



WORLD HEALTH ORGANIZATION
ORGANISATION MONDIALE DE LA SANTE

DISTR.: LIMITED
DISTR.: LIMITEE

WHO/Rab.Res./88.29

ORIGINAL: FRENCH

RAPID RABIES ENZYME IMMUNODIAGNOSIS KIT¹

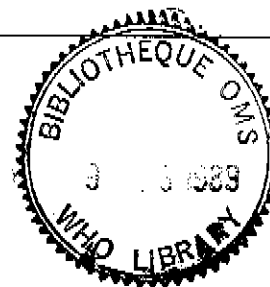
23796

This document describes the components and provides guidance for the use of the Rapid Rabies Enzyme Immunodiagnosis (RREID) kit.

This technique, as well as the kit, have been developed by the WHO Collaborating Centre for Reference & Research on Rabies, Pasteur Institute, Paris.² The specificity and sensitivity of this technique, as well as the simplicity of the kit have been assessed in 1986 and 1987 by the Collaborating Centre through collaborative studies conducted in six European and North American laboratories, and in twelve laboratories located in various countries in Africa, Latin America and Asia (see paragraph 5). The technique was shown to have a specificity and sensitivity comparable to that of the fluorescent antibody test (FAT).

In view of its simplicity and the possibility of reading the results with the naked eye, the kit is particularly useful in large-scale epidemiological surveys and in laboratories which do not use FAT.

This document is primarily intended for the personnel in rabies diagnosis laboratories.



¹By H. Bourhy, P. Perrin and P. Sureau, WHO Collaborating Centre for Reference & Research on Rabies, Pasteur Institute, 28 rue du Docteur Roux, 75724 Paris Cédex 15, France

²P. Perrin, P.E. Rollin and P. Sureau. A Rapid Rabies Enzyme Immunodiagnosis (RREID): A useful and simple technique for the routine diagnosis of rabies, *J. Biol. Stand.*, 1986, 14, 217-222.

This document is not issued to the general public, and all rights are reserved by the World Health Organization (WHO). The document may not be reviewed, abstracted, quoted, reproduced or translated, in part or in whole, without the prior written permission of WHO. No part of this document may be stored in a retrieval system or transmitted in any form or by any means - electronic, mechanical or other without the prior written permission of WHO.

The views expressed in documents by named authors are solely the responsibility of those authors.

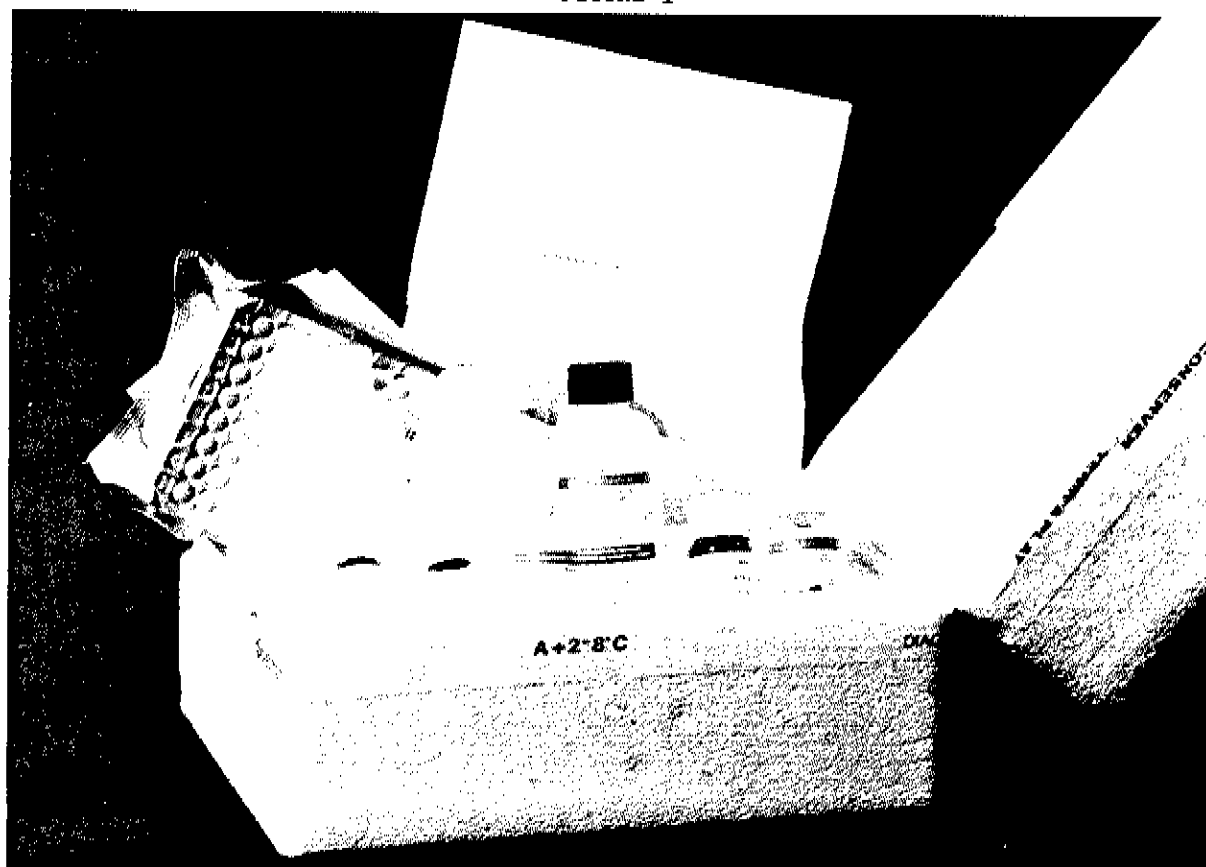
Ce document n'est pas destiné à être distribué au grand public et tous les droits y afférents sont réservés par l'Organisation mondiale de la Santé (OMS). Il ne peut être commenté, résumé, cité, reproduit ou traduit, partiellement ou en totalité, sans une autorisation préalable écrite de l'OMS. Aucune partie ne doit être chargée dans un système de recherche documentaire ou diffusée sous quelque forme ou par quelque moyen que ce soit - électronique, mécanique, ou autre - sans une autorisation préalable écrite de l'OMS.

Les opinions exprimées dans les documents par des auteurs cités nommément n'engagent que lesdits auteurs.

1. THE DIAGNOSIS KIT

1.1 Components

FIGURE 1



The diagnosis kit consists of an insulating polystyrene package containing instructions and the reagents required for carrying out the test (see Figure 1). Characteristics of the reagents are specified below. Each component is labelled with a code (R1 to R10).

- R 1 1 microplate with 96 wells. Each well is sensitized with purified antinucleocapsid antibodies. The plate is wrapped in a vacuum-sealed aluminium envelope. Each plate can be divided into 6 strips of 16 wells each.
- R 2 1 bottle of washing solution (concentrated 20 times).
- R 3 1 bottle of negative control antigen (lyophilized supernatant of homogenized brain from uninfected mice).
- R 4 1 bottle of positive control antigen (lyophilized supernatant of homogenized brain from mice infected with the CVS-strain of the rabies virus).
- R 5 1 bottle of antinucleocapsid antibodies conjugated with peroxidase (10 times concentrated).
- R 8 1 bottle of buffer containing the substrate for the enzymatic reaction.

- R 9 1 vial containing 8 tablets of chromogen (O-phenylenediamine).
R 10 1 vial containing the stopping solution for the enzymatic reaction (4N sulphuric acid).

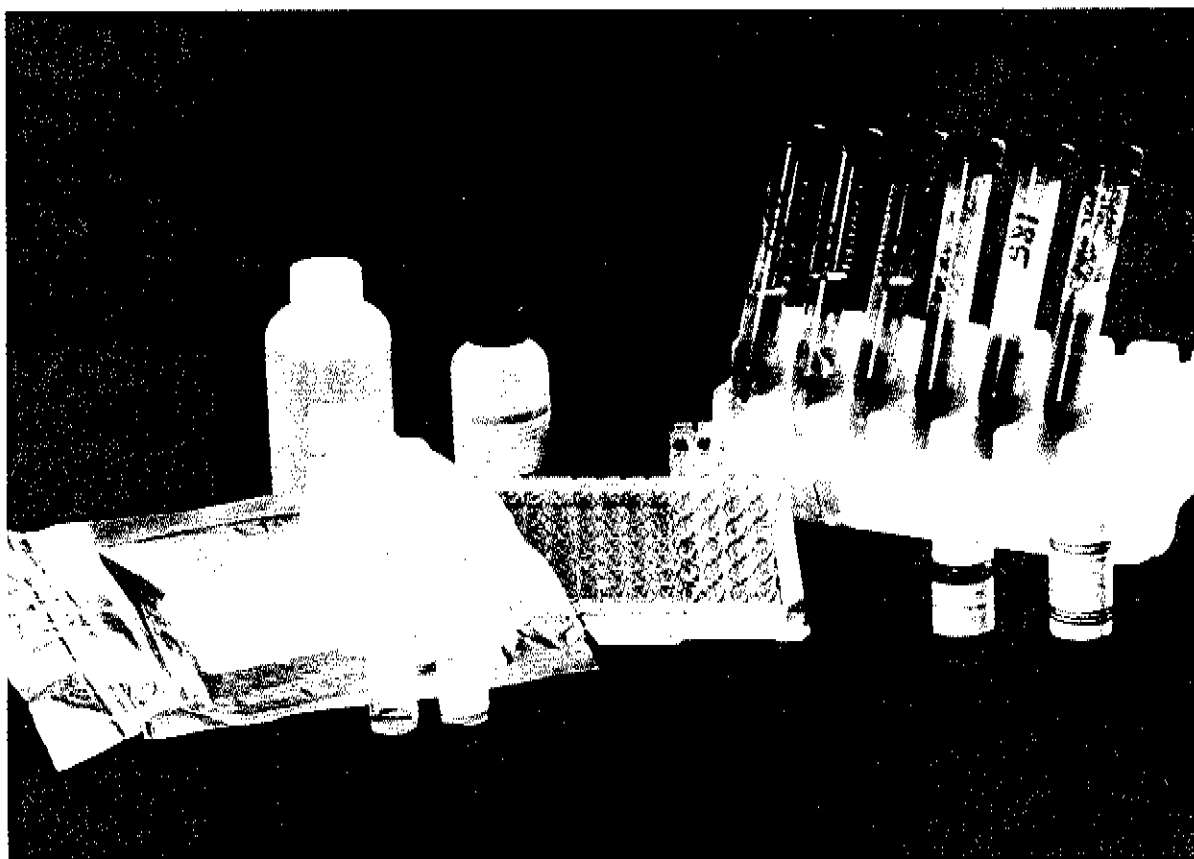
1.2 Storage conditions and shelf-life

The kit should be stored at 4°C. Should it be stored unreconstituted at this temperature, each component remains active till the date shown on the label on the outer package.

2. RECONSTITUTION OF THE REAGENTS

Open the aluminium package containing the sensitized microplate (R1) and remove requisite number of strips. Close the package and keep the unused strips at 4°C (see Figure 2).

FIGURE 2



Dilute washing solution (R2) in 20 parts distilled water before use.

Reconstitute negative control antigen (R3 - inactivated by beta-propiolactone) with 1 ml distilled water.

Reconstitute positive control antigen (R4 - inactivated by beta-propiolactone) with 1 ml distilled water.

Dilute antinucleocapsid antibodies conjugated with peroxidase (R5) in 10 parts washing solution (R2) in the quantity required.

The buffer (R8) - Citrate 0.05 M pH = 5.6; Oxygenated water 0.03%; and Sodium merthiolate 0.01% - is ready for use.

Dissolve one tablet of chromogen (R9 - ortho-phenylenediamine) in 15 ml. citrate buffer (R8), using plastic tweezers to pick up the tablets.

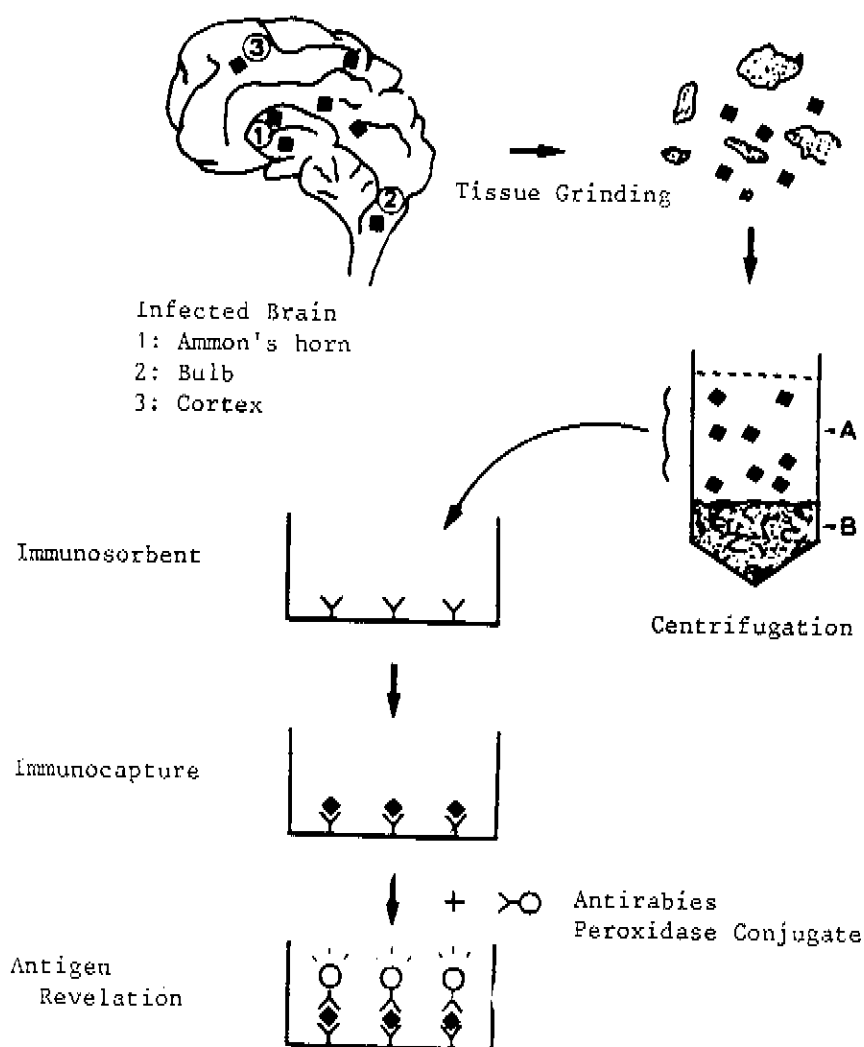
The stopping solution (R10 - 4N sulphuric acid) is ready for use.

3. METHODS

3.1 Principle of the technique (see Figure 3)

The samples are homogenized in a buffered saline solution and centrifuged to eliminate particles of debris. The supernatants are then incubated in wells of microplates that have been sensitized with rabies antinucleocapsid antibodies. The presence of any rabies nucleocapsids in the samples is then revealed by antinucleocapsid antibodies conjugated to peroxidase. The appearance of a yellow colour after introduction of the chromogen substrate mixture shows that rabies nucleocapsids are present in the sample.

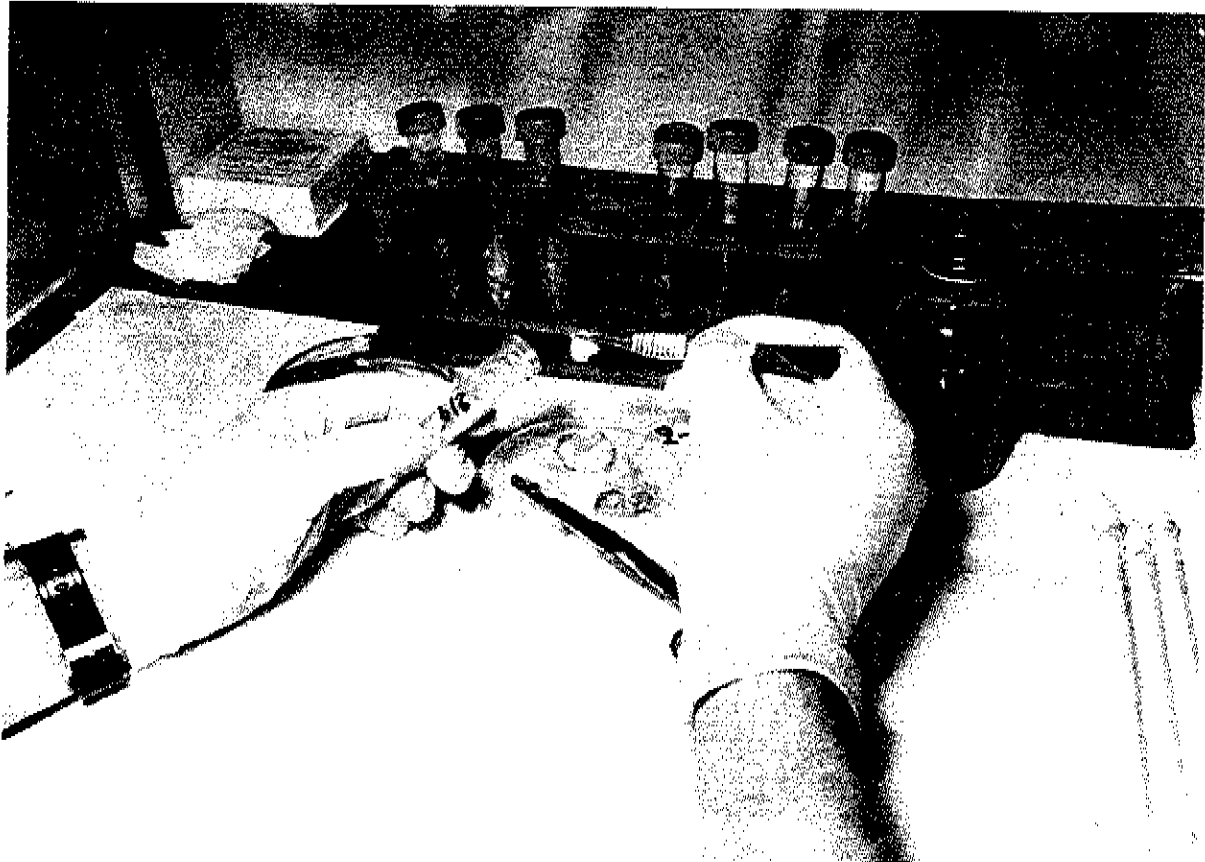
FIGURE 3



3.2 Preparation of samples

A piece of spinal bulb, cortex and Ammon's horn from each brain* are mixed and homogenized at 30% (weight/volume) in culture medium, phosphate buffer pH = 7.2, or washing solution R2 (see Figure 4). The samples are then centrifuged at 3000 rpm for 20 minutes to eliminate gross brain particles. The clear supernatant is used for the rest of the test. This supernatant may be inactivated by heating it in a water bath for two hours at 56°C.

FIGURE 4

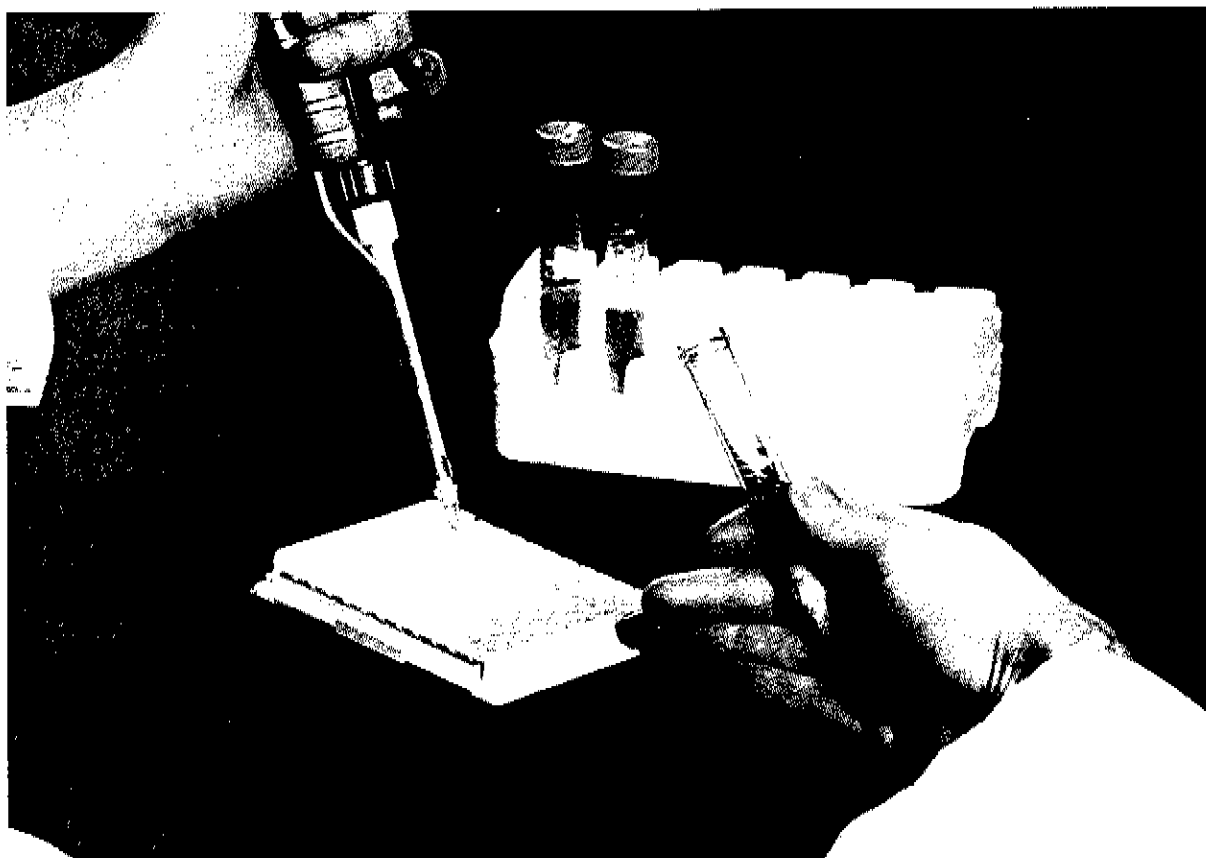


*A carcass believed to be infected with rabies should be handled only by someone vaccinated against rabies, wearing gloves, mask and visor.

3.3 The test

(a) Put 200 μ l of each of the control antigens in one or two wells of the microplate, e.g., negative control (R3) in well(s) 1A/1B and positive control (R4) in well(s) 1C/1D. Then, distribute 200 μ l of the supernatants of the samples to be tested in one or two of the following wells (e.g., wells 1E/1F for specimen S1, 1G/1H for S2, etc.). If an automatic plate reader is used, the first set of wells, which will serve as a blank control for photometric readings, receives 200 μ l of washing solution (R2).

FIGURE 5



(b) Incubate the microplate in a humid atmosphere at 37°C for one hour.

(c) Remove the products from the plate by suction* and wash the wells with washing solution (see Figure 6) at least 5 times.

*Warning : These products are potentially virulent. Recipients for collecting fluids by suction should contain a bleach solution.

FIGURE 6



- (d) Put 200 μ l of peroxidase conjugate (R5) in each well.
- (e) Incubate the microplate in a humid atmosphere at 37°C for one hour.
- (f) Remove the conjugate from the plate by suction and wash the wells with washing solution at least 6 times.
- (g) Put 200 μ l of substrate chromogen mixture (R8 + R9) in each well.
- (h) Allow colour to develop for 5 minutes in darkness and at room temperature.
- (i) Stop the reaction after 20 or 30 minutes, depending on speed of colouration, by adding 50 μ l of stopping solution (R10) to each well.
- (j) Read the colour either with the naked eye or with a photometer at 492 nm depending on requirements and equipment.

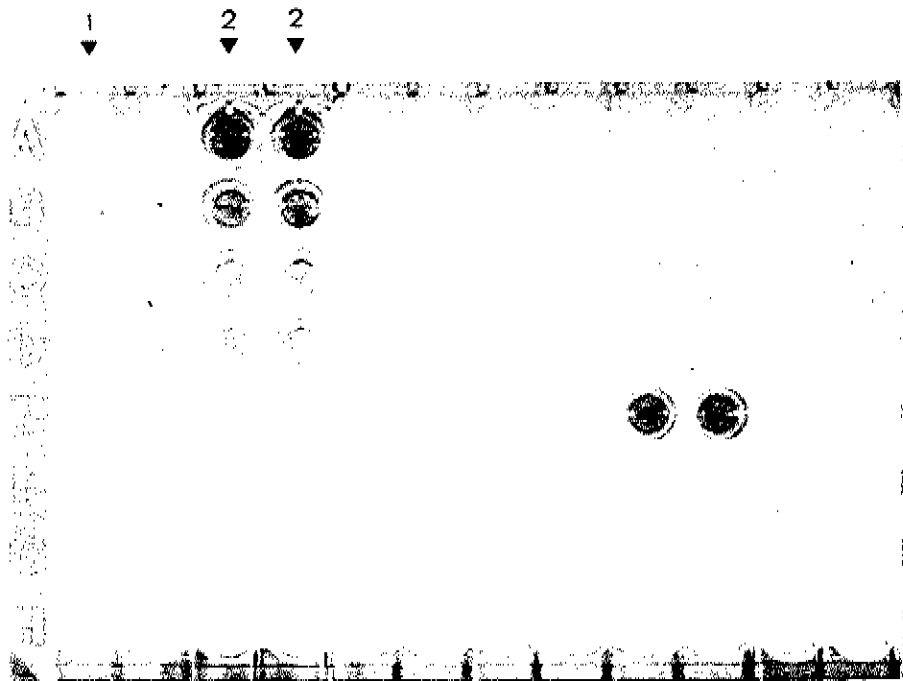
4. INTERPRETATION OF RESULTS

4.1 Reading with the naked eye (see Figure 7)

The positive control is markedly coloured in yellow.

Each sample showing yellow colouration is considered positive (the negative control should not be coloured). This rapid reading is often sufficient for diagnosis of rabies. It has the advantage of not requiring other equipment.

FIGURE 7



- 1 : "Blank" control for photometric readings
- 2 : Successive dilution of the positive control

4.2 Photometer reading

In order to confirm the validity of the test, it must be ensured that the positive control has an optic density (OD) greater than 1.5 unit and that the negative control has an OD less than 0.1 unit. The minimum value for a positive reading is then obtained by adding 0.080 unit to the mean of the OD recorded for the negative control.

5. EVALUATION OF THE TECHNIQUE

5.1 Specificity and sensitivity

Six European and North American laboratories (four of which are WHO Collaborating Centres) tested 1253 samples originating from 27 animal species and from 10 different locations.¹

The results of their examination with the RREID were compared to those obtained when using the fluorescent antibody test (FAT). The two techniques gave the same result for 1220 samples. The concordance between RREID and FAT was 97.4% (see Table 1). However, the RREID was shown to be slightly less sensitive than the FAT.

At the request of WHO, the WHO Collaborating Centre in Paris coordinated a further collaborative study for the evaluation of the technique in rabies laboratories located in African, Asian and Latin American countries. Data from twelve laboratories have already been received (see Table 2). The concordance between the two techniques is 96.31%.² The results confirm those obtained following the first evaluation round.

In addition, the National Centre for Reference on Rabies of the Pasteur Institute in Paris examined 1755 samples for which the concordance between the two techniques was 98.9%. Details are given in Table 3 for the first 1007 samples studied.

5.2 Simplicity of use

These collaborative studies also showed that the RREID technique is easy and quick to carry out, and that reading of results is simple. The time required to obtain the final result, compared to those of the other diagnostic methods, is given in Graph No. 1.

5.3 Cost

In comparison with the FAT, the RREID only requires small investment in specific equipment, if readings are made with the naked eye. In this case, the cost of the laboratory equipment is below US\$ 2500. When a photometer is used, the investment averages US\$ 9000. These costs are represented in Graph No. 2 and compared with those of other techniques currently used for rabies diagnosis.

¹Results of a collaborative study of an experimental kit for the Rapid Rabies Enzyme Immunodiagnosis, P. Perrin and P. Sureau, WHO Bulletin, 4, 1987, 489-493

²Résultats de la technique RREID obtenus par divers laboratoires de diagnostic d'Afrique, d'Amérique latine et d'Asie, P. Sureau & H. Bourhy (submitted for publication in the WHO Bulletin), 1988

A RREID kit (commercialized by Pasteur Diagnosis) costs approximately US\$ 185. The cost of examining one sample (when one well is used) is about US\$ 2. It slightly exceeds that of the FAT. A comparison of these costs according to the diagnostic technique is shown in Graph No. 3.

6. CONCLUSION

The specificity and sensitivity of the RREID is comparable to that of the FAT. The technique requires little time and is simple to perform. The technique can also be used on samples in an advanced state of decay.

As readings taken with the naked eye are satisfactory, the technique requires less investment in specific laboratory equipment than for the FAT.

Utilisation of RREID technique is therefore recommended

- to confirm the FAT, (reading of which is not entirely objective);
- when a large number of samples must be examined (epidemiological survey);
- as an alternative to FAT when no fluorescent microscope is available in the laboratory.

Acknowledgements

The authors wish to thank Miss Florence Durand for the photographs and Dr Jacques Barrat for the graphs.

TABLE 1. CONCORDANCE BETWEEN RESULTS OF RREID AND FAT (FOR SIX EUROPEAN AND NORTH AMERICAN LABORATORIES)

| Laboratory | Total no. of samples | Number of samples | | | | Concordance* % |
|---------------|----------------------|-------------------|-------------|-------------|-------------|----------------|
| | | +FAT +RREID | -FAT -RREID | +FAT -RREID | -FAT +RREID | |
| A | 155 | 79 | 66 | 10 | 0 | 93.3 |
| B | 130 | 39 | 89 | 1 | 1 | 98.5 |
| C | 474 | 115 | 352 | 4 | 3 | 98.5 |
| D | 199 | 150 | 41 | 1 | 7 | 96.0 |
| E | 39 | 34 | 1 | 4 | 0 | 89.7 |
| F | 256 | 234 | 20 | 2 | 0 | 99.2 |
| Grand Total : | 1253 | 651 | 569 | 22 | 11 | 97.4 |

$$*Concordance = \frac{\text{number of positives with both techniques} + \text{number of negatives with both techniques}}{\text{total number of samples examined}} \times 100$$

TABLE 2 COMPARISON BETWEEN RESULTS OF RREID AND FAT (FOR RABIES LABORATORIES OF AFRICA, LATIN AMERICA AND ASIA)

| Laboratory | Total no. of samples | Number of samples | | | |
|---------------|----------------------|-------------------|----------------|----------------|----------------|
| | | +RREID -FAT | +RREID -FAT | -RREID +FAT | -RREID -FAT |
| 1 | 93 | 73 | 0 | 3 | 17 |
| 2 | 94 | 37 | 0 | 3 | 54 |
| 3 | 50 | 28 | 0 | 0 | 22 |
| 4 A | 68 | 47 | 0 | 11 | 10 |
| 4 B | 63 | 31 | 0 | 1 | 31 |
| 5 | 78 | 23 | 1 | 1 | 53 |
| 6 | 87 | 17 | 0 | 0 | 70 |
| 7 | 42 | 28 | 0 | 0 | 14 |
| 8 | 34 | 13 | 0 | 0 | 21 |
| 9 | 30 | 9 | 0 | 1 | 20 |
| 10 | 12 | 8 | 1 | 1 | 2 |
| 11 | 64 | 27 | 0 | 1 | 36 |
| 12 | 103 | 87 | 3 | 0 | 13 |
| Grand Total : | 818 | 428 | 5 | 22 | 363 |

Concordance RREID with FAT : $704/731 = 96.69\%$

Specificity RREID/FAT : $428 \text{ FAT+}/433 \text{ RREID+} = 98.84\%$

Sensitivity RREID/FAT : $433 \text{ RREID+}/450 \text{ FAT+} = 96.22\%$

TABLE 3

Correlation between results of FAT, isolation on neuroblastoma cells (N_{2a}) and RREID

| | | NUMBER OF SAMPLES | | | | | |
|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| | | FAT + | | | FAT - | | |
| N _{2a} + | N _{2a} - | N _{2a} + | N _{2a} - | N _{2a} + | N _{2a} - | N _{2a} + | N _{2a} - |
| RREID+ | RREID- | RREID+ | RREID- | RREID+ | RREID- | RREID+ | RREID- |
| | | TOTAL | | TOTAL | | TOTAL | |
| 169 | 4 | 7 | 9 | 189 | 0 | 0 | 2 |
| | | | | | | 816 | 818 |
| | | | | | | | 1007 |

FAT +/FAT - = Result of fluorescent antibody test on smears.

N_{2a}+/N_{2a}- = Result of isolation on neuroblastoma cells culture (N_{2a}).

RREID+/RREID- = Result of Rapid Rabies Enzyme Immunodiagnosis.

| | RREID | N _{2a} |
|--|-------|-----------------|
| - Concordance with FAT ^(a) | 98.51 | 98.41 |
| - Sensitivity ^(b) (%) | 93.12 | 91.53 |
| - Specificity ^(c) (%) | 99.76 | 100.00 |
| - Positive predictive value ^(d) (%) | 98.95 | 100.00 |
| - Negative predictive value ^(e) (%) | 98.43 | 98.08 |

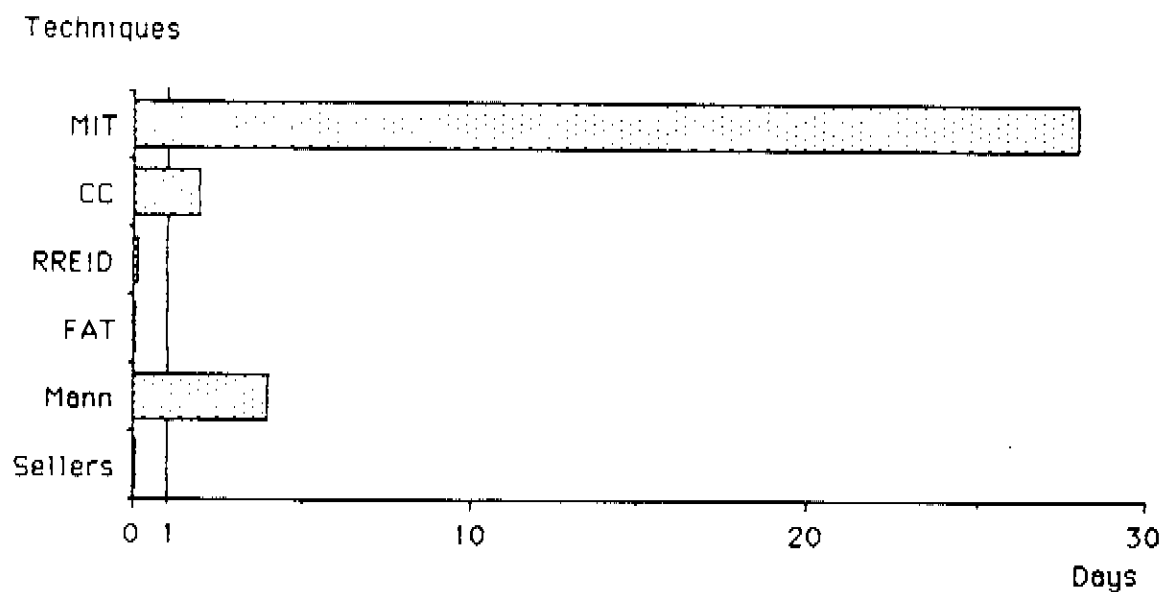
(T.P. stands for true positives, F.P. for false positives, T.N. for true negatives and F.N. for false negatives)

(a) = T.P. + T.N./Number of samples - (b) = T.P./((T.P. + F.N.) - (c) = T.N./((T.N. + F.P.)).

(d) = T.P./((T.P. + F.P.)).

(e) = T.N./((T.N. + F.N.)).

GRAPH No. 1. TIME REQUIRED FOR EXAMINATION OF A SAMPLE
BY DIFFERENT DIAGNOSIS TECHNIQUES



MIT = Mouse inoculation test

CC = Cell culture (Neuroblastoma cells N₂A)

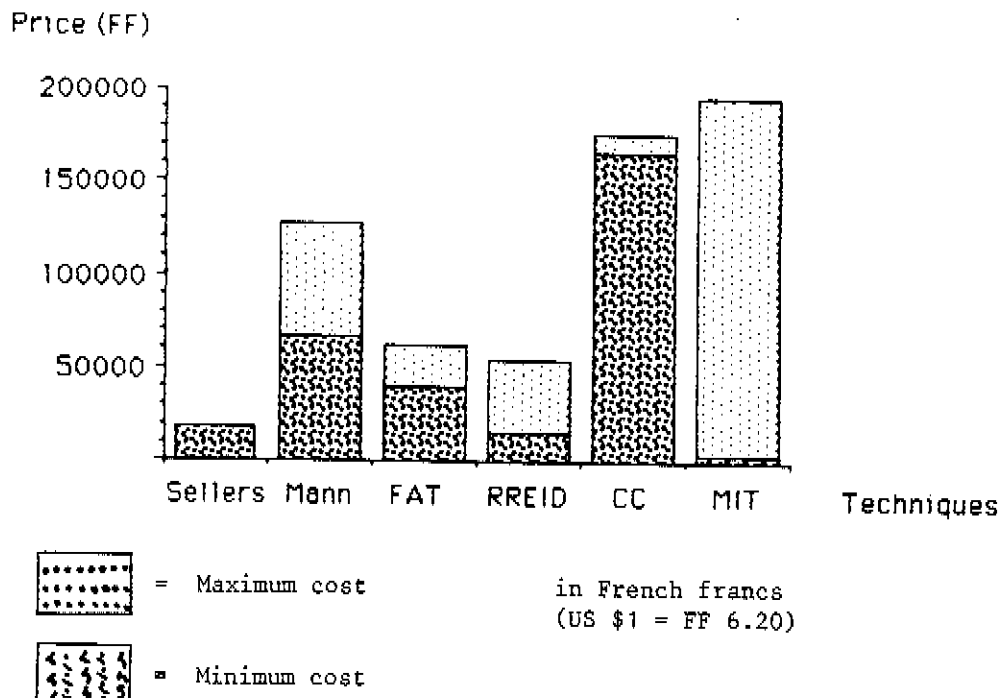
RREID = Rapid Rabies Enzyme Immuno-Diagnosis

FAT = Fluorescent Antibody Test

Mann = Histological techniques

Sellers = Histological techniques

GRAPH No. 2 COST OF LABORATORY EQUIPMENT
REQUIRED FOR DIFFERENT
DIAGNOSTIC TECHNIQUES



GRAPH No. 3 COST COMPARISON OF DIFFERENT
DIAGNOSTIC TECHNIQUES

