Original Research

Comparison of Clinico-Pathological and Immunopathological Techniques for Diagnosis of Rabies in Dogs

A. B. Beigh[1*], B. S. Sandhu[2], S. Bashir[3] and C. K. Singh[2]

[1]Directorate of Extension- Sher-e-Kashmir University of Agricultural Sciences and Technology, J&K-190006, INDIA
[2]Department of Veterinary Pathology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana-141004, Punjab, INDIA
[3]Department of Biochemistry, Hamdard University, New Delhi-110062, INDIA

***Corresponding author:** beighab@gmail.com

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Abstract

The present study was consummated to compare the sensitivity of detection of rabies virus antigen by different immunopathological techniques in specie which have pivotal role in transmission of the disease i.e. dogs. Out of total 41 cases, 22 cases were positive by direct-FAT, indirect-FAT, IHC and 12 cases by demonstration of Negri bodies i.e. histopathology and thus, revealed 68.75% sensitivity in comparison to direct- FAT. While as, indirect-FAT, and IHC revealed 100% sensitivity in comparison to direct-FAT. Percentage of neurons positive for Negri bodies by H & E and IHC were 13.22% and 33.63% and average number of Negri bodies detected per neuron by H & E and IHC were 1.39 and 2.75. Thus, it is concluded that rabies diagnosis in dogs can be accomplished from detection of rabies viral antigen from fixed brain tissues which offers same sensitivity as detection of rabies in impression smears.

Key words: Dogs, FAT, IHC, Histopathology, Rabies

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Introduction

Rabies is a zoonotic viral disease and canines pose serious threats to human beings hence it is of serious concern for medical and veterinary fraternity alike. It is a neuroparalytic viral disease of animal species caused by a neurotropic negative sense single stranded RNA (ssRNA) virus of the genus *Lyssavirus*, Order Mononegavirales and of family Rhabdoviridae. The virus particles have a bullet shaped (*rhabdos* = rod in Greek) structure with a diameter of 75nm and a length of 100-300nm (Meslin *et al.*, 1996), 11-15 kb in size (Murphy *et al.*, 1999). Rabies virus is transmitted when the virus is introduced into bite wounds, into open

cuts in skin, or onto mucous membranes from saliva or other potentially infectious material such as neural tissue (Meslin *et al.*, 1996). Viral replication and dissemination occur widely throughout the CNS before the virus spreads centrifugally to the salivary glands. It is not possible to detect rabies infection during the incubation period, which is generally 1 to 8 weeks (Stein *et al.*, 2010). Rabies in animals occurs in two clinical forms, i.e. furious and paralytic. Rabies in buffaloes and cattle is widely prevalent in India, wherein buffaloes, cattle and other livestock species get infected from rabies virus mainly through bites of rabid dogs and occasionally through bites of rabid carnivores like mongoose, cats, jackals and rodents (Sudarshan, 2004). Dogs are the vectors or reservoirs for rabies and the virus is transmitted through the saliva after infected animal's bites (Krebs *et al.*, 2003).

Diagnosis of clinical rabies is difficult and is often not made until after death of the animal, so early diagnosis of rabies in animals is necessary for timely administration of post-exposure prophylaxis (Zimmer *et al.*, 1990). 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 on brain impression smears and histopathological examination of the brain for Negri bodies. These inclusions are not present in all cases and the use of fresh tissue samples for laboratory examination is hazardous due to possible risk of contamination of the environment with rabies virus. However, in many situations, only formalin-fixed tissue is available for post-mortem diagnosis due to lack of laboratory facilities or presentation of fixed rather than fresh tissues to the laboratory (Abreu *et al.*, 2012). Hence, there is a need for a better method of diagnosis of rabies using formalin-fixed paraffin-embedded tissues. Formalin fixation of tissues simplifies collection, storage and transport of samples, and eliminates hazards and need for bio containment. It also facilitates retrospective studies.

Immunohistochemistry and indirect FAT technique improves diagnostic accuracy by promoting visualization of the distribution of the infectious disease agent in histological sections (Rissi *et al.*, 2008). They provide sufficient amplification of the antibody-antigen interaction to enable detection of antigens immunogenically altered by fixation. So, the present study was envisaged to establish the comparison of sensitivity of routine detection with application of FAT on nervous tissue impression smear with other techniques on formalin fixed nervous tissues.

Materials and Methods

Clinical Cases

Dogs (41) suspected for rabies presented at Rabies Research cum- Diagnostic laboratory, of the Guru Angad Dev Veterinary and Animal Sciences University (GADVASU) was incorporated in the present study.

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Sample Collection

The samples collected were whole brain. Three pieces of each tissue sample were stored in deep freezer at –20°C, in 50% glycerol saline solution, and in 10% neutral buffered formalin solution.

Direct Fluorescent Antibody Technique (dFAT) of Brain Tissue Impression Smears

The dFAT was employed as diagnostic technique because of its sensitivity, accuracy and speed as recommended by World Health Organization (Meslin *et al*., 1996). Lyophilized, adsorbed Anti-rabies nucleocapsid fluorescein isothiocyanate (FITC) conjugate was acquired from Bio-rad Marnes-La-Coquette, France. 0.1ml of conjugate was added on the duplicate impression smears that is one positive and negative control slides. The smears were covered with cover slips and slides incubated at 37°C for 30 minutes by placing in a humidified chamber. The slides were twice washed in 0.01 M phosphate buffered saline (PBS) pH 7.5 for 5 minutes each. Thereafter, air-dried and mounted in 90 per cent buffered glycerol (pH 8.5). The slides were examined using an $AHBT_3$ - RFC reflected light fluorescence attachment (Olympus, Japan).

Indirect-Direct Fluorescent Antibody Technique (Indirect-FAT)

Paraffin embedded tissues cut at 4-5µ thick sections were mounted on Superfrost/ Plus slides (Fisher Scientific, USA).The dewaxing and rehydration of tissue sections was carried out by EZ-AR Common solution at 70°C for 10 minutes in microwave oven followed by antigen retrieval in citrate buffer (0.01 M, pH 6.0-6.2). Then fluorescein isothiocyanate (FITC) conjugate (0.1 ml) was added on paraffin embedded tissue sections and then incubated at 37°C for 60 minutes by placing in a humidified chamber. Then slides were washed with PBS washing buffer (pH 7.2-7.6) for 2 times 5 minutes each. The sections were air dried and cover glasses were applied using aqueous mounting media Fluoromount™ (SIGMA-ALDRICH, Saint Louis, Missouri, USA). The slides were examined using a fluorescent microscope (Nikon, 800*i*, Japan).

Immunohistochemistry Technique

Paraffin embedded tissues cut at 4-5µ thick sections were mounted on slides (Fisher Scientific, USA).The dewaxing and rehydration of tissues sections were carried out by EZ-AR Common solution (BioGenex Laboratories Inc., San Ramon, California, USA) at 70°C for 10 minutes followed by antigen retrieval in EZ-AR 3 solution at 95°C for 5 minutes. The endogenous peroxidase activity was quenched by incubating slides with a solution of 3% H_2O_2 in methanol for 25 minutes at room. Non-specific protein binding was blocked using power block solution (BioGenex Laboratories Inc., San Ramon, California, USA) for 15 minutes. Slides were incubated with polyclonal rabbit anti-rabies antibody (1:500 and 1:1000 dilution in PBS 1% BSA) for one hour at room temperature. Slides were washed with PBS buffer for 3 times, 3 minutes each subsequently. Then sections were incubated with secondary antibody ImmPRESS™ UNIVERSAL kit

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(Vector Laboratories Inc., Burlingame, U.S.A.) for 30 minutes at room temperature. Slides were thereafter washed with PBS washing buffer for 3 times, 3 minutes each. The antigen-antibody-peroxidase reaction was visualized by adding 3, 3 '-diaminobenzidine (DAB) solution on sections for 1-2 minutes. Sections were washed in tap water for 5 minutes and were counterstained with Gill's haematoxylin (Merck, Germany) for 30 seconds and finally washed in running tap water for 5 minutes. The sections were dehydrated in ascending grades of alcohol, cleared in xylene and mounted with DPX. The stained slides were examined under microscope (BX 61, Olympus Corporation, Japan).

Histopathology

All tissues samples viz. cerebellum, cerebrum, hippocampus, pons and medulla oblongata from dead animals were collected in 10% neutral buffered formalin solution.After fixation in 10% neutral buffered formalin, tissue samples were given overnight washings under tap water. These tissues were routinely processed through ascending grades of alcohol, cleared in benzene and embedded in paraffin wax. The paraffin sections were cut at 4 to 5µ thickness and stained by haematoxylin and eosin (H & E) method (Luna, 1968).

Sensitivity Comparison with Direct-FAT

Sensitivity of various techniques was calculated in comparison with dFAT (Perrin and Sureau, 1987).

$$Sensitivity = \frac{True\ Positive}{True\ positive + False\ negative}$$

Result and Discussion

Clinical Signs

In case of rabid dogs, anorexia was found in 81.8% (18/22) cases, followed by paralysis in 77.2% (17/22); behavioral change in 68.18% (15/22); hyper salivation and difficult intake of food in 59% (13/22) and history of biting/aggressiveness in 50% (11/22) cases. However, not recognization of owner in 45.4% (10/22) and pica in 36.36% (9/22) cases**.** Whereas, 22.72% (5/22) cases showed Circling, respectively (Table1). Similar symptoms have been earlier reported (Salem *et al*., 1995, Rissi *et al*., 2008).

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Table 1: Clinical signs in rabid dogs (Total positive cases = 22)

Symptoms	No. of Animals	Percentage
Off feed	18	81.80%
Hyper salivation	13	59%
Difficulty in standing/paralysis	17	77.20%
Fever	10	45.40%
History of	11	50%
biting/aggressiveness		
Difficult intake of food	13	59%
Pica	8	36.36%
Not recognizing owner	10	45.40%
Behavioral change	15	68.18%
Circling	5	22.72%
Vaccination	5	22.72%

Direct FAT

Out of 41 cases, 22 cases (53.6 %) were diagnosed positive for the presence of rabies viral antigen (Table 2). Characteristic apple green immunofluorescence was observed intra-cytoplasmic in neurons as well as in form of diffused fluorescence in the brain tissue smears (Fig. 1). FAT is sensitive, specific, and easy to perform, serves as standard diagnostic procedure and is the preferred test for rabies diagnosis (Whitfield *et al.*, 2001). It serves as a gold standard for assessment of immunohistochemistry, histopathology and modified fluorescent-antibody test on formalin fixed tissues (Awahan *et al.*, 2012; Pedroso *et al.*, 2009).

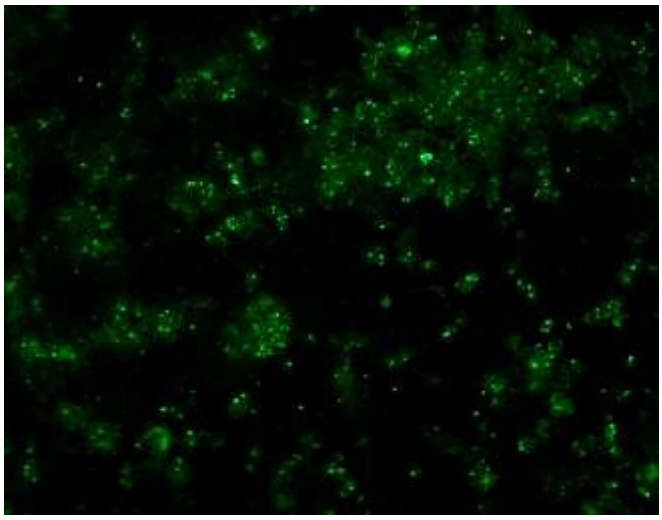

Fig.1: Impression smear drawn from hippocampus of a rabid dog showing apple green fluorescence in neurons. Direct FAT X 165

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Table 2: Comparison of Direct-FAT with other diagnostic techniques for detection of rabies virus antigen

S. no.	Direct-FAT	Histopathology	Indirect-FAT	IHC
1.	-	-	-	-
2.	+	+	+	+
3.	-	-	-	-
4.	-	-	-	-
5.	-	-	-	-
6.	-	-	-	-
7.	-	-	-	-
8.	-	-	-	-
9.	+	+	+	+
10.	+	-	+	+
11.	+	+	+	+
12.	+	+	+	+
13.	+	+	+	+
14.	+	-	+	+
15.	+	-	+	+
16.	-	-	-	-
17.	-	-	-	-
18.	+	+	+	+
19.	+	+	+	+
20.	-	-	-	-
21.	+	+	+	+
22.	+	+	+	+
23.	-	-	-	-
24.	+	-	+	+
25.	-	-	-	-
26.	-	-	-	-
27.	-	-	-	-
28.	+	-	+	+
29.	-	-	-	-
30.	+	-	+	+
31.	+	-	+	+
32.	+	-	+	+
33.	+	+	+	+
34.	+	-	+	+
35.	+	+	+	+
36.	-	-	-	-
37.	-	-	-	-
38.	+	+	+	+
39.	+	-	+	+
40.	-	-	-	-
41.	-	-	-	-
%Test positive	**(22/41) 53.6%**	**(12/41) 29.26%**	**(22/41) 53.6%**	**(22/41)53.6%**

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Indirect FAT

Out of 41 cases, 22 cases (53.6 %) were found positive for rabies virus antigen (Table2) and revealed 100% sensitivity in comparison to direct-FAT on fresh tissue smears (Table 3).

Table 3: Sensitivity comparison of FAT on fixed brain tissue (indirect-FAT) with that on direct FAT.

Test	FAT on Fresh Brain Smears (Positive)	FAT on Fresh Brain Smears (Negative)	Total
FAT on formalin fixed tissue (Positive)	22	0	22
FAT on formalin fixed tissue	0	19	19
Total	22	19	41

$$\text{Sensitivity of FAT on formalin fixed brain tissue sample} = \frac{True\ Positive}{True\ Positive + False\ Negative} \times 100$$

$$= 22/22+ 0 \times 100= 2200/22 = 100\%$$

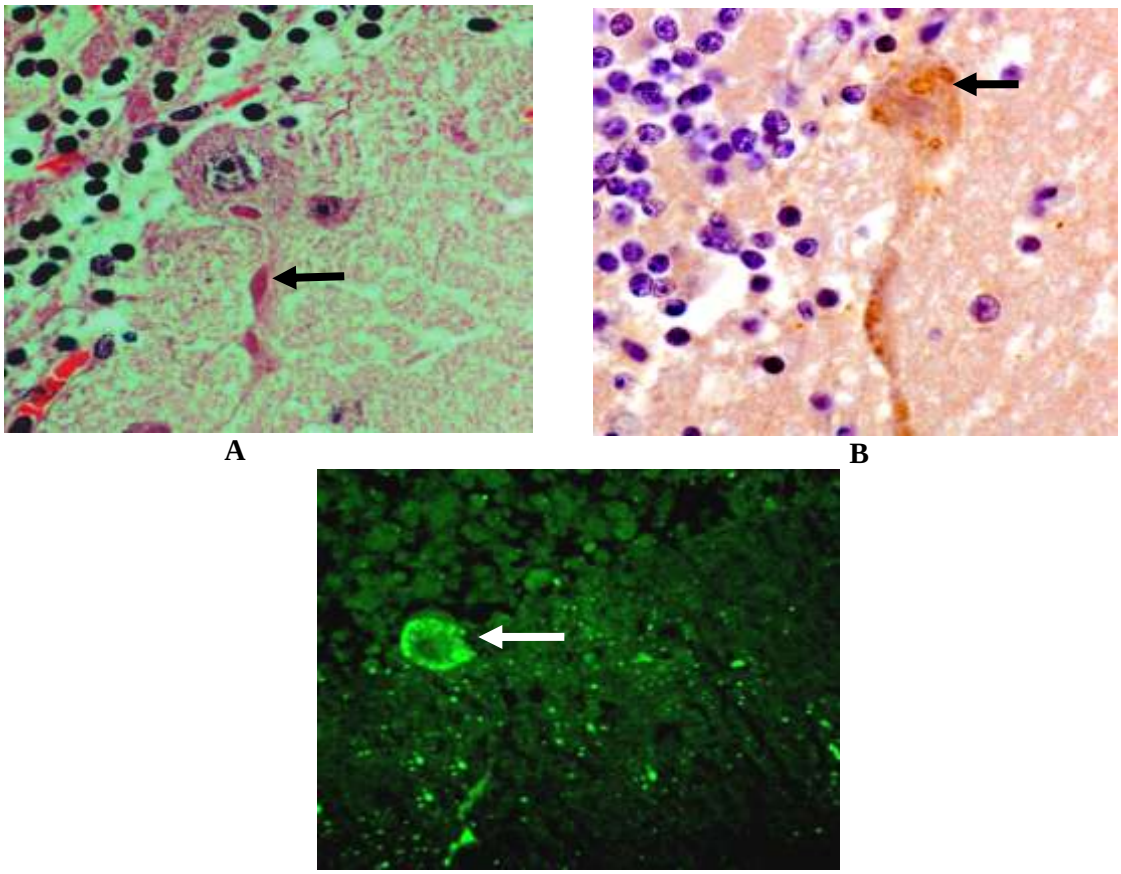

Fig. 3: Section of cerebellum of rabid dog showing few Negri bodies (arrow) with H&E stain- Original magnification x 1000X (A), corresponding IHC stained section - Original magnification x 1000X (B) showing brown coloured Negri bodies (arrow) and corresponding FAT stained section- Original magnification x 400X (C) showing more clearly green coloured Negri bodies (arrow).

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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 (Fig. 3& 4).

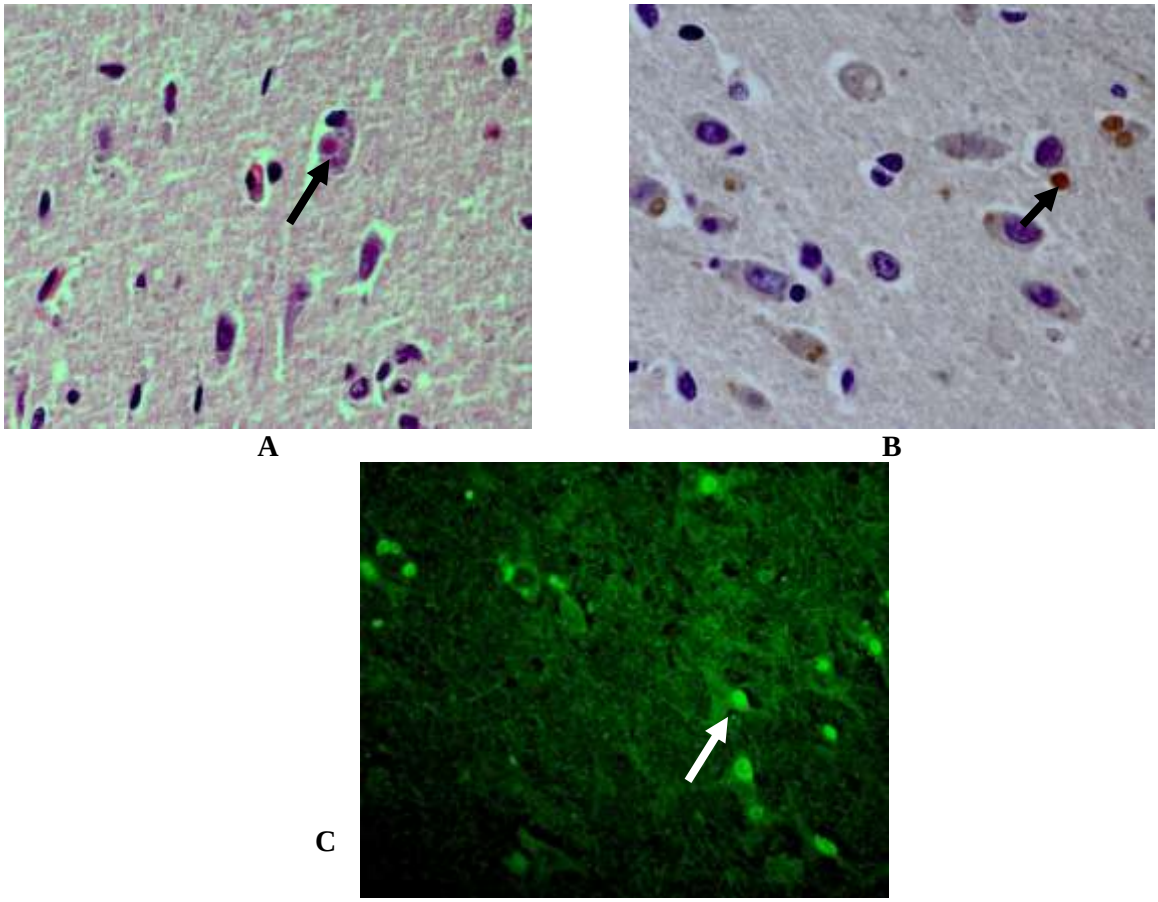

Fig. 4: Section of hippocampus of rabid dog showing faintly stained Negri bodies (arrow) with H&E stain- Original magnification x 1000X (A), corresponding IHC stained section - Original magnification x 1000X (B) showing brown coloured Negri bodies (arrow) and corresponding FAT stained section- Original magnification x 400X (C) showing more clearly green coloured Negri bodies (arrow).

Although FAT is the standard technique for quick rabies diagnosis, it requires the use of fresh samples, which contain live virus and entail public health risk (Woldehiwet *et al.*, 2005). Transporting fresh samples is a problem in countries where diagnostic laboratories are not well established or where lack of refrigeration and high ambient temperatures can interfere with the FAT (Stein *et al.*, 2010). FAT on formalin fixed tissue can be used as an alternative to FAT on fresh tissue with the same sensitivity, when only formalin-fixed tissue is available for post-mortem diagnosis. Detection of viral antigen was almost same in tissues stored in formalin for short and long period of time. Several attempts have been made to detect rabies virus antigen in formalin-fixed tissues using FAT staining procedures (Warner *et al*., 1997; Whitfield *et al*., 2001; Abreu *et al*., 2012).

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Immunohistochemistry

Brain tissues were positive in 22 out of 41 cases (53.6 %) (Table 2), using polyclonal antiserum by immunohistochemistry and it revealed 100% sensitivity in comparison to direct-FAT (Table 4). The controls were negative and free of endogenous peroxidase (Fig. 2).

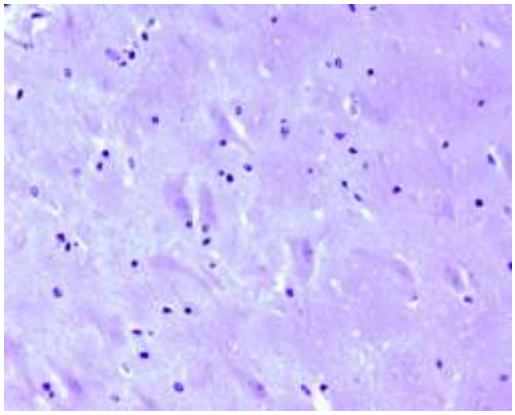

Fig. 2: Negative control of IHC-Section of hippocampus showing absence of reaction. IHC-One step polymer HRPO Technique - Original magnification x 400X

Table 4: Sensitivity of IHC in comparison to direct –FAT

Test	FAT on Brain Smears (Positive)	FAT on Brain Smears (Negative)	Total
IHC on brain (Positive)	22	0	22
IHC on brain (Negative)	0	19	19
Total	22	19	41

$$\text{Sensitivity of IHC on formalin fixed brain tissue samples} = \frac{\text{True positive}}{\text{True positive} + \text{False negative}} 100$$

$$= 22/22+ 0 \times 100= 2200/22 = 100\%$$

A large amount of distinct, granular rabies viral antigen deposits stained as sharply demarcated brown precipitates of variable sizes were found within the Purkinje cells and in the neurons of the hippocampus, in the axons, in the processes of neurons and in the stroma (Fig. 3 & 4). IHC improves diagnostic accuracy by promoting visualization of the distribution of the infectious disease agent (Bourh and Sureau, 1990, Rissi *et al*., 2008). Over the last 2 decades, immunohistochemistry (IHC) has become increasingly popular for detecting numerous antigens in fixed tissues (Jogai *et al.,* 2001; Woldehiwet *et al.,* 2005). Studies have shown that the sensitivity of IHC for rabies is equal to that of FAT (Zimmer *et al.,* 1990). IHC may be even more sensitive in early diagnosis of suspected cases when traditional histological and FAT techniques could not detect viral antigens or lesions (Lembo *et al.,* 2006; Woldehiwet *et al.,* 2005).

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Histopathology

Out of 41cases, 12 cases (29.26 %) were found positive for rabies by demonstration of Negri bodies (Table 2) and thus, histopathology revealed 77.77% sensitivity in comparison to direct-FAT (Table 5).

Table 5: Sensitivity of histopathology in comparison to direct -FAT

Test	FAT on Brain Smears (Positive)	FAT on Brain Smears (Negative)	Total
Histopathology(Positive)	12	0	12
Histopathology(Negative)	10	19	29
Total	22	19	41

$$\text{Sensitivity} = \frac{\text{True positive}}{\text{True positive} + \text{False negative}} \times 100$$

= 12/12+1 0 ×100= 1200/22 = 68.75%

Negri bodies appeared as single or multiple, eosinophilic intracytoplasmic inclusions within the Purkinje neurons**,** in the axons and in the neurons of the hippocampus (Fig. 3 & 4). Histologically, rabies is characterized by a viral encephalitis with Negri bodies in neurons, but these changes can be mild or even absent (Maxie *et al.,* 2001)**.** Negri bodies were seen as intracytoplasmic and eosinophilic type of inclusion bodies inside the Purkinje neurons, in the axons (Pedroso *et al.,* 2008, Rissi *et al.,* 2008). Although the presence of Negri bodies has been considered pathognomonic for rabies, they are absent in 20% to 60% of rabies cases (Stein *et al.,* 2010).

Comparison of Immunohistochemistry and Histopathology

Hundred neurons per case were observed for Negri bodies and number of Negri bodies in positive neurons (Table 6) and a comparison of IHC and histopathology were done (Table 7). With IHC 33.63% neurons were positive for Negri bodies and 13.22% with H & E. It can be concluded that IHC established many more virus infected cells than H & E stained sections. Average numbers of Negri bodies detected per neuron by IHC were 2.75 which were greater than H & E stained brain sections (1.39). The amount of antigen detected with IHC was much more abundant than histopathological findings (Fig. 3 & 4). IHC established many more virus infected cells than H & E stained sections (Awahan *et al.,* 2012). The amount of antigen detected with IHC was much more abundant than histopathological findings (Jogai *et al.,* 2001, Suja *et al*., 2004).

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Table 6: Histopathological and Immunohistochemical evaluation of brain tissues for number of Negri bodies

S. No.	No. of Neurons Positive for Negri Bodies/100 Neurons		No. of Negri Bodies Detected/100 Neurons	
	H &E	IHC	H & E	IHC
1.	26	57	40	117
2.	10	18	11	56
3.	0	7	0	23
4.	0	61	10	73
5.	0	6	0	8
6.	0	23	0	35
7.	0	52	0	77
8.	31	94	44	247
9.	0	36	0	70
10.	54	87	57	152
11.	23	32	27	40
12.	0	55	0	65
13.	57	82	91	439
14.	23	47	35	65
15.	0	17	0	20
16.	22	47	30	100
17.	19	63	19	119
18.	21	53	21	66
19.	0	46	0	129
20.	0	18	0	30
21.	5	22	5	26
22.	16	43	16	80
Total	291	740	406	2037

Table 7: Comparison of histopathology (H&E) and immunohistochemistry (IHC)

Parameter	H& E	IHC
Neurons having Negri bodies (n=2200)	291	740
%age of Neurons positive (Negri bodies)	13.22	33.63
Total number of Negri bodies detected	406	2037
Average number of Negri bodies per	1.39	2.75

Conclusion

IHC was more sensitive than histopathology but as sensitive as either of FAT procedures and proved to be a valid method for rabies diagnosis and can replace FAT where fluorescent microscopy is not available or when fresh samples are not available for FAT. As compared to IHC specificity of FAT on formalin fixed

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tissue was more, this is because of nonspecific binding of polyclonal antibody with nonspecific antigens in case of IHC. There is also enhanced detection of viral antigen due to fluorescence of antigen-antibody complex.

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