

## **Immunofluorescent staining of trypsinized formalin-fixed brain smears for rabies antigen: results compared with those obtained by standard methods for 221 suspect animal cases in Nigeria**

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### SUMMARY

Formalin-fixed samples from 221 animal brains received for rabies diagnosis in Nigeria were digested in 0·1 % trypsin in phosphate buffered saline, pH 7·4, and smears stained for rabies antigen by direct immunofluorescence (IF). The results were compared with those obtained using fresh material from the same animals for Negri body staining, mouse inoculation (MI) and occasionally immunofluorescent staining.

From 191 specimens examined for Negri bodies and by mouse inoculation 51 and 64 respectively proved positive. The IF smear technique under investigation failed to detect 5 of these but showed up as positive 30 which had been recorded as Negri-negative and 19 that had gone undetected by MI too. In a direct comparison with IF staining of fresh tissue from 23 known rabies positive animals the similar staining of trypsin-digested formalized smears failed to give a positive result in 2 out of 23 cases.

Some problems were encountered with smears not sticking to slides. When gelatinized slides were used fluorescence was sometimes poorer.

Where transport and refrigeration are difficult and section-cutting equipment is lacking the technique of IF staining of smears prepared from formalized brain tissue after treatment with trypsin can be a useful adjunct to other diagnostic methods. It also makes for safer working where special facilities are absent.

### INTRODUCTION

In Nigeria and doubtless in many other developing countries in the tropics, at least 10 % of brain samples received at laboratories for rabies diagnosis arrive in a putrid state because refrigeration facilities are inadequate or not functioning continuously. Lewis & Thacker (1974) showed that such deteriorated brain specimens are likely to give false negative results; this is a very serious matter because post-exposure treatment of patients may depend upon confirmation of rabies diagnosis. To avoid this problem we have evaluated the use of smears

prepared from trypsin-digested formalized brain specimens in an immunofluorescent test and compared the results with the methods in current use, i.e. examination for Negri bodies, mouse inoculation and occasionally immunofluorescence. The results in this paper demonstrate such a test can serve an effective purpose and also be much safer to carry out where special handling facilities are lacking. It is known that rabies-infected brain tissue may still be infective after fixation in acetone for 4 h at  $-60^{\circ}\text{C}$  (Fischman & Ward, 1969). It also avoids the need to cut histological sections from paraffin-embedded tissues, another technique not always feasible under difficult conditions.

## MATERIALS AND METHODS

### *Source of specimens*

The present study utilized 221 rabies-suspect brains from animals received either at the Rabies Diagnostic Laboratory, National Veterinary Research Institute at Vom, or the Veterinary Diagnostic Unit at Ahmadu Bello University. The majority, 213, were from dogs, the few others being 5 from cats and 1 each from cattle, goat and a monkey. Specimens of hippocampus, about 3–5 mm cubes, were taken, put into 10% buffered formalin and coded. Immunofluorescent staining was carried out any time from 3 weeks to 2½ years later.

### *Smear preparation*

The specimens were taken out of the formalin and washed in 0.1 M phosphate buffered saline, pH 7.4 (PBS). The tissue was then crushed between two tongue-depressors, or cut into small pieces, and placed in a solution of 0.1 g crude trypsin (Difco Laboratories, Detroit, Michigan, U.S.A.) plus 0.2 g calcium chloride in 100 ml PBS, either for 1 h at room temperature or at  $4^{\circ}\text{C}$  overnight. The tissues could then be crushed further and smears made on glass slides. The smears were fixed in acetone at  $-20^{\circ}\text{C}$  for 1 h, or overnight, and air-dried before staining. An alternative method was tried in some cases so that the smears were made on slides previously coated in gelatin by several dippings into a filtered solution made up from 5 g gelatin (Fisher Scientific Co., Pittsburgh, Pa, U.S.A.) in 800 ml of hot distilled water to which 0.5 g chromic potassium sulphate was added as described by Rowse-Eagle, Watson & Tignor (1981). The smears made on these slides were dried in an oven at  $56^{\circ}\text{C}$  overnight and then washed and air-dried before staining. The object of gelatin-coating slides was to stop the tissue being washed off during the staining processes.

### *Smear staining and examination*

A standard technique for direct immunofluorescent staining (IF) was used throughout (Dean & Abelseth, 1973). This involved an appropriately diluted fluorescein-isothiocyanate-labelled antirabies immunoglobulin (BBL, Cockeysville, Maryland 21030, U.S.A.) with 2 drops of 0.1% Evans Blue dye added to each 1 ml of conjugate as a counterstain. The smears were routinely stained, washed 3 times, allowing 10 min per time, and air-dried. They were then examined for fluorescence using a Dialux microscope with  $\times 10$  ocular and  $\times 100$  objective lens with

epi-illumination and an HBO 100 illuminator. An apple-green fluorescence appearing as discrete dots or conglomerates indicated that rabies antigen was present.

#### *Examination for Negri bodies*

The standard Seller's stain method was used both on suspect brains submitted for diagnosis and on the brains of mice used in the inoculation test.

#### *Mouse inoculation test*

Weanling mice were inoculated intracerebrally with 20% suspect brain suspension in PBS containing 0.75 g bovine serum albumin (Fraction V) per 100 ml (Koprowski, 1973). The mice were observed for 30 days and the brains of dead ones examined for Negri bodies as above. These tests were done in the Rabies Diagnostic Laboratory at Vom.

#### *IF staining of fresh or frozen tissues*

Smears from the hippocampi of 42 suspect rabid animals were fixed in acetone at  $-20^{\circ}\text{C}$  for about 30 min, air-dried, stained by the Dean and Abseleth method described above and examined for fluorescence.

## RESULTS

The results from the various tests performed at Vom and Ahmadu Bello were collated at the Ahmadu Bello University, Zaria. The main findings are shown in Table 1, which compares the diagnostic results obtained by IF staining of trypsin-digested formalin-fixed brain material with those obtained by the classical techniques of Seller's stain for Negri bodies either of the suspect animal brain itself or of mouse brain after intracerebral inoculation of such material. Results were available in 191 cases. The classical techniques found 64 of the 191 suspect animals to be rabies-positive and it is worth noting that 13 of those positive were only detected after intracerebral inoculation of mice. The new technique under test indicated 78 of the same 191 suspect brains as rabies positive. Five of 64 cases diagnosed as positive by one or both of the classical methods were classed as negative by the new test system, a failure rate of 8%. On the other hand, 19 of 127 recorded as negative for rabies in the routine tests turned up positive by the new system, giving an increase of 14 positives on the original 64 detected.

We were able to check 23 brain specimens known to be rabies-positive by IF staining of fresh or frozen tissue with the new IF test which used trypsin-digested formalin-fixed tissues. The latter test indicated 21 positives and two negatives – a 90% agreement. Nineteen brain specimens were negative by both methods.

We were also able to compare trypsin-digested formalized brain smears on gelatin treated slides with those put directly on to glass and fixed in acetone (Table 2). In four out of nine cases the acetone-fixed slides gave better fluorescence and in the other five there was no difference. About 10% of acetone-fixed smears become detached during the staining procedure. The advantages of not having smears washed off gelatin treated slides are offset by sometimes having less fluorescence.

Table 1. *Immunofluorescent staining of rabies antigen in formalin-fixed tissue after trypsin treatment compared with other diagnostic methods*

Combined results of other diagnostic tests		Immunofluorescent staining		
		Positive	Negative	Total
Mouse inoculation	Positive }	48	3	51
Negri bodies	Positive }			
Mouse inoculation	Positive }	11	2	13
Negri bodies	Negative }			
Mouse inoculation	Negative }	19	108	127
Negri bodies	Negative }			
Total		78	113	191

Table 2. *Comparison of immunofluorescent staining when smears were fixed either in acetone or made on gelatin-treated slides and heated at 56 °C overnight*

Specimen number	Quantity of fluorescence*		Comments
	Gelatin-coated slide	Acetone fixation	
37	±	+	Acetone fixation better
43	+	2+	Acetone fixation better
90	4+	4+	No difference
120	±	3+	Acetone fixation better
203	3+	4+	Acetone fixation better
208	±	±	No difference
215	+	+	No difference
243	2+	2+	No difference
248	+	+	No difference

\* ± Fluorescence in 1-3 fields; +, 4-6 fields; 2+, 7-10 fields; 3+, 10-15 fields; 4+, almost every field.

Some subsidiary aspects of the results are worth mentioning:

(a) Of 221 brain samples examined 162 (73%) were submitted because of associated human bites.

(b) All animals which had bitten two or more people were found to be positive by the new test.

(c) Two of the cat brains and the goat brain submitted were rabies-positive when stained for Negri bodies and following mouse inoculation but were negative by the new technique.

## DISCUSSION

It was Huang, Minassian & Moore (1976) who introduced prior trypsinization to improve the immunofluorescent staining of antigens in formalin-fixed, paraffin-embedded tissues. Then Johnson, Swoveland & Emmons (1980) and Umoh & Blenden (1981) reported the detection of rabies antigens by IF staining of such fixed and trypsinized brain material. Many workers use tissue sections for such work

(Huang, Minassian & More, 1976; Johnson, Swoveland & Emmons, 1980) but it would be faster and simpler if direct smears could be made from trypsin-digested formalized material, particularly in developing countries where section-cutting facilities may be absent. Experience gained in the present trial shows that such a direct smear technique works quite well. Gelatin coating the slides to stop the smears being washed off during the staining process is effective but has the disadvantage that fluorescence may be reduced in some cases and is probably not worthwhile. There were some discrepancies between results obtained by Negri-body staining and mouse inoculation and the new system under test. A small number of specimens were positive for rabies by mouse inoculation but negative by IF staining on formalin-fixed tissue. Reid *et al.* (1983) have shown that if the virus titre is low ( $10^3$  MICLD<sub>50</sub>/0.03 ml) false negative results may occur when the IF test is used following trypsin treatment of formalin-fixed brain tissue. Intracerebral inoculation of mice, however, may detect low titres of rabies virus as replication occurs in the brain.

It is known that Negri bodies may be present in only 75 % of rabies cases (Dupont & Earle, 1965) and in our study a significant proportion (30 out of 140 specimens) were positive by the IF technique but negative when examined for Negri bodies. False negative results following mouse inoculation (19/127) were most probably associated with deteriorated specimens but could have been due to the presence of inhibiting substances and auto-interference phenomena.

Lacking immediate explanation are the three cases positive for Negri bodies but negative on the experimental test system and also the 2 out of 23 cases previously found to be positive by IF when fresh/frozen tissue was used instead of trypsinized-formalized tissue. The length of time the brain material was held in formalin did not seem to be a factor.

It is clear that no one of the diagnostic methods used is completely reliable and to give the maximum chance of avoiding false negative diagnosis, which may have mortal consequences for a person bitten but not treated, they should all be employed if possible. When the chances of getting fresh brain tissue in good condition to a diagnostic laboratory are poor, or uncertain, it is well worth while sending part of the material in buffered 10 % formalin. Following trypsin digestion it can then be used for IF staining with the confidence that this technique will detect about 90 % of cases which would have been picked up by the routine diagnostic systems and show up a substantial number of others which would have been missed by Negri body staining and mouse inoculation.

It must be appreciated that there are problems with the technique and more work is needed to increase the sensitivity of the test and to improve the adherence of the smears to the glass slides.

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