50 in half the immunized mice (ED50) for the reference lot was 28.3 ± 3.6 ng. The reference vaccine lot was stable after four years at 4°C and a coefficient of variation of 35% was calculated for the intermediate precision. Another ten vaccines batches tested had a relative potency above 0.9. Nine of these batches also fulfilled the parallelism and linearity tests. The tenth batch did not comply with the linearity criteria. The assay was specific since no response was observed in negative controls. Moreover, the method was sensitive to detect physical changes in the vaccine, as the ED50 increased more than tenfold after thermal stress. Finally, a blind study confirmed the capacity of the method to detect batches with reduced amounts of the active ingredient.

## INTRODUCTION

Research and clinical studies on veterinary vaccines are commonly conducted in the natural hosts, which is always the most relevant model available. However, the use of large species such as cattle and swine in the routine analysis is expensive due to the high costs associated with the animals, the infrastructure, and the appropriately trained personal (1-3). Moreover, these tests often involve significant pain and distress for the animals because severe clinical signs or lethality are used as endpoints. The regulatory framework for vaccine testing encourages the application of 3R´s rules (Reduce, Refine and Replace) (4). Therefore, it is important to find alternatives to reduce the number of animals, or replace them with small laboratory animal models or in vitro tests, to contribute to animal welfare and reduce costs.

Laboratory animals are usually more practical, economical, and time-saving than the natural host. Additionally, these models may contribute to understanding the immune mechanisms associated with protection, the magnitude and duration of the immune response, the ideal route of administration of the target antigen, and to develop different strategies to enhance protective immune responses (5).

Classical Swine Fever (CSF) is a disease of great economic importance, mainly in those countries with high levels of commercialization and consumption of pork meat. In some regions, the virus has remained endemic for decades. The etiological agent, CSF virus (CSFV), is an enveloped positive-stranded RNA virus member of the Pestivirus genus of the Flaviviridae family (6).

Porvac® is a subunit vaccine against CSF. Its active principle is the E2CD154 antigen, formed by the fusion of the viral envelope E2 glycoprotein and porcine CD154 molecule. This vaccine combines the fast onset of protection characteristics of the modified live vaccines (MLV) with the safety profile of subunit vaccines (7-11).

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 lethal challenge with 105 PLD50 of a highly virulent strain was established to release Porvac® batches. This test has been previously used in the batch release process of MLV (1, 12). However, it involves suffering for negative control animals. Furthermore, it requires the handling of large amounts of virus, and, therefore, special containment facilities are needed to avoid dissemination of the virus, increasing the costs of the quality control (3). Finally, the test itself is costly and CSFV seronegative pigs are scarce due to the endemic nature of the disease and the prolonged use of an MLV in the country.

The last edition of the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (13), indicates that the efficacy of veterinary vaccines should be demonstrated through valid statistical analysis in the host species. Nevertheless, challenge experiments in the final species may be unnecessary for batch-to-batch release if data of the predictive value of serological tests are available.

The potency tests required for the approval of each vaccine batch must correlate with the efficacy of the vaccine in the host species (14). Previous work by several investigators has established that the presence of NAb titers equal to or higher than 1:50 is a good correlate of protection against CSF.

BALB/c mice are often used for a variety of immunological studies because Th2 cells are readily activated by immunization so they generate an excellent antibody response (15). Mice are not a natural host to CSFV; however, they were competent to develop neutralizing antibodies against the CSF virus (CSFV) in response to immunizations with different candidates that used the E2 protein as a vaccine antigen (16-17).

Taking into account the above considerations the present work aims to standardize a potency test for Porvac® batch to batch release in BALB/c mice as an alternative for the viral challenge experiment in pigs. Some pre-validation studies of this method will be also presented such as linearity, parallelism, specificity, and intermediate precision.

## METHODS

### Vaccine

The subunit vaccine Porvac® consists of the E2CD154 chimeric protein formulated in an oil in water emulsion with the adjuvant MontanideTM ISA50V2 (SEPPIC, France). The vaccine batches were produced under Good Manufacturing Practices as previously described (7, 18). The E2CD154 concentration in the vaccine was 25 µg/mL. Eleven vaccine lots were studied: P51031, P61011, P61021, P71011, P81011, P81021, P81031, P81041, P81051, P81061, and P81071. The first of them, with demonstrated efficacy in a viral challenge test in pigs, was established as a reference lot.

### Experimental Animals

Eight week-old female BALB/c mice of 18–20 g of weight purchased from the National Center for Laboratory Animal Production (CENPALAB), La Habana, Cuba, and housed in proper animal care facilities at the Bioterio (CIGB) during the experimental period. All experiments were approved by the Institutional Committee for the Care and Use of Laboratory Animals (CICUAL) according to the regulations issued by the Cuban State Center for Drugs Control (CECMED) (19). Plastic tunnels as toys as a form of environmental enrichment were included inside the mice cages to reduce stress.

### Standardization Of The Dose-Response Curve

Several experiments were conducted to find out the optimal range for the dose-response curve. The vaccine containing 25 µg/mL of E2CD154 was diluted with a blank emulsion of MontanideTM ISA 50 V2 to prepare formulations with different concentrations of E2CD154. A range from 2.5 µg to 160 ng of E2CD154 in 50 µl was explored in the first experiment. The rest of the experiments covered a range between 125 ng (a 1/10 dilution of Porvac®) and 7.8 ng.

### Potency Test Methodology

Mice were randomly assigned to five experimental groups per batch. Animals from groups 1 to 5 (ten mice per group) received 50 µL of the vaccine with two-fold decreasing quantities of E2CD154 (from 125 ng to 8 ng). Negative controls (five non-treated and five placebo inoculated mice) were included in all experiments. The vaccine was administered by the intramuscular route at days 0 and 21, the same immunization schedule recommended by the manufacturers for pigs. Mice were bled at day 28 from the tail vein and the NAb titers against CSFV were determined by the Neutralizing Peroxidase Linked Assay (NPLA) test. A NAb titer higher than 1:50 was considered as a positive response. GraphPad Prism V8.2 software (GraphPad Software Inc., La Jolla, USA) was used to adjust the dose-response curves with a non-linear, four parameters regression with variable slope. The effective dose which produces a positive response in the 50% of mice (ED50) was interpolated from the adjusted curves.

### Relative Potency, Linearity And Parallelism Analysis

The relative potency of ten Porvac® production batches were calculated with the ParLin software V4.2 (Quality Control Department, CIGB, Havana, Cuba) which has been validated and registered (registry number: 1485-2004). Batch P51031 was used as a reference lot in this assay and was run in parallel with each batch studied. ParLin software follows the algorithm proposed by Finney (20) to adjust dose-response curves. The data were fitted to parallel lines for reference and unknown samples, using a log transformation. The linearity and parallelism of the dose-response curves between each lot and the reference lot were also assessed.

### Neutralization Peroxidase Linked Assay (NPLA) For Detection Of Neutralizing Antibodies Against CSFV

The NAb titers against CSFV were measured by NPLA following the general procedure described in the OIE manual (13) with some minor changes as previously described (11, 18). The Margarita strain of CSFV was provided by the National Center for Animal and Plant Health (Mayabeque, Cuba) and the horseradish peroxidase-conjugated anti-E2 monoclonal antibody (CBSSE2.3-HRP) was purchased by CIGB of Sancti Spíritus (Sancti Spíritus, Cuba). The presence of viral replication was determined by visual inspection with an optical

microscope. The last serum dilution without any signal of virus replication was considered as the neutralizing titer.

### Intermediate Precision

The coefficient of variation (CV) of the ED50 values of 14 replicates of the reference batch P51031 run on different days by different operators was calculated to estimate the intermediate precision.

### Specificity

The humoral response developed in negative control mice (non-vaccinated and placebo) was assessed to evaluate the specificity of the method.

### Sensitivity Of The Potency Test For Heat-Stressed E2CD154 Protein

E2CD154 was incubated for 60 min at 60°C followed by another 60 min at -70 °C. A water-in-oil emulsion was prepared at a ratio of 40:60 (oil/aqueous phase) with an SD-41 homogenizer (IKA, Germany). The aqueous phase included the E2CD154 antigen in PBS (pH 7.2) and Montanide ISA 50 V2 (SEPPIC, France) adjuvant was the oily phase. The concentration of E2CD154 in the final formulations was 25 µg/mL. The immunogenicity of the heat-stressed antigen was compared with the untreated antigen by inoculating groups of ten mice with 125 ng, 62.5 ng, 31.2 ng and 15.6 ng.

### Sensitivity Of The Potency Test For Quantitative Changes In The Formulation

A blind study was conducted with five different formulations of the vaccine. The antigen concentration in these formulations was 25 µg/mL (correspond to the E2CD154 concentration in Porvac®), 12.5 µg/mL, 6.25 µg/mL, 3.12 µg/mL and 6.56 µg/mL. The formulations were prepared at the laboratory scale as described in epigraph 2.9. A blind potency test was run with all of them in parallel following the procedure described above. The first dilution tested was 1:10 followed by 4 two-fold serial dilutions.

## STATISTICAL ANALYSIS

The statistical analysis was conducted with the package GraphPad Prism 6, Version 8.2, (GraphPad Software Inc., La Jolla, USA). Normality was determined by the Kolmogorov-Smirnov test. The homogeneity of variances was determined by the Levene Test. Kruskal-Wallis test followed by Dunn's multiple comparison tests was applied to compare the antibody titers among the different vaccines lots.

## RESULTS

### Potency Test Standardization

After several attempts, the optimal range for the dose-response curve was established from 125 ng to 7.8 ng per mice using the reference batch P51031 (Figure 1). The antigen dose capable of inducing a protective NAb titer (≥1:50) in half the immunized mice (ED50) for the reference lot was 28.3 ± 3.6 ng.

### Stability Of The Reference Batch And Intermediate Precision

Figure 2A shows the dose-response curves for the reference batch P51031 evaluated at 0, 3, 12, 24, 36, and 48 months. This batch was stable up to four years after the formulation as illustrated by the time course of the

ED50 values and their 95% CI (Figure 2B). The variation coefficient calculated for the ED50 values of the reference batch P51031 run 14 times with two different operators was 35.4, which is an indicator of the intermediate precision of the assay.

### Relative Potency, Linearity, And Parallelism Of Ten Batches Of Porvac®

The dose-response curves of ten batches of Porvac® are represented in Figure 3. The determination coefficients r2 of all curves were higher than 0.97 except for batch P61021. All batches had relative potency values higher than 0.9 (Table I). All batches fulfilled the parallelism criteria (Table1) (ANOVA, $p > 0.05$). All batches, except P61021, fulfilled also the linearity criteria and the confidence intervals for the relative potency were between the accepted limits (0.33-3) (21).

### Specificity

As described above all batches of Porvac® tested induced NAb titers against CSFV. In contrast, animals immunized with placebo and non-immunized mice did not have detectable NAbs in any of the assays (NAb titer < 1:5). A total of 410 mice were evaluated.

### Heat Stress Of E2CD154 Protein

The heat-stressed E2CD154 protein showed a reduced potency in mice as compared to the non-treated protein (Figure 4). In this experiment the ED50 for the control antigen was lower than 16 ng. In contrast, the ED50 value for the heat-stressed antigen was around 250 ng. This represents a more than one order of magnitude reduction in potency after heat-stress. Doses of 61 ng or lower of the heat-stressed protein did not induce a detectable NAb response.

### Capacity Of The Method To Detect Quantitative Changes In The Vaccine Formulation

The results of this experiment are shown in Figure 5. Only the formulations corresponding to the actual concentration of E2CD154 in the vaccine (25 µg/mL) or the immediate dilution (12.5 µg/mL) produced the characteristic dose-response curve in the range of dilutions explored. Formulations with a lower concentration of E2CD154 only induced partial responses or failed to generate a positive NAb response in the animals (titers > 1:50).

## DISCUSSION

Here we described the optimization and some pre-validation studies of an alternative potency test in mice for Porvac® vaccine against classical swine fever. According to regulatory entities, optimization or pre-validation studies is the process by which the most important parameters of an assay are evaluated and adjusted to ensure their consistency.

P51031, a GMP-produced lot of Porvac®, which efficacy had been previously demonstrated in viral challenge experiments in pigs, was selected as the reference lot in the assay. The first objective of the study was the definition of the optimal vaccine concentration range for the test, where a sensitive dose-response curve could be observed. This range was defined between 125 ng and 8 ng, with an ED50 of 29 ng for the reference lot. This very low ED50 value in mice corroborates the high immunogenicity of Porvac®.

Higher values of ED50 (between 65 ng and 250 ng) were reported for Heberbiovac HB, a recombinant vaccine against Hepatitis B (Heber Biotech, La Habana, Cuba) using a similar potency test in mice (22). The active principle of Heberbiovac HB is the Hepatitis B surface antigen produced in yeast, a highly immunogenic particulate protein (23-24).

Another particulate antigen, the papillomavirus virus-like particles formulated in Merck aluminum hydroxy phosphate sulfate adjuvant exhibited an ED50 of 22 ng in mice, very similar to E2CD154 (25). Considerably higher ED50 values of 3.8 µg and 4.3 µg were calculated for the Schistosoma mansoni subunit antigen Sm-TSP-2 formulated in aluminum hydroxide (26).

Although the use of different adjuvants precludes a strict comparison of the potency among the different antigens, it can be concluded that the potency of Porvac® is similar or better than other particulate antigens containing vaccines.

Particulate antigens are known to stimulate more efficiently antigen-presenting cells, a cardinal step in the instrumentation of the immune responses (27-28). Unpublished results in our laboratory indicate that E2CD154 also forms virus-like particles. This fact, together with the presence of the CD154 molecule, a strong activator of the immune response, could explain its elevated immunogenicity.

An arbitrary potency value of 1 was attributed to the reference lot to assess the parallelism and linearity of another ten GMP-produced batches of Porvac® and calculate their relative potency. All these batches except one fulfilled the parallelism and linearity statistical criteria and their lower and upper confidence intervals or the relative potency were within the accepted values (between 0.33 and 3) (21).

Batch P61021 did not comply with the linearity criteria and its upper 95 % interval was higher than 3. Further studies are required to understand why it did not fulfill those criteria. However, since its potency was higher than the reference lot; the immunogenicity of batch P61021 in pigs is expected to be higher or at least equal to the reference lot. In fact, all these ten Porvac® batches passed the viral challenge test in pigs (unpublished results). Based on these results, a relative potency of 0.8 is proposed as a threshold for Porvac® lots to pass the potency test.

Furthermore, the stability of the reference lot stored at 4°C during four years was demonstrated. The data from a single lot tested in several time points by two operators allowed the calculation of the CV of the potency values. A CV of 35.4 is appropriate for the intermediate precision of an in vivo test since CVs above 50 % are often described for this kind of assay (29). Further validation studies must assess the intra-assay CV of the method which as a general rule must be lower than the inter-assay CV.

Viral challenge experiments in the relevant species confirm the efficacy of a vaccine but require large numbers of large animals for significant results and may be relatively insensitive to small changes in the quality or quantity of the active substance (29). In the present work, the strength of the mice potency test to detect qualitative changes in the antigen due to heat stress was demonstrated. Furthermore, the assay was also very

sensitive to detect quantitative changes in the amount of E2CD154 formulated. When the antigen concentration was halved the vaccine still showed a similar potency which demonstrates the robustness of the vaccine formulation. However, lower antigens concentrations failed to produce a dose-response curve or to induce any neutralizing antibody response at all. These characteristics, together with the high specificity of the test, make this assay useful to detect deviations in the vaccine production that could pass undetected in the viral challenge test.

According to Haas et al., (30) increasing the number of doses on the dose-response curve and the number of biological replicates per group could improve the overall performance of biological assays. However, a compromise must be reached between the optimal performance of the assay and the necessity to reduce the number of animals in the biological assays. The five doses used in this potency test seem to be sufficient to cover the useful range of the dose-response curve to calculate the ED50. Additionally, other authors found that a reduction in the number of animals per group from 20 to 10 was acceptable in a similar potency assay for Heberbiovac HB in mice (22). The use of ten animals per group is in agreement with the 3 R principle for the use of animals in research (4) and with CICUAL recommendations (19). Additionally, halving the number of replicates in the assay reduces significantly the associated costs since the number of NPLA assays is also halved. Moreover, the number of mice in the control and placebo groups was limited to five since these samples gave consistently negative results in the NPLA assay.

Animal welfare principles encourage the replacement of larger species for small laboratory animals that are easier to manage and handle and do not suffer the consequences of lethal virus inoculation. The difficulties in the acquisition of CSFV seronegative pigs in a country where all piglets are immunized with a modified live vaccine or Porvac® is another limitation for the viral challenge test, therefore, the transition to another experimental model such as mice is urgently needed.

## CONCLUSIONS

An alternative potency test was established for Porvac® vaccine, with a dose-response range between 125 ng and 8 ng, appropriate intermediate precision, specificity, and the capacity to detect qualitative and quantitative changes in the formulation.

The ED50 determined in this test for Porvac® is equivalent or better than other particulate antigens with demonstrated efficacy.

The relative potency of ten Porvac® batches determined by parallelism studies was above 0.9. A relative potency above 0.8 is thus proposed as a condition for vaccine lots to pass this test.

These results support the applicability of the potency test in mice as part of the quality control of Porvac® batches.

## REFERENCES

1. S. BLOME; A. MEINDL-BOHMER; W. LOEFFEN; B. THUER; V. MOENNIG, Assessment of classical swine fever diagnostics and vaccine performance. Revue Scientifique et Technique-Office International des Epizooties 25 (3), 1025-1038, 2006.
2. V. GERDTS; H.L. WILSON; F. MEURENS; S. VAN DRUNEN LITTEL-VAN DEN HURK; D. WILSON; S. WALKER; C. WHELER; H. TOWNSEND; A.A. POTTER, Large animal models for vaccine development and testing. ILAR journal 56 (1), 53-62, 2015.
3. Z. CAO; H. ZHANG; Q. YANG; H. ZHANG; G. FAN, Establishment of a method for evaluation of the efficacy of a classical swine fever virus subunit vaccine in rabbits. American journal of veterinary research 81 (6), 521-526, 2020.
4. C. LANG; O. KOLAJ-ROBIN; G. CIREFICE; L. TACONET; E. PEL; S. JOUETTE; M. BUDA; C. MILNE; E. CHARTON, Replacement, Reduction, Refinement-Animal welfare progress in European Pharmacopoeia monographs: activities of the European Pharmacopoeia Commission from 2007 to 2017. Pharmeuropa bio & scientific notes 2018, 12-36, 2018.
5. A. BRUN; J. BÁRCENA; E. BLANCO; B. BORREGO; D. DORY; J.M. ESCRIBANO; G. LE GALL-RECULÉ; J. ORTEGO; L.K. DIXON, Current strategies for subunit and genetic viral veterinary vaccine development. Virus research 157 (1), 1-12, 2011.
6. S. BLOME; C. STAUBACH; J. HENKE; J. CARLSON; M. BEER, Classical swine fever—an updated review. Viruses 9 (4), 86, 2017.
7. S. MUÑOZ-GONZÁLEZ; Y. SORDO; M. PÉREZ-SIMÓ; M. SUAREZ; A. CANTURRI; M.P. RODRIGUEZ; M.T. FRÍAS-LEPOUREAU; M. DOMINGO; M.P. ESTRADA; L. GANGES, Efficacy of E2 glycoprotein fused to porcine CD154 as a novel chimeric subunit vaccine to prevent classical swine fever virus vertical transmission in pregnant sows. Veterinary microbiology 205, 110-116, 2017.
8. D. PÉREZ-PÉREZ; Y. SORDO-PUGA; M.P. RODRÍGUEZ-MOLTÓ; T. SARDINA; E. SANTANA; C. MONTERO; J. ANCIZAR; Y. CABRERA; Á. TUERO; P. NARANJO, E2-CD154 vaccine candidate is safe and immunogenic in pregnant sows, and the maternal derived neutralizing antibodies protect piglets from classical swine fever virus challenge. Veterinary Microbiology, 109153, 2021.
9. Y. SORDO-PUGA; D. PÉREZ-PÉREZ; C. MONTERO-ESPINOSA; A. OLIVA-CÁRDENAS; I. SOSA-TESTE; C.A. DUARTE; M.P. RODRÍGUEZ-MOLTÓ; T. SARDINA-GONZÁLEZ; E. SANTANA-RODRÍGUEZ; M. VARGAS-HERNÁNDEZ, Immunogenicity of E2CD154 Subunit Vaccine Candidate against Classical Swine Fever in Piglets with Different Levels of Maternally Derived Antibodies. Vaccines 9 (1), 7, 2021.
10. Y. SORDO-PUGA; M. SUÁREZ-PEDROSO; P. NARANJO-VALDÉZ; D. PÉREZ-PÉREZ; E. SANTANA-RODRÍGUEZ; T. SARDINAS-GONZALEZ; M.K. MENDEZ-ORTA; C.A. DUARTE-CANO; M.P. ESTRADA-GARCIA; M.P. RODRÍGUEZ-MOLTÓ, Porvac® Subunit Vaccine E2-CD154 Induces Remarkable Rapid Protection against Classical Swine Fever Virus. Vaccines 9 (2), 167, 2021.
11. M. SUÁREZ-PEDROSO; Y. SORDO-PUGA; I. SOSA-TESTE; M.P. RODRIGUEZ-MOLTO; P. NARANJO-VALDÉS; T. SARDINA-GONZÁLEZ; E. SANTANA-RODRÍGUEZ; C. MONTERO-ESPINOSA; M.T. FRÍAS-LAPOREAUX; Y. FUENTES-RODRÍGUEZ; D. PÉREZ-PÉREZ; A. OLIVA-CÁRDENAS; C.L. PEREDA; N. GONZÁLEZ-FERNÁNDEZ; E. BOVER-FUENTES; M. VARGAS-HERNÁNDEZ; C.A. DUARTE; M.P. ESTRADA-GARCÍA, Novel chimeric E2CD154 subunit vaccine is safe and confers long lasting protection against classical swine fever virus. Veterinary Immunology and Immunopathology 234, 110222, 2021.
12. OIE, Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 5th Edition, Chapter 2.8.3. 1, 2018.
13. OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, Chapter 3.8.3. Classical swine fever (hog cholera) infection with classical swine fever virus. http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.08.03_CSF.pdf; (accessed 3/4/2020).
14. OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, Chapter 1.1.8. Principles of Veterinary Vaccine.

https://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/1.01.08_VACCINE_PRODUCTION.pdf (accessed 3/7/2020).

15. H. NAKAMURA, BALBC/c Mouse. In Brenner's Encyclopedia of Genetics, Second ed.; S. MALOY; H. HUGHES, Eds. Elsevier Inc: 2013.
16. Y. SORDO; M. SUÁREZ; R. CARABALLO; T. SARDINA; E. BROWN; C. DUARTE; J. LUGO; L. GIL; D. PEREZ; A. OLIVA, Humoral and cellular immune response in mice induced by the classical swine fever virus E2 protein fused to the porcine CD154 antigen. Biologicals : journal of the International Association of Biological Standardization 52, 67-71, 2018.
17. Y. PARK; S. LEE; H. KANG; M. PARK; K. MIN; N.H. KIM; S. GU; J.K. KIM; D.-J. AN; S. CHOE, A classical swine fever virus E2 fusion protein produced in plants elicits a neutralizing humoral immune response in mice and pigs. Biotechnology letters 42 (7), 1247-1261, 2020.
18. M. SUÁREZ; Y. SORDO; Y. PRIETO; M.P. RODRÍGUEZ; L. MÉNDEZ; E.M. RODRÍGUEZ; A. RODRÍGUEZ-MALLON; E. LORENZO; E. SANTANA; N. GONZÁLEZ, A single dose of the novel chimeric subunit vaccine E2-CD154 confers early full protection against classical swine fever virus. Vaccine, 2017.
19. CECMED, Lineamientos para la Constitución y Funcionamiento de los Comités Institucionales para el Cuidado y Uso de los Animales de Laboratorio (CICUAL). CECMED, Ed. Cuba CCpeCdMd. No 64-2013, 2013, 2013.
20. D. FINNEY, The statistical structure of biological assays. 日本統計学会誌 7 (1), 1-9, 1977.
21. W.H. ORGANIZATION, Recommendations to Assure the Quality, Safety and Efficacy of Recombinant Hepatitis B Vaccines. Proposed replacement of: TRS 786, Annex 2 and TRS 889, Annex 4. WHO 2010. Geneva, Switzerland: WHO Press: 2010.
22. M. IZQUIERDO-LÓPEZ; K. COSME-DÍAZ; G. GARCÍA-ILLERA; Z. NÚÑEZ-LAMOTTE; Y. MARTÍNEZ-CUÉLLAR; M. VEGA-SIMÓN; L. COSTA-ANGUIANO; M. QUINTANA-ESQUIVEL; I. ROSALES-TORRES; O. MOSQUEDA-LOBAINA, Establecimiento de un ensayo de potencia in vivo para el antígeno de superficie recombinante del virus de la Hepatitis B, en vacunas monovalentes y combinadas. VacciMonitor 23 (3), 83-90, 2014.
23. M. MPHAHLELE; H. SCHOEMAN; S. ASPINALL; R. BURNETT; T. KYAW, Immunogenicity and safety of yeast-derived recombinant hepatitis B vaccine (Heberbiovac HB) in South African children. South African Medical Journal 94 (4), 280-281, 2004.
24. B. MUMTAZ; M. SIDDIQ, Immunogenicity and Economic Evaluation of Different Recombinant HepatitisB Vaccines in Different Dosages, Schedules, and routes of Administration. Pak J Med Sci 24 (6), 833-837, 2008.
25. M. SHANK-RETZLAFF; F. WANG; T. MORLEY; C. ANDERSON; M. HAMM; M. BROWN; K. ROWLAND; G. PANCARI; J. ZORMAN; R. LOWE, Correlation between mouse potency and in vitro relative potency for human papillomavirus type 16 virus-like particles and Gardasil® vaccine samples. Human Vaccines 1 (5), 191-197, 2005.
26. G. LI; L. HOEWELER; B. KEEGAN; J. PENG; L. SCHOLTE; P. HOTEZ; M.E. BOTTAZZI; D. DIEMERT; J. BETHONY, Potency testing for a recombinant protein vaccine early in clinical development: lessons from the Schistosoma mansoni Tetraspanin 2 vaccine. Vaccine: X, 100100, 2021.
27. Y.R. CARRASCO; F.D. BATISTA, B cells acquire particulate antigen in a macrophage-rich area at the boundary between the follicle and the subcapsular sinus of the lymph node. Immunity 27 (1), 160-171, 2007.
28. C.M. SNAPPER, Distinct immunologic properties of soluble versus particulate antigens. Frontiers in immunology 9, 598, 2018.
29. G. CHALONER-LARSSON; R. ANDERSON; A. EGAN; M.A. DA FONSECA COSTA FILHO; J.F. GOMEZ HERRERA; V. SUPPLY; W.H. ORGANIZATION A WHO guide to good manufacturing practice (GMP) requirements; World Health Organization: 1999.
30. J. HAAS; J. MANRO; H. SHANNON; W. ANDERSON; J. BROZINICK; A. CHAKRAVARTTY; M. CHAMBERS; J. DU; B. EASTWOOD; J. HEUER, In vivo assay guidelines. In Assay Guidance Manual [Internet], S. MARKOSSIAN; A. GROSSMAN; K. BRIMACOMBE, Eds. Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences: 2012.

## TABLES

Table I. Relative potency, linearity, and parallelism of ten batches of Porvac®

| Batches | Relative potency | LL 95% CI | UL 95% CI | Parallelism | Linearity |
|---|---|---|---|---|---|
| | (u) | 0.33 - 3 | | ANOVA p values 0.01 ≤ F ≤ 1 | |
| P61011 | 1.35 | 0.883 | 1.143 | 0.1934 | 0.9879 |
| P61021 | 2.42 | 0.37 | 11.15 | 0.4452 | 0.0085 |
| P71011 | 1.09 | 0.8 | 1.26 | 0.6551 | 0.8621 |
| P81011 | 1.67 | 0.72 | 1.55 | 0.1295 | 0.8732 |
| P81021 | 0.91 | 0.8 | 1.11 | 0.9459 | 0.9996 |
| P81031 | 1.1 | 0.91 | 1.1 | 0.8586 | 0.9995 |
| P81041 | 1.26 | 0.84 | 1.24 | 0.2273 | 0.8145 |
| P81051 | 3.27 | 0.8 | 1.29 | 0.2227 | 0.9974 |
| P81061 | 1.26 | 0.92 | 1.1 | 0.2524 | 0.9988 |
| P81071 | 1.43 | 0.91 | 1.1 | 0.2011 | 0.9967 |

LL: lower limits; UL: upper limits; CI, confidence interval of the relative potency

**Authors: Aymé Oliva-Cárdenas, et. al.**

*Published on IVT Network (www.ivtnetwork.com)*

**JVT Volume 28, Issue 1 – February 2022**

## FIGURES

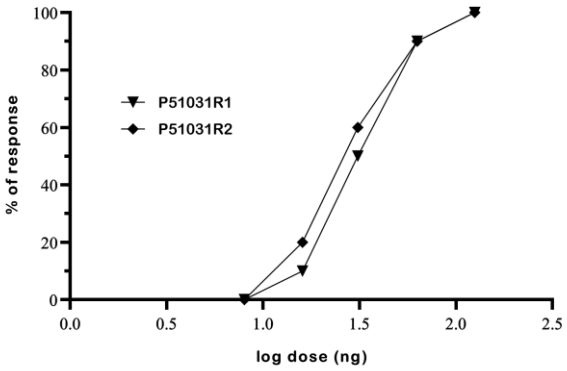


Figure 1. Dose-response curve for reference batch P51031. Five dose groups and 10 mice per group were studied. Seroconversion values were defined as the percent of mice with NAb titers higher than 1:50. Dose values were logarithmically transformed. A non-linear, four parameters regression was applied. Determination coefficients r2 were > 0.999. The ED50 calculated from this model was 28.3 ± 3.6 ng.

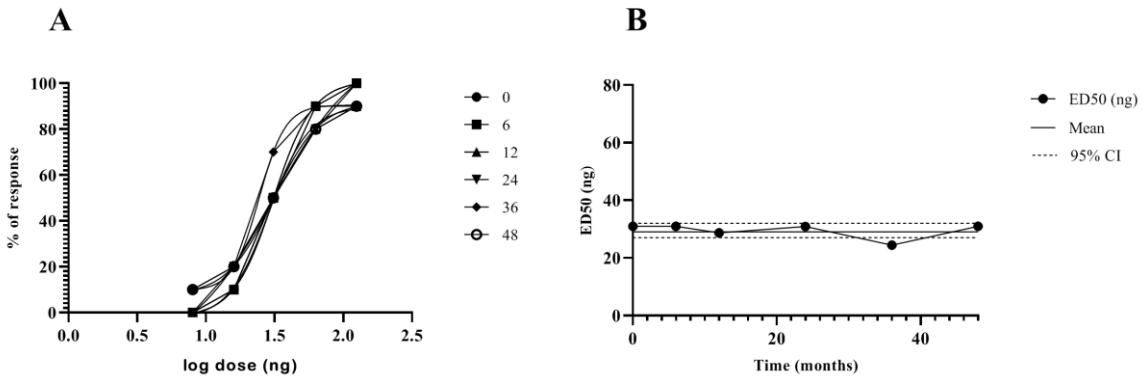


Figure 2. Stability of reference batch P51031. A. Dose-response curves for potency tests. Potency tests with the reference batch were run at months 0, 6, 12, 24, 36, and 48. Five dose groups and 10 mice per group were studied. Seroconversion values were defined as the percent of mice with NAb titers higher than 1:50. Dose values were logarithmically transformed. A non-linear, four parameters regression was applied. Determination coefficients r2 were > 0.999. B. ED50 values were calculated for all time points. The solid horizontal line indicates the average ED50. Discontinued horizontal lines indicate the 95% CI for the ED50.

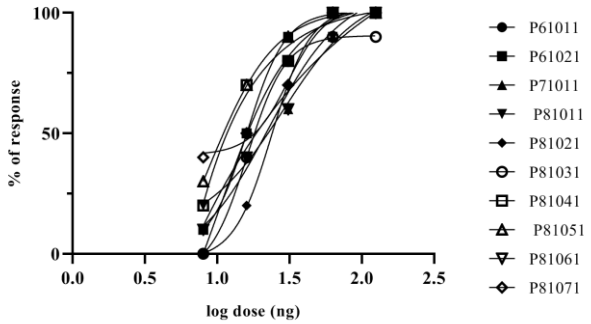

Figure 3. Dose-response curves for ten different batches of Porvac®. Five dose groups and 10 mice per group were studied. Seroconversion values were defined as the percent of mice with NAb titers higher than 1:50. Dose values were logarithmically transformed. A non-linear, four parameters regression was applied. Determination coefficients r2 were > 0.999.

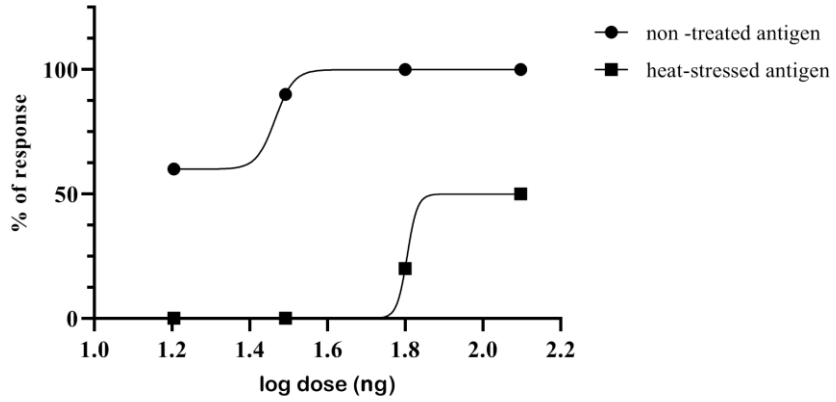


Figure 4. Dose-response curves of heat stressed E2CD154. E2CD154 was treated for 60 min at 60°C followed by another 60 min at -70 °C. The protein was then formulated with Montanide ISA TM50 V2 at a final concentration of 25 µg/mL. The immunogenicity of the heat-stressed antigen was compared with the untreated antigen by inoculating groups of ten mice with 125 ng, 62.5 ng, 31.2 ng and 15.6 ng. Circles: untreated E2CD154 control; Squares: heat-stressed E2CD154.

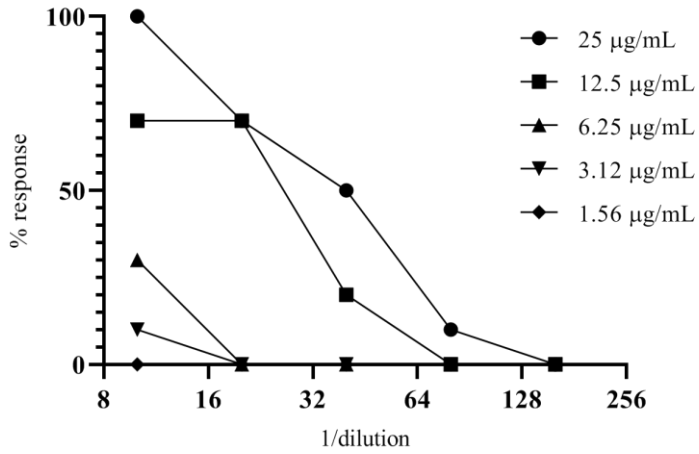


Figure 5. Dose-response curves for five experimental formulations with different amounts of E2CD154. E2CD154 was formulated at 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL, 3.125 µg/mL and 1.56 µg/mL in MontanideTM 50V2 and the potency of these formulations was blindly studied in groups of 10 mice. Y Axis: percentage of positive responses, X Axis 1/dilution of each vaccine formulation. The scale has been log2 transformed.

**ACKNOWLEDGMENTS**

We would like to thank all researchers and technicians from the Bioterio (CIGB, Cuba) for their unconditional support in the handling and care of the animals.

**FUNDING**

This research did not receive financial support from external sources.