

Comparison between the Counter Immunoelectrophoresis Test and Mouse Neutralization Test for the Detection of Antibodies against Rabies Virus in Dog Sera

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The detection of rabies antibodies is extremely valuable for epidemiological studies, determination of immune status in man, animals, and for the diagnosis of the disease. Several serological procedures have been described for this purpose. The present study reports a comparison between counterimmunoelectrophoresis test (CIET) and mouse neutralization test (MNT) in the detection of antibodies against rabies virus from 212 serum samples of vaccinated dogs. The agreement between both techniques was 79.7% and a significative association was demonstrated. The correlation coefficients between MNT and the CIET titers was determined considering 88 samples showing positive results in both techniques [CIET = 2 and MNT = 5 (0.13 IU/ml)] and resulted $r^2 = 0.7926$ ($p < 0.001$). The performance of CIET system was technically simple, cheap and rapid, and thereby it could be useful for serological monitoring of dog vaccination campaigns as well as for individual analysis.

Key words: rabies - rabies antibodies - mouse neutralization test - counterimmunoelectrophoresis test

The most effective way to control rabies is the vaccination of dogs still the most important reservoirs of the disease in several parts of the world. In fact, the resistance to rabies virus infection induced by vaccination is associated with neutralizing antibody production and many procedures have been described for detecting or measuring those antibodies. Mouse neutralization test (MNT), developed by Atanasiu (1967) was used for many years as a standard virus neutralization test which was substituted by microneutralization tests in cell culture as rapid fluorescent-focus inhibition test (RFFIT), developed by Smith et al. (1973), and more recently the fluorescent antibody virus neutralization test (FAVN test) developed by Cliquet et al. (1998).

In 1977 a modified counterimmunoelectrophoresis (CIE) technique has been developed for determining rabies antibodies and was described as a sensitive, simple, inexpensive and relatively rapid procedure (Diaz & Varela-Diaz 1977). The method provided results that correlated well with those obtained by the MNT, the indirect fluorescent-antibody test and the RFFIT (Diaz & Myers 1980, 1981, Diaz 1983). The technique was also used to determine the antigens content in lots of suckling mouse rabies and tissue culture rabies vaccines (Miceli et al. 1992, 1993), and to determine the antibody titer in hyperimmune antirabies sera used for human prophylactic treatment (Diaz & Myers 1984). Initially developed to determine rabies

antibodies in human sera (Diaz & Varela-Diaz 1977, Diaz et al. 1986, Chauhan et al. 1991) it was also used for equine (Diaz & Myers 1984, Diaz et al. 1995), bovine (Albas et al. 1995b, Oliveira et al. 2000), ovine (Sanchez & Rubio 1991), and canine sera (Albas et al. 1995a). The present study reports a comparison between counterimmunoelectrophoresis test (CIET) and mouse neutralization test in the detection of antibodies against rabies virus in sera of dogs immunized along vaccination campaigns.

MATERIALS AND METHODS

Dogs sera and vaccination procedure - Serum samples ($n = 212$) were obtained from 16 non-vaccinated dogs and from 67 vaccinated with 2 ml of Fluenzalida's type vaccine (inactivated suckling mouse brain vaccine) by subcutaneous administration during vaccination campaigns. In order to obtain different level of antibody titers, blood samples were taken from dogs that received different number of vaccine doses during their lifetime and also at different periods of vaccination: one, six, nine and twelve months.

CIE antigen and indicator serum - The strain used was the Challenge Virus Standard (CVS 31/2) obtained from Instituto Biológico, São Paulo, Brazil, passed twice in suckling mouse brain. The antigen was prepared in suckling mouse brain and the indicator serum was produced by the immunization of rabbits with the rabies vaccine according to the procedures established by Diaz (1985).

CIET - The CIET was performed essentially by the procedure described by Diaz (1985). In brief, serial of two-fold dilution beginning from 1:5 to 1:40 was prepared. An equal volume of fixed dilution (1:40) of antigen, determined by chessboard procedure, was added to each dilution of dog sera and incubated for 37°C for 60 min. After

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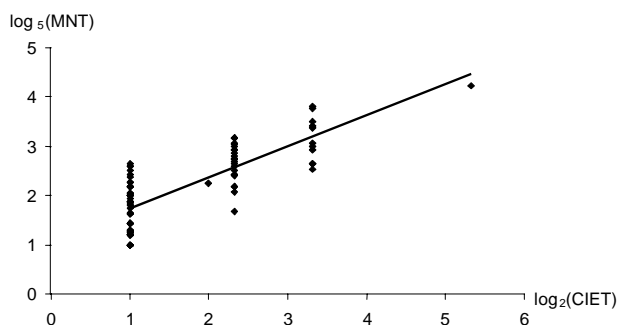
that, the serum-virus mixture in the cathode wells was electrophoresed in agarose gel at 0.9% at a constant current of 10mA per slide during 45 min. Following, the anodal wells were filled with indicator serum (1:4) and further electrophoresed for 120 min at the same current. Negative and standard positive controls were tested simultaneously in each run. The slides were maintained overnight in phosphate buffer saline (PBS) and the precipitin bands were observed with oblique lighting as well as stained with amido black. The highest dilution of each serum showing no precipitation line was taken as endpoint and the titer was expressed as the reciprocal of the dilution.

MNT - Serial five-fold dilution of the test and standard sera were made in a PBS pH 7.3. To 0.4 ml of diluted serum an equal amount of CVS (challenge virus standard) strain, containing 30-50 LD₅₀/0.03 ml was added, and the mixture incubated at 37°C for 90 min. Each dilution of the serum-virus mixture was inoculated intra-cerebrally into each group of six mice of either sex. After an observation period of 15 days, the titer was determined by the Reed-Müench method (Reed & Müench 1938). An international reference serum (WHO Standard for Rabies Immunoglobulin from National Institute for Biological Standard and Control) containing 30 IU/ml was used to calculate the titers in IU (International Units)/ml.

Statistical analysis - For the purposes of statistical analysis MNT titers were expressed as log₅ of the reciprocals and CIET titers as log₂ of the reciprocals. The Pearson linear correlation coefficient (*r*) between two variables was determined considering 88 samples showing positive results in both techniques [CIET = 2 and MNT = 5 (0.13 IU/ml)] and the regression analysis was used to fit a straight line throughout a set of points. The chi-square test was used to check for an association between the titers obtained by both techniques (n = 212).

RESULTS

The positive and negative results were determined with the cutoff bring as ≥ 2 for CIET and ≥ 5 (0.13 IU/ml) for MNT. Table I shows the co-specificity (83.5%) and co-sensitivity (76.5%) of CIET for antibody detection. Table II shows in details, the frequency distribution of results according to the antibodies titer in each test, expressed as IU/ml (MNT) and the reciprocals of serum dilution as described before for CIET. The agreement between CIET



Linear regression line for antibody titers obtained by mouse neutralization test-MNT (log₅) and by counterimmunoelectrophoresis test-CIET (log₂); $y = 1.102839 + 0.631282x$; $r = 0.7926$

TABLE I

Comparative results of the counterimmunoelectrophoresis test (CIET) and mouse neutralization test (MNT) for determining rabies antibodies in vaccinated dogs sera

CIET	MNT		Total
	Positive	Negative	
Positive	88	16	104
Negative	27	81	108
Total	115	97	212

Co-specificity = 83.5%; Co-sensitivity = 76.5%; Agreement = 79.7%; $\chi^2 = 75.865$ ($p < 0.0001$)

and MNT was 79.7% and a significant association between them at 0.01% of probability ($\chi^2 = 75.865$) was demonstrated. The correlation coefficient between the CIET and the MNT for a total of 88 serum samples positive in both techniques was $r = 0.7926$ ($P < 0.001$). The linear regression equation that resulted on the straight line given in the Figure was $y = 1.1028 + 0.6313x$ indicating the minimum estimated MNT value which corresponds to a certain CIET/MNT titer.

DISCUSSION

Dog sera had already been studied by Diaz and Varella-Diaz (1977) in their original description of the CIET for detection of antibodies to rabies virus and by Albas et al. (1995a). In most of the experiments developed to have

TABLE II

Frequency distribution for results of antibodies titer obtained by the counterimmunoelectrophoresis test (CIET) and mouse neutralization test (MNT) in sera of vaccinated dogs

CIET titer ^b	MNT titer ^a					Total
	< 0.13	0.13 – 0.62	0.63 – 3.12	3.15 – 15.62	> 15.62	
< 2	81	27	0	0	0	108
2 - 4	13	38	15	0	0	66
5 - 10	3	1	22	10	0	36
20 - 40	0	1	0	0	1	2
Total	97	67	37	10	1	212

a: titers expressed as IU/ml; b: titers expressed as reciprocals of serum dilutions

those antibodies evaluated, both in human and animal sera, the correlation coefficient ranged from $r = 0.58$, in humans (Diaz & Myers 1980) to $r = 0.85$ in ovine (Sanchez & Rubio 1991) and $r = 0.91$ in bovine (Albas et al. 1995b). Albas et al. (1995a) obtained a correlation coefficient $r = 0.76$ for dog sera which was very similar to the results observed in the present study ($r = 0.79$). According to Sanchez and Rubio (1991) the original description of the CIET mentioned that a correlation coefficient equal or superior to 0.70 indicates an acceptable association.

Positive results was established as those higher than 5 in the MNT, that corresponded to 0.13 IU/ml. The protective titer of 0.5 IU/ml recommended by WHO corresponded in our experiments to a titer of 20 (reciprocal of dilution). The titer in IU/ml was not established for the CIET. All the 16 non-vaccinated dogs showed negative results in both CIE and MN tests. From the 27 sera showing negative results in CIET and positive results in MNT, 52% (14/27) presented the minimum considered titer for the experiment (0.13 IU/ml), 37% (10/27) titers from 6 (0.15 IU/ml) to 10 (0.25 IU/ml) and 11% (3/27) from 11 (0.27 IU/ml) to 25 (0.62 IU/ml) (data not shown on the Tables).

The above mentioned studies did not describe the values obtained for sensitivity, specificity and agreement of CIET in comparison to MNT but most of the results showed a specificity higher than the sensitivity (Diaz & Myers 1980). Using a higher number of serum samples ($n = 212$) and comparing the CIET with the MNT (standard test) the results of this experiments also showed higher values for CIET specificity compared to sensitivity.

Although other recent techniques, as efficient and quick as CIET, could be used for measuring antibody titers to rabies virus (Piza et al. 1999), CIET is described as measuring antibodies directed to the viral glycoprotein and consequently detecting the neutralizing potential of the studied sera (Diaz & Myers 1981). The CIET has the advantages of being a relatively simple, quick and economical method compared to MNT and other methods like RIFFT (Smith et al. 1973) and FAVNT (Cliquet et al. 1998). Furthermore, it could be useful for the evaluation of antibody titer in dog population after massive vaccination campaigns and because of its higher specificity, it might also be useful to individual analysis.

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