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Research Paper

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Consistency of the Neutralizing Peroxidase Linked Assay for Classical Swine Fever and Homologation with an OIE Reference Laboratory

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***Abstract*—**Porvac® is a subunit vaccine against classical swine fever (CSF) that has been recently registered in Cuba. The Neutralizing Peroxidase Linked Assay (NPLA) is the preferred method to measure the levels of neutralizing antibodies (NAb) against classical swine fever virus (CSFV). The purposes of this study were to assess the consistency of the NPLA technique conducted for three years at the Center for Genetic Engineering and Biotechnology (CIGB) and to homologate the performance of the technique with the Nacional Center for Animal Health (CENSA), a reference laboratory for CSF validated by the World Organization for Animal Health (OIE). The consistency of the test was evaluated through two types of systematic controls: the back-titration of the virus stock, and the internal control serum. The 89.3 % of 140 NPLA assays conducted during this period were valid according to the OIE recommendations for the back-titration. The geometric mean of the NAb titre for the internal control was 1628, with lower and upper 95% confidence intervals of 1542 and 1710, respectively. The inter-assay geometric coefficient of variation of the assay was 31.4 %. Finally, a 100% coincidence for CSFV positivity and a statistically significant correlation (Spearman $r = 0.78$, $p < 0.0001$) between NAb titres measured at CIGB and CENSA was found after the parallel evaluation of 136 samples from Porvac® vaccinated pigs. Those results indicate that the NPLA assay instrumented at CIGB is robust, highly reproducible, and comparable to a reference laboratory.

Keywords— NPLA; classical swine fever; neutralizing antibodies, consistency, homologation

I. INTRODUCTION

Classical Swine Fever is a very dangerous viral disease producing considerable socio-economic damage to the swine industry worldwide [1], [2], [3]. The variability of the clinical signs and lesions exhibited by CSFV-infected pigs does not offer concluding evidence for a clear diagnosis [1], [4], [5]. Therefore, an initial diagnosis based on clinical signs and pathological findings must be ratified by laboratory tests. The Neutralization Peroxidase Linked Assay (NPLA) is one of the most useful diagnostic methods for CSF, recommended by the World Organization for Animal Health (OIE) because of its high sensitivity and specificity [6]. The NPLA technique allows not only the detection of specific antibodies against CSFV but also the capacity of these antibodies to neutralize the CSF infection. Various studies have been able to correlate a NAb titre higher than 1:50 with protection against lethal CSFV challenge [7], [8], [9], therefore the NPLA technique has been also very useful to monitor the immune response to CSFV vaccines and to batch release control of vaccines.

NPLA test is carried out in flat-bottom microtiter plates with a permissive cell line, following a constant virus /variable serum methodology. Since CSFV is not cytopathic in culture, its replication must be detected with an indicator system; a combination of a CSFV-specific antibody and a peroxidase-labeled second antibody is generally used. The test is easy to read using inverted light microscopy [6], [10].

II. RELATED WORK

Porvac® is a subunit marker CSFV vaccine that has been recently registered in Cuba. The active principle of this vaccine is E2-CD154, a chimeric protein formed by the viral E2 external protein fused to CD154 antigen from swine. This vaccine confers an early onset of protection, against viral challenge [11], [12], [13], and prevents the

transmission of the CSFV from vaccinated pregnant sows to their descendants [14].

The NPLA technique has been instrumented in our laboratory for the evaluation of the neutralizing capacity of the antibodies elicited in the animals immunized with Porvac® in either controlled clinical trials or open field studies. This method has been also approved for batch release control of the vaccine since the presence of neutralizing antibodies (NAb) in serum is a reliable correlate of protection for CSFV vaccines.

This study aimed to evaluate the consistency of the NPLA technique at CIGB for three years, based on the results of the two internal controls inserted in the methodology according to OIE recommendations: virus back titre and positive control serum [6]. Additionally, we homologate the test with an OIE reference laboratory, through the simultaneous titration of a panel of CSFV positive sera.

III. METHODOLOGY

Collection of swine sera

A total of 136 blood samples from Porvac® vaccinated animals were taken 7 days after the second immunization. The blood was incubated overnight at 4°C in the absence of an anticoagulant and centrifuged for 10 min at 3000 rpm to collect the sera. The complement was inactivated by incubating the sera for 30 min at 56 °C as recommended by the OIE [6]. The sera were preserved at -20°C. Those samples were simultaneously titrated by NPLA at CIGB and CENSA, an OIE reference laboratory for CSF diagnostic.

CSFV strain and cell line

The pork kidney cell line PK-15 (catalog CCL-33TM from ATCC) was used in the NPLA assay [10]. A variant of the CSFV strain Margarita adapted to cell culture, kindly supplied by CENSA, Mayabeque, Cuba, was used. This strain belongs to subgenotype 1.4 [15], [16], [17].

Neutralizing Peroxidase Linked Assay

The NPLA assay was developed according to the instructions in the OIE Manual [6] with some modifications. Briefly, test sera were two-fold serially diluted in 50 µl of Dulbecco Modified Eagle Medium (DMEN, Gibco, Grand Island, NY, USA) supplement with 2% fetal calf serum (Hyclone, Utah, USA) and penicillin-streptomycin (Gibco, NY, USA) in flat-bottomed microtiter plates (Costar, Kennebunk, ME, USA). The first dilution corresponds to 1:5. Control wells with only DMEN were included for cell control. Next, 50 µL of a viral stock containing 100 TCID50 was added to all wells. Plates were incubated at 37 °C for 1 h to facilitate virus-antibody reactions, and another 50 µL containing 8.5 x 103 PK-15 cells were added. The mixture was kept in a CO2 incubator for 72 h at 37 °C and 5% CO2 (Binder, Tuttlingen, Germany). The medium was discarded and the plates were rinsed with phosphate buffer saline (PBS). The plates were incubated at 70–80°C for 1 hour to fix the cells and then washed again with PBS + 0.05 % tween 20. A working dilution of the horseradish peroxidase (HRP)-conjugated CBSS 2.3 anti E2 monoclonal antibody (MAb) (CIGB of Sancti Spíritus, Sancti Spíritus, Cuba) was added in 50 µL of PBS + 0.05 % tween 20 and incubated for 1 h at 37 °C. Each batch of CBSS 2.3-HRP used was previously titrated to determine the dilution with the optimal signal-to-noise ratio. After three final washes with PBS + 0,05% tween 20, the reaction was revealed with 50 µL of the substrate solution, containing 0.5 mg/mL of Amino Ethyl Carbazole (AEC) and 0.036 % hydrogen peroxide in sodium acetate buffer pH 5.5. The titre was visually determined at the optical microscopy. The presence of the virus was indicated by the reddish-brown coloration in the cytoplasm of the cells. NAb titres were calculated following the method of Reed and Muench [18].

NPLA internal controls

Two internal controls were included in every microplate as suggested by the OIE:

(1) Virus back titre: Four dilutions of the viral stock were included to determine the back titre, which according to OIE must be between 30 and 300 for the assay to be valid. The method of Reed and Muench was also used to calculate the back titre.

(2) Positive control serum. A positive serum from a vaccinated animal was used as an in-house reference material (RM) in all determinations.

Consistency of the NPLA test at CIGB

A total of 140 NPLA assays were run in these 3 years. The percent of acceptance of the assays was calculated. The values of the back titre and the positive internal control serum from 125 valid assays conducted over three years were analysed. Median, geometric mean (GM), and 95% confidence intervals of the GM were calculated for virus back titres and the NAb titre of the positive control serum.

Statistical Analysis

All statistical tests were run with the GraphPad Prism 8 statistical package (GraphPad Prism 8 for Windows, Versión 8.02, GraphPad Software, Inc., La Jolla, USA.). Kolmogorov-Smirnov and D'Agostino-Pearson tests were applied to assess the normality of the data. Spearman non-parametric correlation coefficient was calculated to evaluate the degree of association between the NAb titres measured in the two laboratories.

IV. RESULTS

The general performance of the NPLA test at CIGB

Table 1 summarizes the general performance of the NPLA test at CIGB for three years.

Table 1. The general performance of the NPLA test at CIGB

	Years					
	2017	***2018***	***2019***	***Total***	***X***	***STD***
Number of assays	50	45	45	140	46.6	2.8
Valid assays	42	42	41	125	41.3	0.6

Invalid assays	8	3	4	15	5	2.6
Efficiency (%)	84	93.3	91.1	89.3		

X, arithmetic mean, STD, standard deviation

For an NPLA assay to be valid it should comply with the two internal controls included, as suggested by the OIE: the virus back titration and the positive control serum. The overall efficiency of the technique during this period was 89.3 % with 125 valid assays out of a total of 140. These indicators of efficiency were constant in time with a mean and standard deviation per year of 86.5 ± 9.2 %.

Virus back-titration

Figure 1A represents the tracking chart of virus back-titration during the three years studied. The geometric mean was 134 with 95% confidence intervals of 125 and 144.

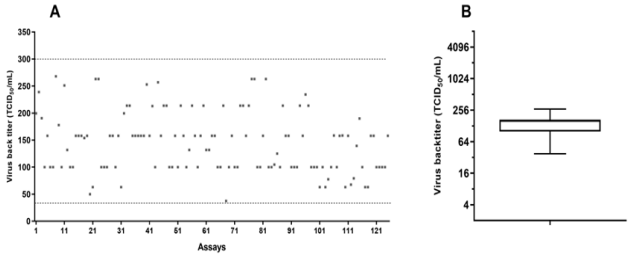

Figure 1. Analysis of the back-titration control during three years. A: Tracking chart for the back-titration in all valid assays within the studied period. Dotted lines represent the upper and lower limits for the assay. B: Box and Whiskers plot showing the median of the back-titres (158), the 95% confidence intervals, and maximal and minimal values (38 and 258, respectively). All back-titres were within the acceptance range for the technique (between 30 and 300).

CSFV positive control serum

Figure 2A shows the tracking chart of the positive control serum values during three years of evaluation. The geometric mean was 1628 with 95% confidence intervals of 1542 and 1719. The median of the positive control (1600) and minimal and maximal values of 800 and 3200, respectively, are represented in figure 2B. A geometric coefficient of variation of 31.4 % was determined for the 125 determinations of the positive serum conducted on different days and with three different operators

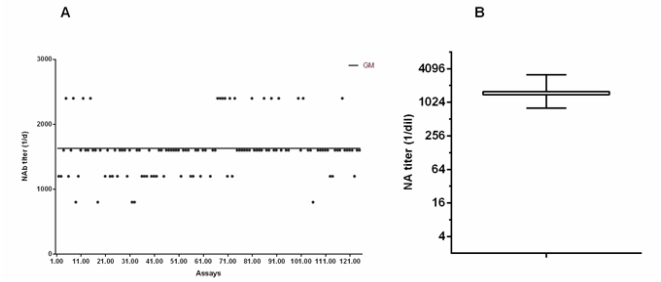

Figure 2. Analysis of the CSFV positive control during three years. A: Tracking chart for the positive control titres in all valid assays within the studied period. The horizontal line represents the geometric mean. B: Box and Whiskers plot showing the median, the 95% confidence intervals, and the maximal and minimal values.

Homologation of the NPLA assay with an OIE reference laboratory

A panel of 136 serum samples from animals vaccinated with Porvac® was simultaneously evaluated in both laboratories: CIGB and CENSA. All samples were positive in both sites for a 100% coincidence. Figure 3 shows the correlation of the neutralization titres measured at CIGB and CENSA. The Spearman non-parametric correlation coefficient of r = 0.78 confirmed the existence of a significant association between both sets of determinations ($p < 0.0001$).

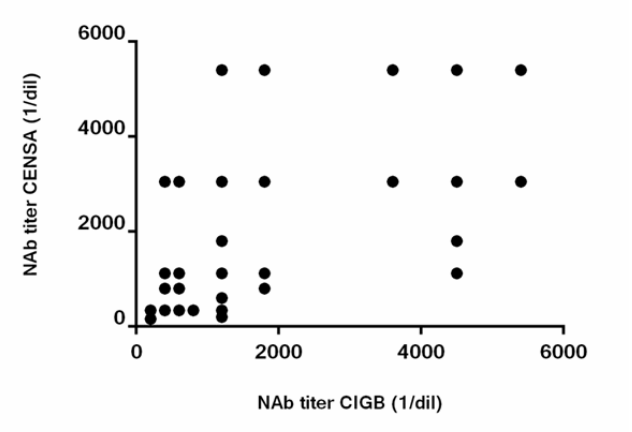

Figure 3. Correlation between neutralization titres for a panel of Porvac® vaccinated pigs measured at CIGB and CENSA. The Spearman correlation coefficient r = 0.78, $p < 0.0001$, indicated a significant association between both sets of determinations. All 136 pairs of determinations were plotted

V. DISCUSSION

The general methodology of the NPLA assay was similar to the one described in the OIE manual, with two modifications. The first was the use of the novel anti-E2 MAb CBSS 2.3 commercialized by the CIGB of Sancti Spíritus, and the second was the direct conjugation of this MAb to HRP which save one step of the technique resulting in a faster assay, with excellent results of specificity and sensitivity.

The consistency of the NPLA technique was assessed through the analysis of the two internal controls of the assay. The virus back-titration confirmed that the virus used in the test had the required concentration in every valid assay (between 30 and 300 $TCID_{50}$ in 50 µl). These results indicate that the back-titration control was consistent during the studied period.

The inclusion of a CSFV positive serum in every assay allowed the estimation of the intermediate precision of the test. All values for the positive control fluctuated between 1:800 and 1:3200; one log2 above or below the median value of 1:1600. These results confirm that the assay had an adequate range of variation for the positive control serum during these three years. The value of 31.4% for the geometric coefficient of variation is acceptable since CVs above 50 % are often described for this kind of assay which uses live cells as substrate.

Next, we conducted a homologation study with the CENSA laboratory. The 100% of coincidence, and the positive correlation found between the neutralizing antibody titres measured at both laboratories, homologate the NPLA technique performed at CIGB with the only laboratory in our country that has been certificated by the OIE reference centre of TiHo-Hannover.

The relevance of the neutralizing antibody titres measured by NPLA as a correlate of protection against CSFV infection has been demonstrated by various groups. A NAb titres equal to or higher than 1:50 is considered protective [9], [19], [20]. A reliable and robust methodology to measure the levels of neutralizing antibodies against CSF is a very important instrument for the release of vaccine batches and the follow-up of vaccination campaigns in the country.

VI. Conclusion and Future Scope

The NPLA technique at CIGB is robust, has rendered consistent and reliable results during three years, and could be homologated with an OIE reference laboratory. Hence, NPLA is a useful and reliable method for both, the evaluation of the neutralizing response of Porvac® immunized animals and the release of vaccine batches. Further work is required to complete the validation of the technique.

References

[1] V. Moennig, G. Floegel-Niesmann, and I. Greiser-Wilke, "Clinical signs and epidemiology of classical swine fever: a review of new knowledge," *Vet J*, **vol. 165, pp. 11-20, 2003**.

[2] V. Moennig and I. Greiser-Wilke, "Classical swine fever virus," *Encyclopedia of Virology*, B. Mahy and M. Van Regenmortel, Eds., Third Edition ed: Oxford: Academic Press, **pp. 525-32, 2008.**

[3] O. Fonseca, K. R. Santoro, M. A. Abeledo, Y. Capdevila, O. Fernández, P. Alfonso, et al., "Spatiotemporal distribution of classical swine fever in Cuba, 2007-2013," *Revista de Salud Animal*, **vol. 38, pp. 30-38, 2016**.

[4] S. Blome, A. Meindl-Bohmer, W. Loeffen, B. Thuer, and V. Moennig, "Assessment of classical swine fever diagnostics and vaccine performance", *Revue Scientifique et Technique-Office International des Epizooties*, **vol. 25, pp. 1025-1038, 2006**.

[5] G. Floegel - Niesmann, C. Bunzenthal, S. Fischer, V. Moennig, and O. R. Kaaden, "Virulence of recent and former classical swine fever virus isolates evaluated by their clinical and pathological signs," *Journal of Veterinary Medicine, Series B*, **vol. 50, pp. 214-220, 2003**.

[6] OIE. "Chapter 3.8.3. Classical swine fever (infection with classical swine fever virus)". *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*, **pp 1-26**, **2019**.

[7] M. Barrera, O. Sanchez, O. Farnos, M. P. Rodriguez, P. Dominguez, H. Tait, et al., "Early onset and long lasting protection in pigs provided by a classical swine fever E2-vaccine candidate produced in the milk of goats," *Vet Immunol Immunopathol*, **vol. 133, pp. 25-32, 2010**.

[8] A. Bouma, A. J. de Smit, E. P. de Kluijver, C. Terpstra, and R. J. Moormann, "Efficacy and stability of a subunit vaccine based on glycoprotein E2 of classical swine fever virus," *Vet Microbiol*, **vol. 66, pp. 101-14, 1999**.

[9] C. Terpstra and G. Wensvoort, "The protective value of vaccine-induced neutralising antibody titres in swine fever," *Vet Microbiol*, **vol. 16, pp. 123-8, 1988**.

[10] C. Terpstra, M. Bloemraad, and A. L. Gielkens, "The neutralizing peroxidase-linked assay for detection of antibody against swine fever virus," *Vet Microbiol*, **vol. 9, pp. 113-20, 1984**.

[11] M. Suárez, Y. Sordo, Y. Prieto, M. P. Rodríguez, L. Méndez, E. M. Rodríguez, et al., "A single dose of the novel chimeric subunit vaccine E2-CD154 confers early full protection against classical swine fever virus," *Vaccine*, **vol. 35, pp 4437-43, 2017**.

[12] Y. Sordo-Puga, M. Suárez-Pedroso, P. Naranjo-Valdéz, D. Pérez-Pérez, E. Santana-Rodríguez, T. Sardinas-Gonzalez, et al., "Porvac® Subunit Vaccine E2-CD154 Induces Remarkable Rapid Protection against Classical Swine Fever Virus," *Vaccines*, **vol. 9, 2, pp 167, 2021**.

[13] M. Suárez-Pedroso, Y. Sordo-Puga, I. Sosa-Teste, M. P. Rodriguez-Molto, P. Naranjo-Valdés, T. Sardina-González, et al., "Novel chimeric E2CD154 subunit vaccine is safe and confers long lasting protection against classical swine fever virus," *Veterinary Immunology and Immunopathology*, **vol. 234, p. 110222, 2021**.

[14] S. Muñoz-González, Y. Sordo, M. Pérez-Simó, M. Suarez, A. Canturri, M. P. Rodriguez, et al., "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*, **vol. 205, pp. 110-116, 2017**.

[15] H. Diaz de Arce, J. I. Nunez, L. Ganges, M. Barreras, M. Teresa Frias, and F. Sobrino, "Molecular epidemiology of classical swine fever in Cuba," *Virus Res*, **vol. 64, pp. 61-7, 1999**.

[16] L. Ganges, M. Barrera, J. I. Nunez, I. Blanco, M. T. Frias, F. Rodriguez, et al., "A DNA vaccine expressing the E2 protein of classical swine fever virus elicits T cell responses that can prime for rapid antibody production and confer total protection upon viral challenge," *Vaccine*, **vol. 23, pp. 3741-52, 2005**.

[17] A. Postel, S. Schmeiser, C. L. Perera, L. J. P. Rodríguez, M. T. Frias-Lepoureau, and P. Becher, "Classical swine fever virus isolates from Cuba form a new subgenotype 1.4," *Veterinary Microbiology*, **vol. 161, pp. 334-338, 2013**.

[18] L. J. Reed and H. Muench, "A simple method of estimating fifty per cent endpoints," *American Journal of Epidemiology*, **vol. 27, pp. 493-497, 1938**.

[19] R. H. Hua, H. Huo, Y. N. Li, Y. Xue, X. L. Wang, L. P. Guo, et al., "Generation and efficacy evaluation of recombinant classical swine fever virus E2 glycoprotein expressed in stable transgenic mammalian cell line," *PLoS One*, **vol. 9, p. e106891, 2014**.

[20] P. Biront, J. Leunen, and J. Vandeputte, "Inhibition of virus replication in the tonsils of pigs previously vaccinated with a Chinese strain vaccine and challenged oronasally with a virulent strain of classical swine fever virus," *Veterinary Microbiology*, **vol. 14, pp. 105-113, 1987**.

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Elaine Santana received her BSc Microbiology titer in 1995 from Havana University and MSc in Virology in 1999 from the Tropical Medicine Institute "Pedro Kourí", Havana, Cuba. She has 27 years of research experience in veterinary virology and molecular biology. She has co-authored more than 20 articles in reputed international journals and more than 100 presentations in 63 international meetings.

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