

Development and standardization of an indirect ELISA for the serological diagnosis of classical swine fever¹

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ABSTRACT.- Paredes J.C.M., Oliveira L.G., Braga A.C., Trevisol I.M. & Roehe P.M. 1999. Development and standardization of an indirect ELISA for the serological diagnosis of classical swine fever. *Pesquisa Veterinária Brasileira*, 19(3/4):123-127. Equipe de Virologia, FEPAGRO- Centro de Pesquisas Veterinárias Desidério Finamor, Caixa Postal 2076, Porto Alegre, RS 90001-970, Brazil.

An indirect enzyme linked immunoassay (ELISA-I) was developed and standardized for the serological diagnosis of classical swine fever (CSF). For the comparison, nine hundred and thirty-seven swine serum samples were tested by serum neutralization followed by immunoperoxidase staining (NPLA), considered as the standard. Of these, 223 were positive and 714 negative for neutralizing antibodies to classical swine fever virus (CSFV). In relation to the NPLA, the ELISA-I presented a 98.2% sensitivity; 92.86% specificity, 81.11% positive predictive value, 99.4% negative predictive value and a 94.1% precision. Statistical analysis showed a very strong correlation ($r=0,94$) between both tests. When compared to a commercially available ELISA kit, the performance of both, in relation to the NPLA, was similar. It was concluded that the ELISA-I is suitable for large scale screening of antibodies to classical swine fever virus, although it does not distinguish antibodies to classical swine fever virus from those induced by other pestiviruses.

INDEX TERMS: Classical swine fever, classical swine fever virus, ELISA, serology.

RESUMO.- [Desenvolvimento e padronização de um ELISA indireto para o diagnóstico sorológico de peste suína clássica.] Um ensaio imunoenzimático do tipo ELISA indireto (ELISA-I) foi desenvolvido e padronizado para o diagnóstico sorológico de peste suína clássica. Na comparação foram utilizadas novecentas e trinta e sete amostras de soros suínos, as quais foram testadas pelo teste de soroneutralização seguido de revelação por imunoperoxidase (NPLA), tomado como padrão, resultando em 223 amostras positivas e 714 negativas. Em relação ao NPLA, o ELISA-I apresentou sensibilidade de 98,21%, especificidade de 92,86%, valor preditivo positivo de 81,11%, valor preditivo negativo de 99,4% e precisão de 94,1%. A análise estatística dos resultados revelou uma correlação muito forte ($r=0,94$) entre os dois testes.

Quando comparado com um "kit" de ELISA disponível comercialmente, a performance de ambos em relação ao NPLA foi similar. Concluiu-se que o ELISA-I é um teste apropriado para triagem em larga escala de soros para a detecção de anticorpos contra o Vírus da Peste Suína Clássica (VPSC), embora não seja capaz de diferenciar entre anticorpos induzidos pelo VPSC ou outros pestivírus.

TERMOS DE INDEXAÇÃO: Peste suína clássica, vírus da peste suína clássica, ELISA, sorologia.

INTRODUCTION

Classical swine fever virus (CSFV), the agent of classical swine fever (CSF), is a member of the family *Flaviviridae*, genus

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Pestivirus (Francki et al. 1991). CSF is a major concern to the swine industry, being perhaps the most epizootically dangerous disease to the species in present days (Liess 1987, Terpstra 1991). As such, there is still great need to improve diagnostic methods, in order to identify outbreaks of the disease as promptly as possible and so minimize economical losses. Despite the great damage caused by CSF in Europe in recent outbreaks (Schneidereit 1998), the burden of CSF is felt particularly in developing countries, where losses due to epizootic plagues are felt at its worst, and where laboratory facilities are limited. In situations where large populations are to be examined, the need for serological tests capable of detecting the infection in massive numbers of samples is clear (Jornal Oficial das Comunidades Europeias, 1991). When eradication of a disease is being pursued, such as CSF in Brazil (MAARA 1992), rapid and reliable serological testing is mandatory (Terpstra 1991, Pearson 1992). In this country, however, none of the so far available serological tests for CSFV antibody detection is produced locally, increasing considerably the costs of serological CSF monitoring.

In the present study we describe the development and standardization of an indirect enzyme linked immunoassay intended for use in the serological detection of CSFV antibodies.

MATERIALS AND METHODS

Cells. Cells of the lineage SK6 (Kasza et al. 1972) were used throughout. Cells were multiplied in Eagle's minimal essential medium (E-MEM; Inlab) supplemented with 5% fetal calf serum (Nutricell). All sera and media were previously tested to ensure the absence of pestiviruses or antibodies to pestiviruses. Cells were multiplied and maintained following standard procedures (Roche 1991).

Serum samples. Nine hundred and thirty seven swine serum samples were selected from the laboratory stocks. Their antibody status to CSFV was determined by the NPLA described below. Two hundred and twenty three sera were found positive for neutralizing antibodies to the virus, whereas the remaining 714 sera were neutralizing antibody-negative.

Viruses. The CSFV strain Alfort 187 (Dahle et al. 1987) was obtained from the Central Veterinary Agency, Addlestone, Weybridge, Surrey, UK. Virus stocks were prepared by the inoculation of preformed monolayers of SK6 cells at a multiplicity of infection of 0.1 to 1.0 infectious units per cell. After four days of incubation, cells were frozen once, the supernatant clarified by low speed centrifugation and stocked at -70 °C. Titrations were carried out on microtitre plates using an immunoperoxidase monolayer assay as previously described (Saunders 1977). Titres of stocks were typically $10^{6.0}$ to $10^{6.75}$ tissue culture infective doses 50% per 50 ml (TCID₅₀) of viral suspension.

Neutralizing peroxidase linked assay (NPLA). The neutralizing peroxidase linked assay (NPLA) was performed essentially as described (Jensen 1985), modified as follows: dilutions (1/10 and 1/20) of test sera were prepared in microtitre plates in E-MEM supplemented with 5% pestivirus-free FCS and mixed with an equal volume (50ml) of a suspension containing 100 TCID₅₀ of the CSFV strain Alfort 187. The mixture was incubated for 1 hour at 37°C. Next, a suspension containing 3×10^4 SK6 cells was added to each

well and the plates incubated at 37°C in a 5% CO₂ atmosphere. After four days of incubation, the plates were fixed in 20% acetone in PBS (8.5 g NaCl, 1.55 g Na₂HPO₄, 0.23 g NaH₂PO₄, H₂O q.s.p. 100ml, pH 7.4;) for 10 minutes at room temperature, dehydrated for 4 hours at 37°C and stored at -20°C. For immunostaining, the plates were rehydrated with 100ml of PBS containing 0.5% Tween 80 and subjected to immunoperoxidase as described (Saunders 1977).

Production of ELISA antigen. The antigen for the ELISA-I was prepared essentially as described by Leforban et al. (1990), modified as follows. Roller bottles with SK6 cells at about 90% confluency were infected with 5 ml of the CSFV strain Alfort 187 with a titre of $10^{6.75}$ TCID₅₀. After one hour at 37°C for adsorption, the bottles were refilled with 145 ml of E-MEM with 5% FCS and incubated at 37°C for 72 hours at low rolling speed (0.6 cycles per minute). The supernatant was then removed, cells scraped off the bottles and resuspended in 2.5 ml of 0.2% n-octyl-glucopyranoside (OGP) in PBS for one hour at 4°C under slight agitation. Subsequently, the suspension was sonicated at 60 mA for three periods of 30 seconds and then clarified by centrifugation at 14 000 x g for 5 min. After clarification, the pellet was removed and the supernatant stocked at -70°C, titrated and used as the ELISA-I antigen.

Optimization of the ELISA. Antigen, conjugate, serum dilutions and timing of the test were optimized based on the dilutions where the distinction between positive and negative sera was most evident.

ELISA procedure. ELISA polystyrene flexible microplates (Hemobag) were coated overnight at 4°C with 100ml/well of an appropriate dilution of antigen (1/160 in 1.59 g Na₂CO₃, 2.93 g NaH₂CO₃, q.s.p. 1 litre, pH 9.6). Plates were either used in the subsequent day or stored at -20 for further use. Before use, plates were washed three times with wash liquid (PBS-T20; 5% Tween 20 in PBS); subsequently, the wash liquid removed and an appropriate dilution of each test serum (1:40) was added to each of two wells on the plate (100ml/well). After an incubation period of one hour at 37°C, the plates were washed three times as above and 100 ml of an appropriate dilution (typically 1/2000) of rabbit anti-swine IgG/peroxidase conjugate (Dako) were added to each well. Following further incubation of 1 hour at 37°C, the plates were again washed three times and 100 ml of ortho-phenylene-diamine (OPD) diluted as recommended (Harlow & Lane 1988) and 0.003% H₂O₂ was added. After five minutes (previously determined as the optimum time for stopping), the reaction was stopped by adding 50 ml of 2M H₂SO₄ to each well. Plates were read on a Titertek Multiskan ELISA reader at an optical density of 492 nm.

Determination of the percentual optical density of the sera. The OD of each sera was expressed as the percentual optical density (OD%), which was calculated with basis on the negative control serum in each microplate, in order to minimize variation between plates. The OD% was calculated according to the formula:

$$OD\% = (OD \text{ test serum} / OD \text{ negative control serum}) \times 100$$

Calculation of the cut-off point. The cut-off point was calculated with basis on the arithmetical mean of the OD% of the 714 sera found negative for neutralizing antibodies by the NPLA (mDO%), plus three standard deviations (s). Thus, Cut-off point = mDO% + 3s.

Analysis of validity of the test. The validity of the test (i.e. determination of sensitivity, specificity, positive and negative predictive values and calculation of the correlation coefficient) was determined using the method described by Coggon et al. (1983).

Comparison with a commercially available ELISA. A comparison was made between the ELISA-I and a commercially

available ELISA kit (ELISA-PPC; Sanofi Diagnostics Pasteur). The test consisted of a blocking ELISA, originally developed by Leforban et al (1990). The commercial test was performed as recommended by the manufacturers, with 678 serum samples, 90 of them positive and 588 negative for antibodies to CSFV, as determined by the NPLA. The results obtained with both ELISAs were calculated in relation to the NPLA and compared.

RESULTS

Performance of the ELISA-I. The differences in OD% observed between the positive and negative samples were quite marked. In all plates considered valid presented differences in OD% between positive and negative control sera within the range of 2 to 3.5-fold. In no case a valid test presented differences in OD% from positive to negative sera outside these limits. In the few instances (3 plates) in which controls were not between these limits, the tests were repeated and the expected limits were then attained.

Determination of the cut-off point. The average of the OD% of the 714 antibody-negative sera was 97.66%. The calculated cut-off point of the ELISA-I was determined at an OD% = 149.53, so as to include three standard deviations ($s = 17.29$) or 99.96% of the negative population of sera (González 1974). Serum samples whose OD% were above the cut-off point were considered positive; sera with OD% values below the cut-off were considered negative. All samples with values of OD% close to the cut-off point were considered as suspect and were retested.

Table 1. Comparison of the results obtained with the indirect ELISA (ELISA-I) and the neutralizing peroxidase linked assay (NPLA) on 937 swine serum samples and analysis of validity of the test

ELISA-I ^a	NPLA		Total
	Positive	Negative	
Positive	219	51	270
	(a)	(b)	(a+b)
Negative	4	663	667
	(c)	(d)	(c+d)
Total	223	714	937
	a+c	(b+d)	(a+b+c+d)

^aAnalysis of validity of the ELISA-I (based on the NPLA results):

Sensitivity $(a/a + c) \times 100 = 219 / 219 + 4 \times 100 = 98.21\%$;
 Specificity $(d/b + d) \times 100 = 663 / 51 + 663 \times 100 = 92.86\%$;
 Negative predictive value $(d/c + d) \times 100 = 663 / 663 + 4 \times 100 = 99.4\%$;
 Positive predictive value $(a/a + b) \times 100 = 219 / 219 + 51 \times 100 = 81.11\%$;
 Precision $(a + d/a + b + c + d) = 219 + 663 / 219 + 51 + 4 + 663 \times 100 = 94.1\%$.

Comparison between the ELISA-I and the NPLA and analysis of validity of the test. In Table 1 the results of the comparison between the ELISA and the NPLA are shown. The analysis of the validity of the test (Cogoon et al. 1983) is at the footnotes in Table 1. The correlation coefficient " r " revealed a very strong correlation ($r = 0.941$) between the ELISA and the NPLA. (Fig. 1).

Table 2. Validation of the results obtained with the two immunoassays (ELISA-I and ELISA-PPC^a)

	ELISA-I	ELISA-PPC
Sensitivity $(a/a + c \times 100)^b$	97.7%	90.0%
Specificity $(d/b + d \times 100)$	94.8%	98.1%
Positive predictive value $(a/a + b \times 100)$	74.6%	88.0%
Negative predictive value $(d/c + d \times 100)$	99.6%	98.4%
Precision $(a + d/a + b + c + d \times 100)$	95.3	97.0%
Correlation coefficient (r)	0.94	0.93

^aSanofi Diagnostics Pasteur.

^bCalculi according to Coggon et al. (1993).

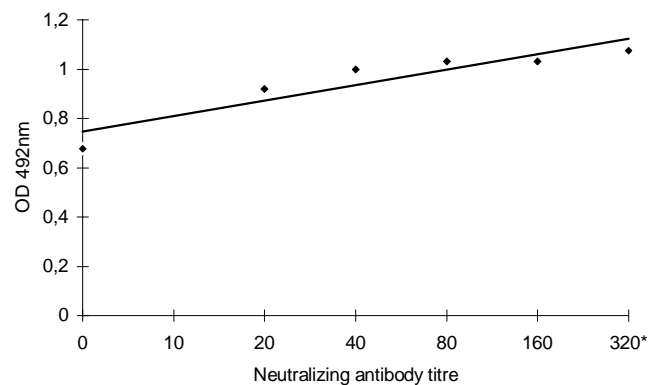


Fig. 1. Correlation between optical density (OD) obtained with the indirect ELISA (ELISA-I) and neutralizing antibody titres in the neutralizing peroxidase linked assay (NPLA). ^a Titres expressed as the reciprocal of the neutralizing antibody titre in the NPLA. Equation of the regression curve: $y = 0.0625x + 0.6853$.

Comparison with the commercial ELISA kit. The results obtained at the comparison between both ELISA-I and ELISA-PPC (Sanofi Diagnostics Pasteur) are shown in Table 2.

DISCUSSION

In this study, we described the development and standardization of an indirect ELISA (ELISA-I) for the serological diagnosis of CSFV infections. The indirect ELISA was designed for use as a large scale screening test for the detection of antibodies to CSFV. The test was found very practical and simple to perform, providing in most cases a clear distinction between positive and negative sera. Most positive and negative sera could actually be identified visually; only in a few instances visual inspection was not enough to distinguish between positive and negative samples, as immediately confirmed by OD analysis.

The sensitivity, specificity positive and negative predictive values, and correlation coefficient calculated for ELISA-I in comparison with the NPLA, adopted as the "golden standard" in this study, were quite comparable to those of similar tests described in the literature (Have 1987, Leforban et al. 1987, Leforban et al. 1990, Wensvoort et al. 1988). In addition, the ELISA-I presented the advantage of a simpler design than si-

milar tests which involve additional steps (Leforban et al 1987, 1990, Wensvoort et al. 1988). The need for less handling shortened the time required for completion of the test (approximately 3 hours for the ELISA-I, as opposed to 4-5 hours with others). This may be a substantial benefit when large numbers of samples are to be tested. In addition, the test was shown to be very reliable in that exhaustive repetitions invariably lead to the same (positive or negative) results (data not shown), despite some expected variation in the OD% obtained.

One of the critical points in designing an ELISA is the preparation of the antigen (Bolton 1981, Crowther & Smith 1987), particularly when CSFV antibodies are the target, since pestiviruses usually multiply to low titres *in vitro*. The method here described for the preparation of the ELISA antigen was highly effective, since it provided a good discriminative capacity between antibody-positive and negative samples. We believe that the association of a virus suspension of relatively high titre, the use of OGP as recommended previously (Have 1987) and the sonication of the antigen preparation, were the determinant factors for the antigen's efficacy.

The sensitivity and specificity of the ELISA test is necessarily related to the "cut-off point" (Coggon et al. 1983). In the ELISA-I, the cut off was determined with basis on the OD% of the actual negative population of sera. The adoption of three standard deviations as the "rule of thumb" to distinguish negative from positive samples would theoretically include 99,96% of the population of the actual antibody-negative samples (González 1974). Therefore, an allowance was made for the test to detect 0.006 % of the negative samples as positives and, as such, a small percentage of false-positive samples is expected. These will have to be retested in a more specific test, such as the NPLA. In addition, as the ELISA-I does not distinguish between antibodies induced by the different pestiviruses, positive samples would have to be again examined in differential tests, in order to determine to which of the pestiviruses the antibodies were more likely induced.

In order to establish a direct comparison with a widely used, commercially available ELISA, the ELISA-I was compared with the ELISA-PPC (Sanofi-Pasteur), which, at the time of performing the comparisons described here, was the only test authorised for use in CSFV serology in the country (Silva 1997).

In the comparison, the ELISA-I presented a sensitivity (95.5%) slightly superior to the ELISA-PPC (90,0%). However, the specificity of the ELISA-PPC was slightly higher (98,1%) than that of the ELISA-I (94,8%). The positive predictive value was smaller for the ELISA-I (81%) than for the ELISA-PPC (88 %). This was due to the fact that the number of "false positives" was larger for the ELISA-I (30 sera) than for the ELISA-PPC (11 sera, not shown). These false positives in the ELISA-I should not become a major problem, since positive sera will have to be retested by the standard NPLA. On the other hand, the number of "false negative" sera at the ELISA-I (2 sera) was smaller than that obtained with the ELISA-PPC (9 sera, not shown). Although no significant differences were found

between the negative predictive values of both tests (Table 2), the ELISA-PPC actually allowed a slightly larger number of positive sera to be considered "negative". In such case, the false negative results may be more compromising than false positives, since the former are not usually submitted to retesting, so the error introduced might compromise control or eradication efforts. This could be pointed out as the greatest disadvantage of the ELISA-PPC, since false negative sera would not be retested by the NPLA and would actually be considered as negative sera, when they were in fact antibody-positive.

As regards the other validation criteria examined, the results obtained indicated that both ELISAs displayed similar performances. All other indicators calculated (sensitivity, specificity, regression analysis, positive and negative predictive values and precision) gave rise to similar results.

A growing number of enzyme immunoassays for the serological diagnosis of CSFV infections are being marketed around the world. However, to date, none of the diagnostic kits available for the serological diagnosis of CSF are produced in Brazil, leading to the need for importations, thus increasing substantially the costs of serological testing. In searching for an alternative to the imported kits, the ELISA-I was developed. The test showed an adequate performance in comparison to the NPLA. As a screening test, it was shown to be useful in CSFV serology, and thus may be used in support to CSF control or eradication programs. However, the ELISA-I must not be relied upon to establish a definitive diagnosis of CSF. For such, the ELISA-I must be followed by the use of a reference test, such as the NPLA, or a pestivirus differential immunoassay (Wensvoort et al. 1988), in the same way as positive sera would have to be retested when screened with the commercially available ELISA kit used for comparison.

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