

SIMPLIFIED FLUORESCENT INHIBITION MICROTEST FOR THE TITRATION OF RABIES NEUTRALIZING ANTIBODIES

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SUMMARY

A simplified fluorescence inhibition microtest (SFIMT) was standardized for the evaluation of antirabies serum neutralizing antibodies based on the rapid fluorescent focus inhibition test (RFFIT) and the fluorescence inhibition microtest (FIMT).

The simplified test showed reproductibility similar to that of the FIMT with advantages as easier execution and quicker reading.

A simple pre-treatment of Brazilian microplates produced for immune enzymatic assays (PROSIL) gave equivalent results and substantial coast reduction, in relation to imported plates (DIFCO).

The simplified test can be easily implemented in less sophisticated laboratories, as alternative to the mouse serum neutralization test, still the most largely employed in Brazil, or even to others as RFFIT and FIMT.

KEY WORDS: Simplified Fluorescence Inhibition Microtest; rabies antibodies

INTRODUCTION

In 1935 WEBSTER & DAWSON⁽⁹⁾ developed an "in vitro" test for the detection of antirabies neutralizing antibodies. These antibodies were associated with protection⁽¹⁰⁾, and the mouse serum-neutralization test (MNT) was thereafter accepted as a good parameter for the evaluation of antirabies immunity. Nevertheless, it has some important drawbacks such as the requirement of a large number of animals, its high cost and a long observation period until the obtention of final results. Furthermore the reproducibility of the test depends on many factors, being one of these the difference in susceptibility of the mice⁽⁹⁾.

To overcome these difficulties several "in vitro" tests were developed, such as the indirect immunofluorescence (IFT)⁽⁸⁾, contraimmunoelectroforesis (CIET)⁽¹⁾, complement fixation (CFixT)⁽⁵⁾, passive hemagglutination (PHAT)⁽²⁾, immuno adherence (IAT)⁽¹¹⁾ and rapid fluorescent focus inhibition (RFFIT)⁽⁷⁾. In comparative studies the RFFIT was selected as the most appropriate because unlike the others it detects exclusively neutralizing antibodies, it correlates strictly with

MNT, being more rapid to perform and presenting a better reproductibility. Based on these observations and principles of RFFIT, ZALAN et al.⁽¹⁰⁾ developed a micro technique they named fluorescence inhibition microtest (FIMT) associating the advantages of RFFIT with those of the microtechniques.

These authors clearly showed that FIMT presents a close agreement with MNT but, differing at times from IFI. Thus, such results allowed to classify this test in the group of those capable to evaluate only neutralizing antibodies in which RFFIT is also included.

In our country, the detection and evaluation of antirabies antibodies rely mainly on tests such as, MNT, IFT and CIET but, all these are inferior to seroneutralization in cell culture by the afore mentioned reasons.

The objective of the present study is the simplification of the FIMT introducing few modifications, without loss of its practical and reproducible

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features, aiming its routine easier use, even in more modest laboratories. For the evaluation of the obtained results, similar parameters to the previous FIMT standardization⁽¹⁰⁾ were investigated as to permit a better comparison between the new SFIMT and the original FIMT.

MATERIAL AND METHODS

Sera

Thirty human serum samples were collected from individuals with no previous history of antirabies immunization and no detectable antibodies by IFT, and were tested twice in different days by SFIMT. Ninety nine human serum samples collected in different periods after immunization were also tested twice in different days by the same techniques.

As standard serum, a hiperimmune antirabies serum was prepared at the Institut Pasteur of Paris for utilization in human treatment, lot R 5672, containing 200 IU/ml. This serum was titred 36 times in different days, 18 times on two different types of microplates.

Virus

The Pasteur Virus strain, kindly provided by Dr. Pierre Perrin from Institut Pasteur of Paris, was propagated in BHK-21 cell monolayer⁽⁶⁾. The viral suspension ($10^{3.39}$ $DL_{50}/0.03$ ml) was used in a 1:4 dilution, corresponding to a 0.07 $DL_{50}/cell$.

Cell culture

The BHK-21 cell lineage grown in Minimum Eagle medium containing 10% bovine fetal serum was used for viral propagation as well as in the microplates.

Microplates

Two types of 96 well microplates were supplied by:

1- Prosil Indústria e Comércio Ltda. (Petropolis, Rio de Janeiro): the microplates are produced for enzyme immune assay and made of flexible PVC with a thin thickness enabling to focus in a microscope immersion objective. The plates were immersed in HCl 0.1N for 12 hours

and then in neutral soap solution for an equal period. After rinsing for 2 hours in running water, they were again rinsed in distilled water and subsequently in deionized water, dried at 37°C and stocked. They were sterilized by U.V. in a laminar flux before used. We are still using, without problems, Prosil microplates treated 18 months ago.

2- Difco Comércio e Indústria, for cell culture.

Simplified fluorescence inhibition microtest (SFIMT)

a) Serum dilutions

The sera were distributed into two series of two fold dilutions beginning from 1/5 and 1/7.5 in a volume of 100µl per well. The standard sera had initial dilutions of 1/800 and 1/1200. A 50 µl of an optimal viral suspension previously titered was added to each well, followed by an incubation period of 90 minutes at 37°C. A 50 µl of cell suspension (10^6 cells/ml) was then added for each well. The microplates were reincubated at 37°C for 24 hours in a CO₂ atmosphere. A satisfactory cellular growth was obtained in the CO₂ provided by the addition of 0.5g anti-acid tablet (Sonrisal-The Sidney Ross Co) in H₂O, as the microplates⁽¹⁴⁾ were kept in a hermetically closed pack of 3000 cm³. A viral antigen titring with double dilutions from 1/1 to 1/8 was performed in each test.

b) Fluorescence antibody staining

Before cell fixation the medium was withdrawn by careful aspiration and the microplates were cooled on ice. Eighty per cent acetone in water (stocked at -20°C) was added. After 10 minutes, the plates were emptied by inversion and dried at 37°C. The staining was carried out by adding 40 µl of an optimal dilution of the antirabies fluorescent conjugate. After an 1 hour incubation at 37°C the microplates were washed by immersion in PBS for 5 minutes and then in distilled water.

To each well it was added pH 8,5 glicerol (carbonate-bicarbonate buffered) in a volume sufficient to cover the cells (about 40µl).

c) Reading

The Difco plates were read in inverted position in a microscope without a "charriot", with

X100 magnification. The Prosil plates were cut, mounted in inverted position over microscope slides and read with immersion objective.

The results expressed the dilution corresponding to the well with 50% decrease of infection. The comparison of the results of unknown sera and of standard serum served as basis for obtaining the titres in I.U.

Indirected Immunofluorescence test (IFT): the IFT was performed according to THOMAS et al⁽⁹⁾.

RESULTS

The serum samples obtained from individuals with no history of rabies vaccination showed negative results by seroneutralization assay in cell culture, in two different occasions.

Table 1 shows the distribution of the medium antibody titres of 99 duplicated sera from vaccinees expressed in terms of dilution and I.U.

The individual results were arranged according to the percentage of deviation from the arithmetic means as shown in table 2. As can be seen, 138 (70.1%) and 188 (94.8%) of the results had a mean deviation of less than 33.3% and 45.5%, respectively.

Table 3 presents the results obtained with the standard serum in 36 tests performed in different days, 18 with each type of plate used. The deviations from the arithmetic mean were always inferior to 33.3%.

DISCUSSION

Our data indicate that the FIMT test, standardized by ZALAN et al⁽¹⁰⁾ taking RFFIT as basis, was simplified (SFIMT) without impairing its fundamental qualities.

Brazilian (Prosil) microplates could be used after a simple, a rapid and non-expensive pre-treatment in acid solution. The treated microplates may probably be kept for an indefinite period requiring only sterilization just before their use.

Employing antiacid tablets we managed a CO₂ production in levels that sufficed the cellular growth and viral replication, obviating the use of HEPES buffer, even when a CO₂ incubator is not available.

We adopted the reading standards according to LAFON⁽⁴⁾, which is simpler and quicker than those established for the FIMT. The occasional loss of precision was compensated by the utilization of 2 alternating series of twofold serum dilutions.

The results were found to be reproducible, since a constant pattern was seen even when obtained in different days and by different observers. There was no advantage of one type of microplate over the other for readings. However the Brazilian plate permitted a better visualization for details and the process of setting each serum titration on a microscope slide eliminated occasional mistakes.

Associating the simplified reading with the type of serum dilutions here adopted, the reproducibility of the SFIMT was maintained in the comparable levels to that described for the FIMT, i.e. approximately 70% of the titres in duplicate showed deviations of the arithmetic mean lower than 33.3%. The results of 36 titrations the standard serum in different days did not exceed this level.

Some sera, mainly the visibly lipemic, altered the cell morphology in the first dilutions (1/5 and 1/7.5) without hampering the cellular infection. Such effect, more evidenced in the Prosil plates, disappeared at all in the subsequent dilutions.

The data here obtained suggest that SFIMT is a simple, quick and low cost assay with a reproducibility similar to that of the FIMT, and may be carried out with advantage in the routine quantitative evaluation of antirabies neutralizing antibodies.

RESUMO

Microteste de inibição de fluorescência simplificado para titulação de anticorpos antirábicos

No presente trabalho foi padronizada uma microtécnica simplificada para a avaliação de anticorpos neutralizantes anti-rábicos com base no RFFIT e no FIMT.

Este teste mostrou reprodutibilidade comparável a dos testes originais, sendo de execução mais simples e de leitura mais rápida.

Uma microplaca de fabricação nacional para testes imuno-enzimáticos (PROSIL Ind. e Com.) pode ser utilizada com resultados equivalentes aos de

Table 1
Medium antibody titres of 99 duplicated sera from vaccinees expressed in dilution and IU/ml.

DILUTION RANGES	IU/ml	Nº OF SERA
20 —— 30	0,20 —— 0,31	4
30 —— 40	0,31 —— 0,42	5
40 —— 60	0,42 —— 0,62	10
60 —— 80	0,62 —— 0,83	14
80 —— 120	0,83 —— 1,25	18
120 —— 160	1,25 —— 1,66	15
160 —— 240	1,66 —— 2,50	15
240 —— 320	2,50 —— 3,33	10
320 —— 480	3,33 —— 4,99	4
480 —— 640	4,99 —— 6,66	2
> 640	> 6,66	2

Table 2
Correlation between the percentage deviations from the arithmetic means and cumulative frequency deviations

PERCENTAGE DEVIATIONS FROM ARITHM. MEANS (RANGES)	SERA			
	Nº	%	CUMULATIVE FREQUENCY	
			Nº	%
6.0 —— 14.3	66	33.3	66	33.3
14.3 —— 20.0	32	16.1	98	49.4
20.0 —— 33.3	42	21.2	140	70.6
33.3 —— 45.5	50	25.2	190	95.8
45.5 —— 50.0	4	2.0	194	97.8
50.0 —— 60.0	2	1.0	196	98.8
60.0 —— 68.4	2	1.0	198	99.8

Table 3

Evaluation of two different types of microplates by 36 titrations of the standard serum carried out in different days.

MICROPLATE	Nº TESTED	TITER	IU/ml	DEVIATION FREQUENCY
DIFCO	3	12800	133	33.3
	12	19200	200	-
	3	25600	266	33.3
PROSIL	6	12800	133	33.3
	6	19200	200	-
	6	25600	266	33.3

microplacas Difco ou similar, com substancial redução de custos, mediante tratamento simples e rápido.

O teste estudado pode ser implantado facilmente em laboratórios menos sofisticados substituindo com grandes vantagens a prova de soro-neutralização em camundongos ainda a mais empregada rotineiramente no Brasil.

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