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Heat induced epitope retrieval for rabies virus detection by direct fluorescent antibody test in formalin-fixed dog brain tissues

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Abstract

There is a great need for a chemical method of tissue preservation that would allow sample storage for extended periods at room temperature. This study aimed at retrieving and detecting rabies virus antigen by direct fluorescent antibody test (DFAT) in formalin-fixed dog brain tissues. Forty fresh dog brain specimens were collected as paired samples from rabies suspected cases that were received for postmortem detection of rabies in the Central Diagnostic Laboratory, National Veterinary Research Institute, Vom. One portion of each paired sample was prepared for fresh fluorescent antibody testing and the other portion was prepared for epitope retrieval and fluorescent antibody testing following fixation in 10% neutral buffered formalin. DFAT on formalin-fixed tissue exhibited a sensitivity of 100% in comparison to DFAT on fresh-tissue. No false positive result was obtained in formalin-fixed DFAT procedure, demonstrating 100% specificity. There was no apparent difference in the intensity of fluorescence in DFAT on fresh sample and formalin-fixed DFAT following heat induced epitope retrieval (concordance = 98%; 95% C.I. 0.9660 to 0.9903). The strength of agreement between DFAT on formalin-fixed and DFAT on fresh tissue was very good (Cohen's kappa coefficient value= 1.000; 95% C.I. 1.000-1.000). This study provides new information on the retrieval of rabies antigen by heat induced epitope retrieval for DFAT on formalinized tissues. Formalin could therefore, be used henceforth to fix tissues of rabies suspected cases for routine diagnosis, transportation or archival purposes. The heat induced epitope retrieval can be routinely used to retrieve rabies virus antigen for DFAT in cases where only formalin-fixed tissues are available or when preservation by freezing is difficult.

Keywords: Direct fluorescent test, Formalin-fixed brain tissues, Heat induced epitope retrieval, Rabies virus.

Introduction

Rabies affects central nervous system (CNS) and death is inevitable once clinical signs set in (Dietzschold *et al.*, 2008). It is caused by rabies virus, a negative-stranded ribonucleic acid (RNA) virus of approximately 12 kilo base pairs (kbp) (Zhou *et al.*, 2013; Kia *et al.*, 2018) with cylindrical morphology (bullet-shaped). Rabies virus which is the type species of the genus *Lyssavirus* and family *Rhabdoviridae* has a relatively simple, modular genome organization and encodes five structural proteins: a RNA-dependent RNA polymerase (L), a nucleoprotein (N), a phosphorylated protein (P), a matrix protein (M) and an external surface glycoprotein (G) (Finke and Conzelmann, 2005; Dietzschold *et al.*, 2008). Lozano *et al.* (2012) reported a decrease in mortality due to rabies mostly in Asia and Africa from approximately, 54,000 deaths in 1990 to 26,000 in 2010. In India alone,

about 20,000 people die annually from rabies (Harris, 2012). In 2015, approximately 6,000 rabies cases were reported in China and about 5,600 cases were recorded in the Democratic Republic of the Congo (Hampson *et al.*, 2015). The main host for the transmission of rabies virus in most endemic countries especially Asia and Africa is dog and there are no stringent laws in place to ensure compulsory vaccination of dogs. More than 10,000 Nigerians are exposed to rabies annually, and about 1,000 annual cattle mortality had been reported (Tekki *et al.*, 2016). In Nigeria, human and animal rabies cases are on the increase annually despite the availability of vaccines for its control and prevention (Ogunkoya, 2008). Some of the advantages of formalin-fixed procedures are the ease of sample preservation and the reduced risk involved in transporting and processing samples which may contain infectious rabies virus (Wunner and Jackson,

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2010). A potential limitation of the procedure to work with formalin-fixed preparation is the inability to cultivate and amplify the virus from an inactivated sample (Shankar, 2009). Immunofluorescence method applied to formalin fixed tissues was previously significantly less sensitive than the direct fluorescent antibody test (DFAT) method on fresh brain tissues. With recent modifications to achieve better immunofluorescence (Warner *et al.*, 1997, 1999), this procedure may now be as sensitive as DFAT on fresh tissues (Whitfield *et al.*, 2001). The direct rapid immunohistochemistry test (DRIT) was recently developed by the US Centers for Disease Control and Prevention (CDC). It is a rapid tests and results are available approximately one hour. The principle of this promising test is based on the detection of rabies N protein in formalin-fixed brain smears by the use of very concentrated monoclonal antibodies with streptavidin peroxidase and a substrate coloring agent. This test uses light microscope for visualization of rabies antigen, therefore, can be implemented in the field (Dürr *et al.*, 2008; Fooks *et al.*, 2009; Madhusudana *et al.*, 2012). Although DRIT procedure may be cumbersome, the sensitivity and specificity could reach 100% when compared to DFAT (Lembo *et al.*, 2006; Madhusudana *et al.*, 2012). There is a great need for a chemical method of tissue preservation that would allow sample storage for extended periods at room temperature. If this were available, a higher proportion of decisions regarding post-exposure immunization could be based upon laboratory data; cumulative surveillance data would be more representative and meaningful; and the infection hazard to laboratory workers would be reduced (Umoh and Blenden, 1981). It was generally felt that chemical preservation with formalin makes DFAT examination virtually impossible. However, with the modification to achieve better immunofluorescence (Warner *et al.*, 1997), this procedure may now be as specific a diagnostic method as DFAT on fresh tissue. Therefore, this study aimed at retrieving and detecting rabies virus antigen by DFAT in formalin-fixed dog brain tissues.

Materials and Methods

Sample collection

Forty fresh dog brain specimens were collected as paired samples from rabies suspected cases that were received for postmortem detection of rabies in the Central Diagnostic Laboratory, National Veterinary Research Institute, Vom, over a period of three months. Samples were collected from different locations, viz. hippocampus, cerebellum, and brain stem respectively. One portion of each paired sample was prepared for fresh fluorescent antibody testing and the other portion was fixed in 10% neutral buffered formalin (NBF) at room temperature for a minimum period of three days. The tests were performed in blinded schedules. The

DFAT rabies status analysis of fresh- tissue samples and those of formalin- fixed tissue samples were done blindly until all tests were done (Whitfield *et al.*, 2001).

Heat induced epitope retrieval and the detection of rabies virus antigen in formalin-fixed tissues

Sodium citrate (2.35 g) and citric acid (0.42 g) were dissolved in 200 ml of distilled water. Then, the total volume of the mixture was made up to one litre with distilled water in a volumetric flask. The sodium citrate buffer was heated to 92°C in a water bath. Formalin fixed brain tissues were removed and washed thoroughly with PBS (pH 8.5). To prepare smears, the samples were crushed in a mortar after which impression smears were made on clean slides. The slides were air dried at room temperature and then fixed by placing in a coplin jar containing acetone at -20°C for 30 minutes. The slides were removed and air dried at room temperature. The slides were then immersed in the staining dish containing the pre-heated sodium citrate buffer (0.01 M; pH 6.0) and incubated at 92°C for 30 minutes in a water bath. The staining dish was then removed and slides were allowed to cool at room temperature for 20 minutes. Thereafter, slides were rinsed in PBS (pH 8.5) and air dried at room temperature. The slides were transferred to a humidified chamber and a drop of 150 µl of fluorescein-labeled monoclonal anti-rabies immunoglobulin (Fujirebio Diagnostics, Inc., U.S) was used for staining. These were then incubated at 37°C for 30 minutes. After incubation, the slides were washed three times with Phosphate Buffered Saline (PBS) (pH 8.5). The slides were then air-dried at room temperature and arranged in a slide carrier. A drop of 50% mounting buffered glycerol and a cover slip were applied on each smear. The slides were visualized under a fluorescent microscope (Zeiss International, Germany). Presence of Bright/dull/dim apple green oval or ellipsoid fluorescing intracellular accumulations was considered positive. Fluorescence was scored by two separate individuals using a three-plus scoring system (scores were as follows: 3 +++ bright apple green fluorescence; 2 ++ dull apple green fluorescence; 1 + dim but detectable apple green fluorescence). DFAT was also carried out on formalinized brain tissues without treatment with sodium citrate buffer.

Rabies antigen detection in fresh brain tissues

Rabies antigen detection by DFAT was done according to Dean *et al.* (1996) and Okoh *et al.* (2018).

Data Analysis

The results were presented using simple descriptive statistics involving percentages, tables and charts. The concordance coefficient and simple Cohen's kappa coefficient value were used for statistical comparison of the diagnostic tests. All statistical procedures were done using the MedCalc Software (MedCalc Software bvba, Version 17.8).

Results

Heat induced epitope retrieval and the detection of rabies virus antigen in formalin-fixed tissues

The combined results of the preliminary experiment on formalin-fixed tissues were presented in Table 1. Out of the 40 samples tested, 32 (80%) brain samples were positive by DFAT after treatment with sodium citrate buffer and none tested positive without treatment with sodium citrate buffer. The results were not affected by the length of time the tissues were left in formalin.

Table 1. Results of Rabies virus antigen detection in formalin-fixed tissues.

DFAT	with sodium citrate buffer treatment	without sodium citrate buffer treatment
Number Examined	40	40
Positive	32 (80%)	0 (0%)

(DFAT): Direct fluorescent antibody test.

Detection of rabies virus antigen in Fresh dog brain tissues

Fresh brain tissues (Hippocampus, brain stem and cerebellum) were positive in 32 (80%) cases out of the 40 cases tested using DFAT for the presence of rabies virus antigen. Eight brain samples were negative for rabies virus antigen (Table 2).

Table 2. Rabies antigen detection in fresh brain tissues by direct fluorescent antibody test.

Part of brain examined	Number examined	Number positive	Positive (%)
Hippocampus	40	32	80
Brain stem	40	32	80
Cerebellum	40	32	80

Comparison of DFAT on fresh tissue and DFAT on formalin fixed tissue

As shown in Table 3, 32 brain tissues were positive for rabies antigen by both the DFAT on fresh tissue and the DFAT on formalin-fixed tissue. Thus, DFAT on formalin-fixed tissue exhibited a sensitivity of 100% in comparison to DFAT on fresh-tissue. No false positive result was obtained in formalin-fixed DFAT procedure, demonstrating 100% specificity. We found 100% agreement (32 positives and 8 negatives) between the two techniques. The concordant scores of the two techniques were shown in Table 4. There was no apparent difference in the intensity of fluorescence in DFAT on fresh sample and formalin-fixed DFAT (concordance = 98%; 95% C.I. 0.9660 to 0.9903) (Fig. 1 and 2).

The strength of agreement (Cohen's kappa coefficient) between DFAT on formalin-fixed and DFAT on fresh tissues was 1.000; 95% C.I. 1.000-1.000.

Table 3. Sensitivity and Specificity comparison of DFAT on fresh and formalin-fixed tissues.

Category	DFAT on formalin-fixed tissues		Total
	Positive	Negative	
DFAT on fresh samples (Positive)	32	0	32
DFAT on fresh samples (Negative)	0	8	8
Total	32	8	40
Sensitivity	100%		
Specificity	100%		

Table 4. Concordant Scores for Rabies virus detection by direct fluorescent antibody test in fresh and formalin-fixed dog brain tissues.

Concordant Score	DFAT _a	DFAT _b
3 (+++)	24	26
2 (++)	8	6
1 (+)	0	0
0 (Negative)	8	8
Total	40	40

(DFAT_a): Direct fluorescent antibody test on formalin-fixed dog brain tissues. (DFAT_b): Direct fluorescent antibody test on fresh dog brain tissues.

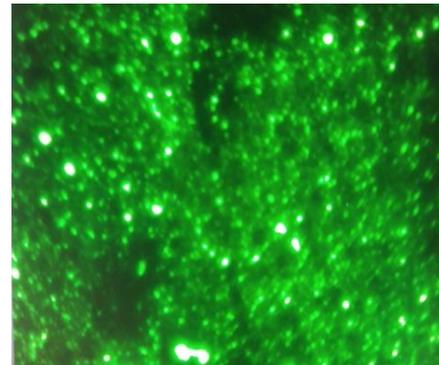


Fig. 1. Apple green fluorescing intracellular accumulation of fresh brain smear. Stained with Fluorescein-labeled monoclonal anti-rabies immunoglobulin ×400.

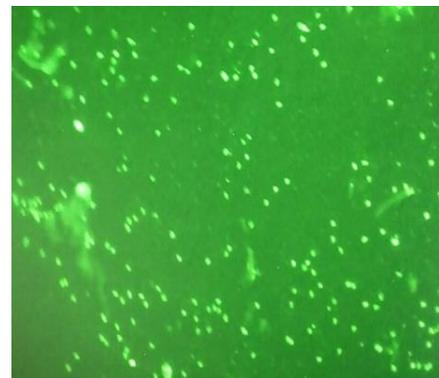


Fig. 2. Apple green fluorescing intracellular accumulation of formalin-fixed brain smear after treatment with heated sodium buffer (0.01M, pH 6.0). Stained with Fluorescein-labeled monoclonal anti-rabies immunoglobulin ×400.

Discussion

Several researchers have made attempts to detect rabies virus antigen in formalin-fixed tissues using DFAT staining procedures (Bourhy and Sureau, 1990; Warner *et al.*, 1997; Whitfield *et al.*, 2001). All of these studies reported challenges in the tests without the use of antigen retrieving agents. The results presented in this study demonstrated 100% sensitivity and specificity of the formalin-fixed DFAT procedure. This agrees with the finding of Whitfield *et al.* (2001) who recorded 99.8% sensitivity and 100% specificity of formalin-fixed DFAT.

However, a lower sensitivity of formalin-fixed DFAT following enzymatic digestion with trypsin was reported in previous study (Umoh and Blenden, 1981). This suggests that heat induced epitope retrieval (HIER) procedure could be a better alternative to enzyme digestion for the retrieval rabies virus antigen in DFAT procedure on formalin-fixed.

Formalin fixation forms protein cross-links that mask the antigenic sites in tissues, thereby giving weak or false negative result for DFAT rabies detection (Warner *et al.*, 1997). Antigen retrieval with heated sodium citrate buffer breaks these protein cross-links and exposes antigenic sites, allowing antibody to bind. In this study, 80% of formalin-fixed tissues tested positive for rabies virus antigen by DFAT after treatment with heated sodium citrate buffer (pH 6.0-6.2). All the fixed tissues tested negative to rabies virus antigen by DFAT without sodium citrate buffer treatment. This shows that HIER procedure can be routinely used to retrieve rabies virus antigen for DFAT in cases where only formalin-fixed tissues are available or when preservation by freezing is difficult.

Formalin-fixed DFAT would be useful in instances where rabies is not considered in the diagnosis of an encephalitic condition until after tissue samples have been placed in buffered formalin (Umoh and Blenden, 1981; Mani and Madhusudana, 2013). The technique should also be especially useful in developing countries where poor refrigeration facilities and tropical heat combine to cause rapid deterioration of tissue samples. It is also important to note that the shipping and handling of formalin-preserved tissues is safer for all persons who come into direct or indirect contact with them (Umoh and Blenden, 1981).

In this study, fresh brain tissues were positive in 80% cases tested for the presence of rabies virus antigen by DFAT. Although, the intensity of fluorescence showed no apparent difference in the different parts of the brain, the size of fluorescence varied and ranged from large oval to small dust like particles. This finding was also observed by Raju *et al.* (2008) who also suggested that thorough examination of impression smears of various anatomic location of the brain is needed for a reliable diagnosis by DFAT. A concordance coefficient of 98%

(95% C.I. 0.9660 to 0.9903) was recorded for DFAT on fresh and formalin-fixed brain tissues which indicate no apparent difference in the intensity of fluorescence of DFAT on fresh sample and formalin-fixed DFAT. The Cohen's kappa coefficient value of DFAT on formalin-fixed tissue relative to DFAT on fresh tissue was 1.000 (95% C.I. 1.000-1.000) which shows a very good strength of agreement between the two techniques. These further attest to the accuracy and reliability of formalin-fixed DFAT.

Conclusion

Although the HIER has been used for antigen retrieval from formalin-fixed tissues in Immunohistochemistry, this study provides new information on the retrieval of rabies antigen by HIER for DFAT on formalinized tissues. Thus, formalin can henceforth be used for the preservation of rabies suspected tissues and subsequently retrieved by HIER procedure when preservation by freezing may be challenging or whenever there are safety concerns during transportation. Our study also indicates that HIER procedure can be routinely used to retrieve rabies virus antigen for DFAT in cases where only formalin-fixed tissues are available. The HIER procedure is easy to apply and suitable for tropical countries with limited electricity since it does not require refrigeration. This study also shows that formalin-fixed DFAT is as valid and reliable procedure for rabies diagnosis as the DFAT on fresh samples and would be useful in cases where the current standard techniques cannot be used.

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Conflict of interest

The authors declare that there is no conflict of interest.

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