

A comparative rabies laboratory diagnosis: peculiar features of samples from apparently healthy dogs in Nigeria

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Abstract

Many diagnostic methods have been used to detect rabies virus antigen. The preferred method for routine rabies diagnosis in fresh brain tissue is fluorescent antibody test (FAT). In this study, FAT was used to evaluate the presence of rabies virus antigen in the brain (hippocampus) of fifty apparently healthy dogs. Mouse inoculation test (MIT) and Microscopic examination for Negri bodies (MEN) were also employed to compare agreement, if any, between these employed methods. FAT detected 13 (26%), while MIT detected 10 (20%) samples positive for rabies virus antigen. Of the 10 samples positive by MIT only one sample was FAT negative all the remaining 9 samples were FAT positive. In all, 14 (28%) samples were positive by the two methods (FAT & MIT) combined. Out of these, 3 (21.4%) were positive by MEN and only those samples with the 3+ distribution of fluorescing viral antigen by FAT as well as positive by MIT showed Negri bodies. Despite the high sensitivity of FAT and the good agreement (Kappa = 0.72) between the two methods; there is need to employ MIT on samples from apparently healthy dogs that showed FAT negative. MEN is not a reliable test for samples from apparently healthy dogs; but it was suggested that any sample from apparently healthy dog that is positive by FAT with 3+ or more distribution of fluorescing viral antigen should be presumed MEN positive.

Key words: Comparative, Rabies Antigen, Healthy Dog, Diagnosis

Introduction

Reports in Africa and elsewhere in the world have shown that rabies in dogs is not invariably fatal, but cases occurred in which dogs do not die but recovered and became carriers (Bell, 1975; Gribencha, 1975; Fekadu and Baer, 1980; Fekadu, 1988). The laboratory diagnosis has fundamental importance so that control measures can be taken, and human treatment decisions may be recommended. Therefore, a reliable diagnostic method must be applied, utilizing adequate and recommended techniques, taking into consideration the limitations and failures of each method. Many diagnostic methods have been used to detect rabies virus antigen (OIE Methods, 2000). The Microscopic examination for Negri bodies (MEN), Mouse inoculations test (MIT), and fluorescent antibody tests (FAT) are some of the most recommended techniques for rabies diagnosis

(Dean *et al.*, 1996; Koprowski, 1996; Tierkel and Atanasiu, 1996). The MEN is a fast and economical procedure, and is recognized worldwide as specific for rabies, since the presence of these Negri bodies always indicates the infection (Tierkel and Atanasiu, 1996). The FAT is the sensitive and preferred method for the detection of the rabies antigen in fresh samples (Meslin and Kaplan, 1996). The isolation of the rabies virus through MIT, confirms the results obtained by other techniques; though its use is expensive and slow. This study compares the sensitivity and agreement among the three diagnostic techniques usually employed in Nigeria, in order to detect rabies virus antigen in the brain of apparently healthy dogs as well as to proffer interpretations on peculiar features of the tests from the obtained results.

Materials and Methods

Location of Study Area

The study was carried out in the northwestern states of Sokoto and Katsina, Nigeria.

Brain Sample Collection

Fifty dog heads from apparently healthy dogs were collected at 'mammy markets' of the army barracks in the respective states where bars and restaurants exist in which dogs are slaughtered as a delicacy for human consumption. The brain (hippocampii) samples were removed as described by Atanasiu (1975) and each sample was placed in pre-labeled bottles (50 in number). The samples were stored at -20° C until used.

Laboratory Analysis

The fifty brain (hippocampii) specimens were subjected to F.A.T and M.I.T. Specimens that were positive by the two methods were then subjected to M.E.N. test.

Fluorescent Antibody Test (FAT) Procedure

This was carried out as described by Dean *et al.* (1996) with minor modifications. Briefly, Light diagnostic rabies fluorescent antibody assay DFA (monoclonal antibody FITC-conjugate) catalog No.5100 reagent from Chemicon International Inc. 1-800-437-7500 was used. Aliquots of DFA reagent diluted at 1:40 in working phosphate buffered saline PBS pH (8.6) for testing were stored in freezer. A known positive dog brain sample (positive control +) and a three weeks old normal mouse brain sample (negative control -) were gotten and used from National Veterinary Research Institute Vom, Nigeria. A thin impression smear of the test brain specimen (T) was made at the centre of a glass slide, while those of the + and - controls were made at left and right sides of the T smear respectively. Two slides were made for each test brain sample concurrently. The smears were air-dried for 30 minutes, fixed in cold acetone contained in Coplin jar for 30 minutes in freezer. The slides were then rinsed in PBS pH (8.6) for 1 minute and oven dried for 5 minutes at 37° C and the smears were encircled with wax pencil. The slides were then placed onto a moist chamber to prevent drying. Two drops each of the diluted aliquot DFA reagent were added to cover the smears within the encircled areas of the slides in the moist chamber and were incubated for 30 minutes at 37°C. The slides were then washed twice in working PBS pH (8.6) for three minutes to remove the unbound DFA reagent before being rinsed in distilled water for 1 min. The slides were then air dried and examined by ultraviolet microscopy. The microscope used was a Leitz Ortholux microscope from Germany. The smears were examined with X20 magnification. The presence of clear cut

rounded dots or clusters of apple green fluorescence indicated the presence of rabies antigen. The findings in the T smears were compared with the findings in the + and - control smears. A 5-point scoring system was used depending on the quantity and intensity of fluorescence in the smears examined.

++++, a massive infiltration of large and small brilliant apple green shining inclusions/dots/particles at varying sizes and shapes in almost every area of the smear (only the + control showed this grade).

+++, a brilliant apple green shining inclusions/dots/particles of varying sizes and shapes are found in almost every microscopic field, the number of inclusions per field varies, but inclusions are numerous on most fields.

++, a brilliant apple green shining inclusions/dots/particles of varying sizes and shapes are present in 10% to 50% of the microscopic field and most fields contain only few inclusions.

+, a brilliant apple green shining inclusions/dots/particles of varying sizes and shapes are present in < 10% of the microscopic field and only few inclusions are found per field (usually only one or two inclusions per field). This category 1+ was not considered true positive in this study, it was considered doubtful due to human and experimental errors.

-, negative when there was no brilliant apple green shining inclusions/dots/particles in any microscopic field.

Mouse Inoculation Test (MIT)

Six 21 day-old specific pathogen free (SPF) Swiss albino laboratory mice, weighing 14g average, were each inoculated intracerebrally with 0.03ml of a suspension of each brain sample at 10% of phosphate buffered saline (PBS) pH 7.2, two drops to the suspension were added of 500 I.U penicillin/ml and 1560 I.U Streptomycin/ml. The mice were observed daily for 30 days. The appearance of the symptoms (circling & spinning movement, bristling of the fur, agitation, paralysis and death) were monitored and recorded.

Microscopic Examination for Negri Bodies (MEN)

The survey for Negri bodies was carried out from impressions of the hippocampii using Sellers' stain (two part of 2% methylene blue in methanol to one part of 1% basic fuchsin in methanol). Two slides were made for each sample. The slides were washed in running water, air-dried for 30 minutes at room temperature and observed under oil immersion (x100). The Negri bodies were observed in the cytoplasm and dendrites of the neurons, presenting an acidophilic stain, with basophilic internal granulations.

Statistical Analysis

For statistical analysis, a sample was defined as positive when at least one of the three applied techniques resulted positive. To compare the result of the percentage of sensitivity and the agreement between two methods (MIT and FAT); a Kappa test for agreement was employed using two by two table as described by Noordhui Zen et al (1997).

Results

Of the total fifty brain specimens tested 13 (26%) were positive by FAT, while 10 (20%) were positive by MIT. Similarly, of the 10 samples that were Positive by MIT only one sample was FAT negative, all the remaining 9 samples were also FAT positive. In other words 14 (28%) of the 50 samples were positive for rabies antigen by the two techniques combined. Finally, of the 14 positive samples only 3 (21.4%) were positive by MEN and only those with grade 3+ intensity & quantity of

fluorescence by FAT as well positive by MIT, showed Negri bodies (Table 1).

Of the 50 samples tested, 23 samples showed fluorescence of varying intensity by FAT (table 1). Ten (43.5%) were of low intensity and quantity (+) of fluorescence for viral antigen, also 10 (43.5%) samples were of medium intensity and quantity (++) of fluorescence. Small proportion of samples 3 (13.0%) appeared to be of moderately high intensity and quantity (+++) of fluorescence and it was the samples in this category that were Negri body positive. There was none 0 (0%) with high (++++) intensity and quantity of fluorescence in the study except the positive control. Those sample with ++ and +++ intensity and quantity of fluorescence i.e. 13 samples were considered positive for rabies viral antigens by F.A.T (Table II). The remaining 10 samples with + intensity and quantity of fluorescence were doubtful and not considered positive; because of human and experimental errors; these same 10 (+) samples were also M.I.T negative.

Table 1

A comparative summary of samples positive by FAT, MIT and MEN.

Test	No. of samples tested	Total no. positive	% positive
FAT	50	13	26
MIT	50	10	20
MEN	14	3	21.4

Key:

%= percentage

FAT= Fluorescent antibody test

MIT= Mouse inoculation test

MEN= Microscopic examination for Negri bodies

Table 2

Distribution of intensity and quantity of fluorescence of the 23 brain samples by F.A.T

Intensity of Fluorescence	No. of Samples	Percentage
+	10	43.5 (doubtful; not considered)
++	10	43.5
+++	3	13
++++	0	0
Total	23	100%

Kappa = 0. 72, good agreement; sensitivity = 69.2%; specificity = 97.3% of M.I.T as compared with F.A.T.

Discussion

Laboratory confirmation of the clinical suspicion of rabies is essential in epidemiologist, and in prescribing anti-rabies treatment after exposure. On the other hand in cases lacking clinical suspicion or laboratory confirmation, such as samples from apparently healthy rabies carrier dogs; the prescribed treatment is generally delayed or interrupted. Therefore, this laboratory diagnosis must be trustworthy in terms of sensitivity and

specificity, and also be quickly obtainable. From the results of this work it is obvious that FAT is the most sensitive, fast and reliable test for specimens from apparently healthy dogs in Nigeria. This is in agreement with the assertion of other workers that FAT is very sensitive on fresh samples (Mc Queen *et al.*, 1960; Meslin and Kaplan, 1996). However, despite this high sensitivity of FAT and good agreement (Kappa = 0.72) seen between FAT & MIT in this study; there is need to always employ MIT on samples from apparently healthy dogs that

turns out to be FAT negative. This is because at least one sample was positive for rabies antigen by MIT, but negative by FAT in the present study. The other 4 samples that were FAT positive but MIT negative could have proved MIT positive if longer period of observation was probably allowed over 30 days used in this study, though internationally accepted. This is because Baer and Cleary (1972) have reported up to 120 days in experimentally infected mice before symptoms appeared. The ten samples with 1+ intensity and quantity of fluorescing particles; that were not considered positive by FAT in this study, were also not positive by MIT (Table 2). This suggests that the samples were truly not positive for rabies viral antigen. The fluorescence seen may be due to non-specific binding of antibody to components of inflamed tissue or artifacts of tissue decomposition as suggested by Kissling (1975). The MEN proves to be non-reliable technique for samples from apparently healthy dogs. However, the three samples that were positive by MEN happens to be same samples that were positive by FAT with 3+ intensity and quantity of fluorescence as well positive by MIT. These suggest that those three samples were either full blown non-fatal rabid or recovering from rabies; thus, the presence of Negri bodies in these samples. Based on these arguments and the peculiarity of this finding; it was suggested that any sample from apparently healthy dog that shows FAT positive with 3+ or more intensity and quantity of fluorescence should be presumed MEN positive and necessary measures such as post-exposure prophylaxis be instituted. The general implication of these findings is that persons or other animals are liable to exposure from bite by unrecognized rabies carrier apparently healthy dogs. Hence, the application of the MEN as a diagnostic tool on sample from apparently healthy dogs is not advocated. FAT is highly recommended, but, where materials are not in place then MIT should be resorted to.

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