Indian Journal of Animal Sciences **84** (2): 159–161, February 2014/Short communication

Sensitivity comparison of immunofluorescent techniques for detection of rabies virus antigen from brain specimens

A B BEIGH[1], B S SANDHU[2], C K SINGH[3], K GUPTA[4] and N K SOOD[2]

Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab 141 004 India

Received: 16 September 2013; Accepted: 18 October 2013

Key words: Animals, FAT, Formalin fixed tissue, Impression smear, Rabies

Rabies, an infectious disease caused by a virus of genus *Lyssavirus*, is considered to be one of the greatest economic problems related to livestock and public health. At necropsy, rabies is usually diagnosed by subjecting fresh or formalin fixed nervous tissue samples to pathological examination and the routine diagnostic methods used are fluorescent antibody test (FAT) on brain impression smears and histopathological examination of the brain for Negri bodies. However, the occurrence of rabies virus infection and Negri bodies were not always found correlated and the use of fresh tissue samples for laboratory examination is hazardous due to possible risk of contamination of the environment with rabies virus (Gunawardena and Blackmore 2007).

In many situations only formalin-fixed tissue is available for postmortem diagnosis due to lack of laboratory facilities or fresh tissues to the laboratory (Warner *et al.* 1997, Abreu *et al.* 2012). Hence there is a need of better diagnostic method which can be applied on formalin fixed tissue samples. There were numerous attempts to find a satisfactory method to detect rabies virus antigen in chemically-preserved tissue, or for retrospective immunofluorescent (IF) examination of tissue that was formalinized at the time of necropsy (Swoveland and Johnson 1979, Johnson *et al*.1980, Reid *et al*. 1983, Umoh *et al*. 1985, Bourhy and Sureau 1990). However, FAT on fresh tissue is most satisfactory and standard procedure for detection of rabies antigen (Goldwasser and Kissling 1958). In the present study, sensitivity and reliability of FAT procedures on 100 fresh and formalin fixed brain tissues were compared.

Tissue source: Brain samples (100) were collected from rabies suspected cases that were received for postmortem detection of rabies in the Department of Veterinary Pathology, GADVASU, Ludhiana. Samples were collected from different locations, viz. hippocampus, cerebellum, and brain stem respectively. Three pieces of each tissue sample were stored at –20°C, in 50% glycerol saline solution, and in 10% neutral buffered formalin (NBF) solution respectively.

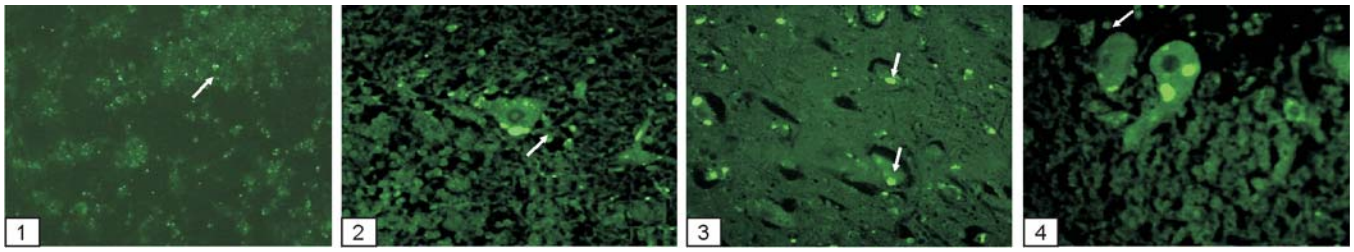
Tissue preparation: The brain tissues were collected as paired samples. One of the portions was prepared for formalin-fixed FAT testing and other portion for fresh FAT testing. Impression smears were made for fresh tissue specimen and fixed in acetone as described previously (Goldwasser and Kissling 1958). Formalin fixation of tissue was done at room temperature in 10% NBF. Formalin fixation was done for minimum of 24 h and maximum of 60 days.

Detection of rabies virus antigen in fresh tissue impression smear using fluorescein-labeled antibody: The detection of rabies virus antigen in impression smear was done similarly as described previously by Awahan *et al.* (2012).

*Detection of rabies virus antigen in formalin-fixed tissues using fluorescein-labeled antibody:*The FAT of formalin-fixed tissues was performed as per Warner *et al.* (1997) with the following modifications: The dewaxing and rehydration of tissue sections was carried out by EZ-AR common solution at 70°C for 10 min in microwave oven; antigen retrieval was done in citrate buffer (0.01 M, pH 6.0–6.2) EZ-Retriever^R System V.2.1 at different time and temperature combinations-2 cycles–95°C for 10 min and at 98°C for 5 min, respectively. Thereafter, slides were washed with PBS washing buffer (pH 7.2–7.6) for 3 times, 5 min each; and allowed to dry at room temperature. Fluorescein isothiocyanate (FITC) conjugate (0.1 ml) was added on every paraffin embedded tissue sections. The slides were incubated at 37°C for 60 min by placing in a humidified chamber. Slides were washed with PBS washing buffer (pH 7.2–7.6) for 2 times 5 min each, twice more in deionized water at room temperature. The sections were air-dried and cover glasses were applied using aqueous mounting media fluoromount. The slides were examined using a fluorescent microscope.

Comparison of test results: Sensitivity of FAT on formalin

Present address: [1] Ph D Student, Veterinary Pathology, SKUAST-K, Srinagar 190 006 (BeighAB@gmail.com). [2,4]Associate Professor (bhupisandhu@redifmail.com, kgupta1968@yahoo.com), [3,5]Senior Veterinary Pathologist (rabiesck@gmail.com, nareshsood47@yahoo.com).



Figs 1–4. **1.** Impression smear drawn from hippocampus of a rabid dog showing apple green fluorescence in neurons. Direct FAT ×165. **2.** Section of cerebellum of rabid cow showing sharply demarcated green colored Negri bodies (arrow) in Purkinje cell and green colored small viral particles distributed in the stroma. Direct FAT. ×400. **3.** Section of hippocampus of rabid dog showing sharply demarcated green coloured Negri bodies (arrows). Direct FAT.× 400. **4.** Section of cerebellum of rabid buffalo showing sharply demarcated green colored Negri bodies (arrow) in Purkinje cell. Direct FAT ×400.

Table 1. Results with FAT on fresh tissue smears

Species	Total no. of animals	Direct FAT positive	FAT on formalin fixed tissue positive
Dog	41	22	22
Buffalo	28	17	18
Cow	15	9	9
Horse	4	2	3
Mongoose	3	3	3
Cat	3	2	2
Rabbit	1	0	0
Samber	1	0	0
Monkey	1	0	0
Jackel	1	1	1
Hyena	1	1	1
Ratel	1	1	1
Total	100	58 (58%)	60 (60%)

fixed brain tissue samples was calculated in comparison with direct FAT on brain impression smears as per Perrin and Sureau (1987). The results of both FAT procedures were compared. Out of total 100 cases, 2 cases showed false negative results with FAT on fresh tissue smears (Table 1).

FAT on fresh tissue smears: Brain tissues were positive in 58 cases (58%) out of 100 cases for the presence of rabies viral antigen. Characteristic apple green immunofluorescence was observed intra-cytoplasmic in neurons (Fig.1) as well as in the form of diffused fluorescence in the brain tissue smears. FAT is sensitive, specific, easy to perform, serves as standard diagnostic procedure, and is the preferred test for rabies diagnosis (Velleca and Forrester 1981, Smith 1999, Whitfield *et al.* 2001).

FAT on formalin fixed tissue: Out of 100 cases, 60 (60%) were positive for rabies virus antigen and revealed 100% sensitivity in comparison to FAT on fresh tissue smears (Table 2). The viral antigen in formalin fixed tissue was visible as distinct apple green coloured intracytoplasmic inclusion bodies and finely granular particles along dendritic arborization, axonal tracts and in the stroma (Figs 2–4). Detection of viral antigen was almost same in tissues stored in formalin for short and long period of time.

Table 2. Sensitivity of FAT on formalin fixed brain tissue samples in comparison to FAT on brain tissue smears

Test	FAT on fresh brain smears (positive)	FAT on fresh brain smears (negative)	Total
FAT on formalin fixed tissue (positive)	58	2	60
FAT on formalin fixed tissue (negative)	0	40	40
Total	58	42	100

$$\text{Sensitivity of FAT on formalin fixed brain tissue samples} = \frac{\text{True positive}}{\text{(true positive + false negative)}} \times 100$$

$$= 60/58+2 \times 100 = 6000/60 = 100\%$$

Comparison of FAT on fresh tissue smear and FAT on formalin fixed tissue: FAT on formalin fixed brain tissue samples revealed 100% sensitivity in comparison to FAT on brain tissue smears (Table 2). Two animals were positive by FAT on formalin fixed tissue but negative with FAT on fresh tissue. With either of the FAT procedures, the most likely explanation of apparent false-negative results is that the rabies antigen may not have been evenly distributed, thus no antigen was present in the sections examined or the disease could have been due to some other pathogen showing similar symptoms like that of rabies. In either of the FAT procedures no false positive results were observed. Proper antigen retrieval and optimum working dilution of anti-rabies antibody are the 2 key factors determining desirable results. FAT on formalin fixed tissues showed comparable results to that of fresh tissue specimens. Thus, present study observed that FAT on formalin fixed tissue is better than FAT on brain tissue smears and can replace FAT on fresh tissue smear for rabies diagnosis.

SUMMARY

The fluorescent antibody test (FAT) is most preferred method to visualize fine particles of rabies viral antigen in rabies infected tissues. In present study, formalin-fixed brain

tissues from 100 clinically rabid animals were heat treated and tested for the presence of rabies virus antigen by direct immunofluorescent (IF) staining. The results were compared with those obtained from direct IF staining of acetone- fixed smears. The evaluation of rabies status between fresh and formalin – fixed tissue was in agreement in 100% of the cases. These results suggested that this technique is a valid procedure for routine diagnosis of rabies, particularly in situations in which fresh tissue is not available.

ACKNOWLEDGEMENTS

Authors thank the Director of Research, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana for providing financial support to conduct this study. We also thank Mr Dan Singh, Mr Kewal Singh, Mr Dilchain Singh and staff of rabies diagnostic and histopathology laboratory for their help, cooperation and support extended during the period of study.

REFERENCES

Abreu C C, Nakayama P A, Nogueira C I, Mesquita L P, Lopes P F, Varaschin M S, Seixas J N, Ferreira E and Bezerra P S Jr. 2012. Domestic microwave processing for rapid immuno-histochemical diagnosis of bovine rabies. *Histology and Histopathology* **27**(9): 1227–30.

Awahan S, Sandhu B S, Singh C K, Gupta K, Sood N K and Kaw A. 2012. Comparison of clinico-pathological, immuno-histochemical and immunofluorescent techniques for diagnosis of rabies in animals. *Indian Journal of Animal Sciences* **82**(1): 3–8.

Bourhy H and Sureau P. 1990. Laboratory Methods for Rabies Diagnosis. *Collection of Institute Louis Pasteu*r. pp.154 –197. Paris, France.

Goldwasser R A and Kissling R E. 1958. Fluorescent antibody staining of street and fixed rabies virus antigen. *Proceedings of the Society for Experimental Biology and Medicine* **98**: 291–323.

Gunawardena G S P de S and Blackemore W F. 2007. Immuno-histochemical detection of rabies virus antigen in the brainstem and spinal cord of rabid dogs in Sri Lanka. *Proceedings of the Peradeniya University Research Sessions* **12**(1): 168.

Johnson K P, Swoveland P T and Emmons R W. 1980. Diagnosis of rabies by immunofluorescencein trypsin-treated histologic sections. *Journal of American Medical Association* **244**: 41–43.

Perrin P and Sureau P. 1987. A collaborative study of an experimental kit for rapid rabies enzyme immunodiagnosis. *Bulletin WHO* **65**(4): 489–93.

Reid F L, Hall N H, Smith J S and Baer G M. 1983. Increased immunofluorescent staining of rabies-infected, formalin-fixed brain tissue after pepsin and trypsin digestion. *Journal of Clinical Microbiology* **18**: 968–71.

Smith J. 1999. Rabies virus. *Manual of Medical Microlbiology*. (Eds) Murray, P R, Baron E J, Pfaller M A, Tenover F C and Yolken R H. 7th edn, pp. 1099–106. American Society for Microbiology, Washington D C.

Swoveland P T and Johnson K P. 1979. Enhancement of fluorescent antibody staining of viral antigens in formalin-fixed tissues by trypsin digestion. *Journal of Infectious Diseases* **140**: 758–64.

Umoh J U, Ezeokoli C D and Okoh A E. 1985. 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. *Journal of Hygiene* **94**: 129–34.

Velleca W M and Forrester F T. 1981. *Laboratory Methods for Detecting Rabies*. U S Government Printing Office, Washington DC.

Warner C K, Whitfield S G, Fekadu M and HO H. 1997. Procedures for reproducible detection of rabies virus antigen mRNA and genome *in situ* in formalin-fixed tissues. *Journal of Virological Methods* **67**: 5–12.

Whitfield C G, Fekadu M, Shaddock J H, Niezgoda M, Warner C K and Messenger S L. 2001. A comparative study of the fluorescent antibody test for rabies diagnosis in fresh and formalin-fixed brain tissue specimens. *Journal of Virological Methods* **95**: 145–5.