

# A Comparison of Serological Methods for Detecting the Immune Response After Rabies Vaccination in Dogs and Cows From Rabies-Endemic Areas in Brazil

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## ABSTRACT

During a serosurvey of domestic dogs and cows in Brazil, a fluorescent antibody virus neutralization (FAVN) test, liquid-phase competitive enzyme-linked immunosorbent assays (LPC-ELISA), and sandwich competitive ELISA (SC-ELISA) were used to measure rabies antibodies in vaccinated animals. Post-vaccination titers measured by LPC-ELISA for vaccinated dogs and cows correlated closely with those found by FAVN ( $r = 0.893$ ). The LPC-ELISA presented relative specificity of 98.00%, sensitivity of 99.49%, and accuracy of 100% when the titers were compared with those obtained by FAVN for the dog testing sera. Thereafter, for the cow sera, the same accuracy was observed, and the relative specificity of 86.90% and relative sensitivity of 98.43% were lower. These results suggested that LPC-ELISA applied here, using polyclonal immunoreagents,

could be valuable for the detection of rabies antibodies, with low cost and reliable results during vaccination campaigns, especially in undeveloped countries.

## INTRODUCTION

Rabies is a fatal zoonotic viral infection of the central nervous system that is transmitted by the bite of a rabid animal and is capable of infecting all mammal species. In most modern laboratories, it is usually diagnosed through the detection of viral antigen in the brain by using a direct fluorescent antibody test (FAT).<sup>1,2</sup> Considerable progress has been achieved in rabies prophylaxis in Brazil, mainly by the use of screening a large number of clinical samples by FAT and by increasing the vaccination programs, resulting in a marked decrease in cases of urban and rural rabies.<sup>3</sup> Tissue culture techniques have long been applied in studies related to rabies virus, and there are now a number of continuous cell lines used in research on pathogenesis, vaccine produc-

tion, diagnosis of rabies, and measurement of the respective immune response.<sup>4-8</sup>

Post-assessment of the efficacy of rabies vaccination campaigns requires blood sampling of vaccinated animals to measure the respective immune response.<sup>9</sup> The most commonly used techniques for this purpose are the serum neutralizing methods performed on mice or cell cultures. The World Health Organization recommended the mouse neutralization test (MNT) and the fluorescent antibody virus neutralization (FAVN) test performed on BHK-21 cells as standard techniques.<sup>3,10-13</sup>

The virus neutralization (VN) test is a sensitive and highly specific test for VN antibodies to rabies virus in serum. Generally, the rapid fluorescent focus inhibition test and microneutralization test, both VN-based tests, have been widely used for detecting and measuring antibodies to rabies virus.<sup>11</sup> However, these tests are tedious and complicated to perform, making them unsuitable for large-scale seroepidemiologic surveillance studies.<sup>14</sup> Initially, to overcome this disadvantage, several enzyme-linked immunosorbent assays (ELISAs) have been developed for detecting antibodies to rabies virus, using monoclonal based techniques.<sup>15-18</sup> In this study, we employed 2 unconventional ELISA competitive methods using polyclonal antibodies, and the results were compared with those obtained by fluorescent antibody virus neutralization (FAVN) tests to rapidly and reliably detect antibodies against rabies virus from compulsorily vaccinated dogs and cows sera,

## **MATERIALS AND METHODS**

### **Cell Culture and Medium**

The chicken embryo related (CER) line, passage 45, was kindly obtained from Laboratory of Viral Immunology, University of Campinas, São Paulo, Brazil. The cells were cultured with Dulbecco's modified Eagle's medium nutrient mixture F-12 (Sigma #D-8900), supplemented with 2 mM glutamine, 10% (v/v) fetal calf serum (Sigma #F-2442) and 1× antibiotic/antimycotic solution (Sigma

#A-5955) at 37°C in a moist atmosphere containing 5% (v/v) CO<sub>2</sub>. Prior to use in the virus propagation procedure, the cells were trypsinized with 0.25% (w/v) trypsin-EDTA solution (Sigma #T-4049) and resuspended at concentrations indicated below.<sup>12</sup>

### **Virus Propagation**

The challenge virus standard (CVS) strain of rabies virus was derived from reference stocks held by the Institute Pasteur, São Paulo, Brazil. Confluent monolayers of CER cells were cultured in 96-well tissue culture plates (Corning #25860). For virus production, 500 µL of stock CVS suspension containing 10<sup>6.7</sup>-tissue culture infectious dose 50% (TCID<sub>50</sub>) were added to a confluent monolayer containing 3 × 10<sup>5</sup> cells pre-filtrated through 0.25 µm.<sup>12</sup> After 60 min at 37°C, 5 mL of medium with 2% fetal serum were added and the cultures incubated for 5 days after infection at 37°C in 5% CO<sub>2</sub>. The respective CER infection was done twice. The supernatant was removed for virus detection by mouse inoculation test and respective monolayers were fixed with 80% (v/v) cold acetone for 15 min in an ice bath. The cells were stained for 1 hour at 37°C with a rabies anti-ribonucleoprotein conjugate (Sanofi Diagnostics Pasteur, Steenvoorde, France) diluted 1/20 in a phosphate buffer solution (PBS) containing 1/200 Evans blue solution. After rinsing with buffered glycerine, the plates were examined with a 100× oil-immersion objective.

### **Virus Purification and Antibodies Production**

Preparation of purified CVS is described elsewhere.<sup>19</sup> The trapping antibody was produced in goats. Briefly, 2 adult goats were inoculated subcutaneously with 250 µg (in 1.0 mL) of purified CVS emulsified with an equal volume of Freund's complete adjuvant. The immunization was performed 3 consecutive times; 28 days after the first injection, the respective animals were boosted with 150 µg of purified CVS with no adjuvant. After an additional 14 days, the animals were bled and the serum was sepa-

rated and stored at  $-20^{\circ}\text{C}$ . The detector antibody was produced in Swiss mice (males weight = 20 g each) and was prepared by the inoculation of 20 animals with 1.0 mL of Flury HEP vaccine (supplied by Institute Pasteur, São Paulo, Brazil). After 28 days, the goats were boosted as described above. Again, after an additional 14 days, all animals were bled out, and the serum was separated, inactivated, and stored at  $-20^{\circ}\text{C}$ .

### **Samples**

The assays were optimised for individual serum samples taken from domestic dogs and cows vaccinated against rabies (147 urban dogs and 64 cows;  $n = 211$ ) and unvaccinated dogs and cows (50 dogs and 34 cows;  $n = 84$ ) from rabies-free areas. Serum samples were stored at  $5 \pm 3^{\circ}\text{C}$  for no more than 5 days. For prolonged storage, the samples were frozen at  $-20^{\circ}\text{C}$ . Each serum was heat inactivated at  $56^{\circ}\text{C}$  for 30 min prior to use. Positive control serum from dog origins titrated to 132 IU/mL, obtained by the Centers for Disease Control (CDC, Atlanta, USA), stored at  $-20^{\circ}\text{C}$  and diluted to 5 IU/mL, was used for all assays.

### **Fluorescent Antibody Virus Neutralization (FAVN) Test**

Fluorescent antibody virus neutralization test was a method adapted from that previously described<sup>11</sup> and performed in 96-well microplates (Corning #25860) using the CER monolayer infection. Brief, serial 3-fold dilutions (1:5, 1:25, and 1:125 of both serum samples, and positive and negative controls) were prepared in the microplates, each serum dilution being added in 4 adjacent wells. A 50- $\mu\text{L}$  amount of a dilution of CVS strain containing 50-200 TCID<sub>50</sub>/mL was then added to each serum dilution well. After 90 min of incubation at  $37^{\circ}\text{C}$  in a humidified incubator with 5% CO<sub>2</sub>, a 50- $\mu\text{L}$  volume of cell suspension containing  $3 \times 10^4$  cells/mL was added to each well, and the plates were incubated for 1 day at  $37^{\circ}\text{C}$ . The slides were fixed in acetone, dried, and stained with fluorescein isothiocyanate-labelled anti-rabies immunoglobulin (Becton Dickinson,

Cockeysville, MD). For each serum dilution, 20 microscopic fields in a single well were evaluated for the presence of virus-infected cells using a Zeiss fluorescence microscope at 160 $\times$  magnification. Titers were calculated using the Reed Muench method. The titers of a standard reference serum diluted to contain 5 IU/mL were titrated in each test. By comparison to the reference serum, results of test sera were reported as positive (test serum  $\geq$  titer of reference serum at 0.5 IU/mL) or negative (test serum  $<$  titer of the reference serum at 0.5 IU/mL).

### **Enzyme Linked Immunosorbent Assay (ELISA)**

Each ELISA was performed as described before for foot-and-mouth disease, with some modifications for the quantification of anti-rabies antibodies.<sup>14,20-23</sup> With regard to the percentage of competition of each test sera, each ELISA was performed using flat-bottom 96-well microtiter plates (Sigma # I-0448) sensitized overnight at  $4^{\circ}\text{C}$  with goat anti-CVS virus antibody (trapping polyclonal antibody) diluted in 0.05 M carbonate-bicarbonate (pH 9.6) coating buffer. Briefly, optimal dilutions of goat anti-CVS serum, test sera, non-purified antigen (crude antigen), mouse anti-rabies serum (detector antibody), and the commercial rabbit anti-mouse conjugated antibody (Sigma #A-9044) were determined using checkerboard titration. All further steps of ELISA were performed at either  $37^{\circ}\text{C}$  for 60 min and using PBS (pH 7.4, 0.01-M PO<sub>4</sub>, 0.14-M NaCl) added to 1% of Tween 20 and 10% of bovine serum albumin (Sigma #A-9418). All reagents were delivered in 50- $\mu\text{L}$  volumes. On each plate, 22 wells were reserved for the antigen control with no test sera added, and they were used to define the mean optical density corresponding to a 100% detector antibody bound to the antigen (maximum OD). The degree of blocking or inhibition for each test serum was then calculated by the following formula: percentage of inhibition (PI) = (maximum OD - sample OD)/(maximum OD)  $\times$  100.

### **Sandwich Competition ELISA**

The sandwich competition ELISA (SC-ELISA) was performed as described before.<sup>22-24</sup> After sensitization of the ELISA plates (described previously) with 12.5 µg/well of trapping antibody, the crude antigen (CVS propagated in CER cells and inactivated) were diluted 1:10 in PBS and incubated for 1 hour at 37°C, followed by the addition of test sera and the mouse anti-rabies virus at the same time, diluted 1:5 in PBS. The microplates were incubated overnight at 4°C. The dilutions were chosen based on the capacity of mouse anti-rabies serum to saturate the CVS virus antigen in the absence of any other specific antibody. The capacity of mouse anti-rabies virus to react with the virus antigen was measured using 0.006% H<sub>2</sub>O<sub>2</sub>, and 0.4 mg of o-phenylenediamine/mL in 0.1 M citric acid buffer (pH 5.0) was added to all the wells; the reaction was allowed to develop for 15 min at room temperature. The reaction was then stopped by the addition of 2-M HCl. Plates were read spectrophotometrically at 490 nm on an ELISA plate reader.

### **Liquid Phase Competition ELISA**

The liquid phase competition ELISA (LPC-ELISA) was performed as described previously with some modifications.<sup>10,14,16,22,23</sup> The crude antigen (CVS propagated in CER cells) was inactivated and diluted in PBS 1:10, and was then incubated overnight at 4°C in sterile bacteriological “non-ELISA” plates (Sigma #M-9655) with the test sera and mouse anti-rabies virus diluted 1:5 and 1:100, respectively. The ELISA plates were then sensitized with goat anti-CVS virus antibody, 12.5 µg/well. Both ELISA and non-ELISA plates were incubated overnight at 4°C. After incubation, the mixture of crude antigen, test sera, and mouse anti-rabies virus was transferred to the ELISA plates after the block phase with 200 µL of 10% of blood serum albumin. The plates were then incubated for 120 min at 37°C, and the presence of mouse antibody, which had reacted with the trapped virus, was detected using rabbit anti-mouse immunoglobulins conjugate as described above.

### **Calculation of ELISA’s Cut-Off Point**

A duplicate set of dilutions of the reference positive serum was included in each test run with an initial dilution of 1:5. The final dilutions of reference positive serum (1:5, 1:10, 1:30, 1:100, 1:300, 1:1000, and 1:3000) equate to test sera with FAVN titers of 5.67, 2.13, 0.56, 0.22, 0.056, and 0.023 IU/mL. The cut-off point was determined by 2-graph receiver operating characteristic (TG-ROC) analysis, which is, briefly, a plot of the test sensitivity and specificity against the cut-off (threshold) obtained by percentage of competition (PC), the latter known as the point of equivalence, where the assay sensitivity is equal to the assay specificity. This point was selected as the cut-off point (CTP) for every ELISA applied in this study. The titers expressed as PC equal to or greater than the CTP were regarded as positive.<sup>23</sup>

### **Data Analysis**

The results were analysed to determine relative sensitivity and specificity, predictive values, and accuracy. Sensitivity was defined as the proportion of positive results obtained by standard technique that were correctly identified by all serological methods applied in this study. Predictive values (positive and negative) were defined as the proportion of 2 tests, both positive and negative, which were corrected. Fisher’s exact test was used to compare the sensitivity and specificity of 2 tests. The ELISA values were linearly regressed on standard method values, and the correlation coefficient (Pearson’s *r*) was obtained. Kappa was calculated to measure the strength of the agreement between the methods. The likelihood ratio at 95% confidence interval (CI) was used to express the probability that both ELISA and cell culture results came from dogs and cows with opposed standard ones. For this purpose, the likelihood ratio for a positive test was defined as sensitivity/(1-specificity) and the likelihood ratio for negative test was defined as (1-sensitivity)/specificity. StatsDirect (CamCode, Ashwell, England) and EXCEL 97 (Microsoft, Bellevue, Wash.) were used for the calculations.<sup>23</sup>

**Table 1.** Frequency tabulation on 197 dogs (147 vaccinated and 50 non-vaccinated or naive) tested by the FAVN reference method and the sandwich (SC) and liquid phase competitive (LPC) ELISA method using 20% and 25% PI as the cut-off value.

		FAVN test result 0.5 IU/ML cut-off			
		Sera from naive dogs (n = 50)		Sera from vaccinated dogs (n = 197)	
ELISA		Negative	Positive	Negative	Positive
SC-ELISA*	Negative	45	2	3	10
	Positive	3	0	4	180
	Total	48	2	7	190
LPC-ELISA†	Negative	49	1	0	0
	Positive	0	0	1	196
	Total	49	1	1	196

\*Relative sensitivity =  $180/190 \times 100 = 94.73\%$

Relative specificity =  $45/48 \times 100 = 93.75\%$

Accuracy =  $180/197 \times 100 = 91.37\%$

Positive predictive value =  $180/(180+4) \times 100 = 97.82\%$

Negative predictive value =  $45/(45+2) \times 100 = 95.74\%$

†Relative sensitivity =  $196/197 \times 100 = 99.49\%$

Relative specificity =  $49/50 \times 100 = 98\%$

Accuracy =  $196/196 \times 100 = 100\%$

Positive predictive value =  $196/(196+1) \times 100 = 99.49\%$

Negative predictive value =  $49/(49+1) \times 100 = 98\%$

**Table 2.** Frequency tabulation on 148 cows (64 vaccinated and 84 non-vaccinated or naive) tested by the FAVN reference method and the sandwich (SC) and liquid phase competitive (LPC) ELISA method using 20% and 25% PI as the cut-off value.

		FAVN test result 0.5 IU/ML cut-off			
		Sera from naive cows (n = 84)		Sera from vaccinated cows (n = 64)	
ELISA		Negative	Positive	Negative	Positive
SC-ELISA*	Negative	60	11	3	0
	Positive	11	0	0	61
	Total	71	11	3	61
LPC-ELISA†	Negative	73	6	0	0
	Positive	5	0	1	63
	Total	78	6	1	63

\*Relative sensitivity =  $61/64 \times 100 = 95.31\%$

Relative specificity =  $60/84 \times 100 = 71.42\%$

Accuracy =  $61/64 \times 100 = 95.31\%$

Positive predictive value =  $61/(61+0) \times 100 = 100\%$

Negative predictive value =  $60/(60+11) \times 100 = 84.51\%$

†Relative sensitivity =  $63/64 \times 100 = 98.43\%$

Relative specificity =  $73/84 \times 100 = 86.90\%$

Accuracy =  $63/63 \times 100 = 100\%$

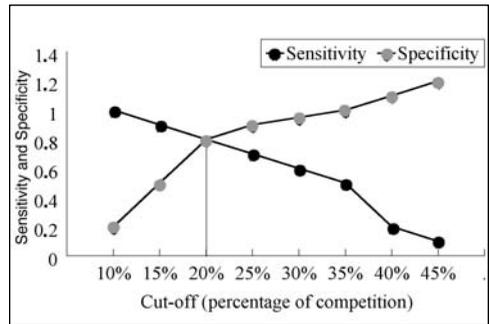
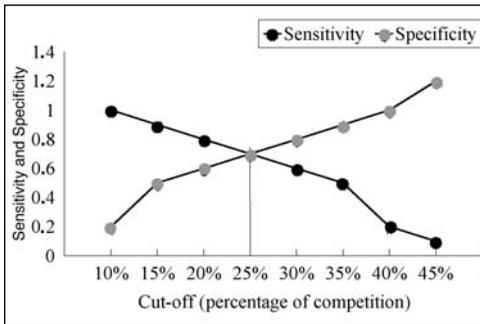
Positive predictive value =  $63/(63+1) \times 100 = 98.43\%$

Negative predictive value =  $73/(73+6) \times 100 = 92.40\%$

## RESULTS

The trapping polyclonal antibody produced in goats presented non-specific reactivity. The trapping antibody tested by indirect ELISA at a 1:5 dilution (12.5 µg/mL) showed a mean

OD of 0.094 at 490 nm (respective blank mean, OD = 0.024). As shown in Tables 1 and 2, the proportion of positive sera by FAVN and SC-ELISA (titer  $\geq 0.5$  IU/mL) was 91.37% for dogs and 95.31% for cows. The



**Figure 1.** Curves of relative sensitivity and specificity of the SC-ELISA (A) and LPC-ELISA (B) using the TG-ROC analysis. The accuracy level used was 95%.

sensitivity and specificity curves of the SC- and LPC-ELISA as functions of the cut-off used are shown in Figure 1 (A and B). By TG-ROC analysis, the intersection point of the 2 curves indicates a cut-off point of 25% and 20% (percentage of competition) for the SC- and LPC-ELISA, respectively. As shown in Tables 1 and 2, the SC-ELISA presents a relative sensitivity and specificity of 93.75% and 94.73%, respectively, for dog sera; 95.31% and 71.42% for cow sera. On the other hand, the LPC-ELISA presents a sensitivity and specificity of 99.49% and 98%, respectively, for dog sera; 98.43% and 86.90% for cow sera.

The relationship between the SC- and LPC-ELISA and FAVN test are shown also in Tables 1 and 2. Of all dog sera tested by SC-ELISA, 45 were negative and 180 were positive. Two dog sera (4%) were positive only with the FAVN, whereas 3 (6%) were positive with SC-ELISA (Table 1), when naive sera were tested. With regard to vaccinated dogs ( $n = 197$ ), 4 (2%) samples presented negative for FAVN and positive for SC-ELISA. The relative sensitivity of SC-ELISA was 94.73% and specificity was 93.75%; accuracy between them was 91.37% ( $P < 0.0001$ ). The positive (97.82%) and negative (95.74%) predictive values also are shown. When the LPC-ELISA was applied with the same purpose, the relative specificity was 98.00% and sensitivity was 99.49%. The accuracy between LPC-ELISA and FAVN test was 100%, higher than SC-ELISA (91.37%), when the dog sera were tested.

When the cow sera were tested, the best accuracy was observed between LPC-ELISA and FAVN test (100%). The relative specificity observed by the use of SC-ELISA was 71.42%, when 11 sera were positive by FAVN standard method and negative by SC-ELISA (13%). However, the LPC-ELISA relative sensitivity was 98.43%, relative specificity 86.90%, positive predictive value 98.43%, and negative predictive value 92.40%.

There was good agreement between the LPC-ELISA and FAVN test ( $r = 0.893$ ), higher than that observed between SC-ELISA and FAVN test ( $r = 0.773$ ). By convention, kappa values of 0.8 to 1.0 express almost perfect agreement between the 2 tests.

## DISCUSSION

The analysis of the serological response against rabies makes use of the MNT, serum neutralization (or VN) test, and the ELISA methods.<sup>2,13</sup>

Attempts have been made to relate the results obtained, in particular those from FAVN, to the protective immune response of the donor animals of the sera. In the present paper, the antibodies from dogs and cows vaccinated against rabies virus were analysed using 2 different ELISA methods of which the LPC-ELISA demonstrated close relationship with the FAVN (accuracy of 100%).<sup>10,11,16</sup>

In fact, if quality control measures are maintained for all reagents used in a serological test for rabies, many different methods

can accurately measure an immunological response to rabies vaccine. It has been proved that rapid fluorescent focus inhibition test and FAVN can adequately measure antibody levels for animals being vaccinated.<sup>11,12,22</sup> Certain ELISA methods, in particular “competitive” ELISA, may have a number of advantages over serum neutralization test, but no test that has been studied produced results that could be unequivocally interpreted as indicative of protection against challenge. Moreover, ELISA methods do not measure protection against rabies “in vivo,” since they are incapable of measuring the immune response required for that “in vivo” protection.<sup>22</sup> The protective immune response against rabies virus relies on the interaction of virus-specific antibody with the virus glycoprotein described before.<sup>25</sup>

Regarding the LPC-ELISA performed here, the use of polyclonal instead of monoclonal antibody demonstrated usefulness in measuring dog and cow antibodies. Polyclonal immunoreagents are cheaper than monoclonal antibodies for the competitive ELISA methods and also easy to use, dispensing special skills and showing adequate applicability, as observed in our study, for detecting sera from vaccinated dogs and cows. These findings were confirmed by the sensitivity, specificity, and accuracy determined here.

Therefore, it should be noted that some epitopes of virus glycoprotein induce production of non-neutralizing antibodies.<sup>25</sup> Based on the fact that virus glycoproteins induce the neutralizing antibodies, serological tests that measure the vaccination response should have a good correlation with FAVN. The LPC-ELISA described above, together with TG-ROC analysis for cut-off determination, showed statistically significant test indices compared with those from earlier studies, particularly a close correlation coefficient and a high level of agreement with FAVN.<sup>24,25</sup> Finally, the LPC-ELISA demonstrated to be a very useful method and able to replace FAVN in seroepidemiologic surveys in undeveloped countries, where the rapid large-scale process-

ing of sera is necessary for the understanding of vaccination campaigns efficiency.

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