DIAGNOSIS OF RABIES IN BUFFALOES: COMPARISON OF CLINICO-PATHOLOGICAL, IMMUNOHISTOCHEMICAL AND IMMUNOFLUORESCENT TECHNIQUES

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ABSTRACT

The present study was envisaged to compare the sensitivity of detection of rabies virus antigen by application of Fluorescent Antibody Technique on fresh impression smear (Direct-FAT) and that on formalin fixed nervous tissue (Indirect-FAT), histopathology and immunohistochemistry (IHC) in the species which is highly significant for the economics of the dairy farmer i.e. buffalo. A total of 28 cases of buffaloes suspected for rabies were presented. Out of 28 cases, 18 (64.28%) cases were positive by direct-FAT, indirect-FAT, IHC and 60.71% (17/28) by demonstration of negri bodies and thus, histopathology revealed 94.4% 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 and E and IHC were 59.35% and 78.88% and average number of Negri bodies detected per neuron by H & E and IHC were 1.8 and 3.01, respectively. Important clinical signs in rabid animals were anorexia, circling/Head pressing, behavioural change and bellowing. Thus, it is concluded that rabies detection in animals can be accomplished from diagnosis of rabies from fixed brain tissues which offers same sensitivity as detection of rabies in impression smears.

Keywords: buffaloes, FAT, IHC, formalin fixed, rabies

INTRODUCTION

Rabies is a fatal zoonotic disease of worldwide concern caused by a neurotropic negative sense single stranded RNA (ssRNA) virus of the genus *Lyssavirus*, Order Mononegavirales and of family Rhabdoviridae. 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. 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 Warner

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et al. (1997) and Abreu *et al.* (2012). Hence, there is a need for a better method of diagnosis of rabies using formalin-fixed paraffin-embedded tissues. Immunohistochemistry, indirect FAT and histopathology can be performed on such samples. Immunohistochemistry and indirect FAT technique improves diagnostic accuracy by promoting visualization of the distribution of the infectious disease agent in histological sections Deborah *et al.* (1991). 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 tissue.

MATERIALS AND METHODS

A total of 28 cases of buffaloes suspected for rabies were presented at Rabies Research-cum-Diagnostic laboratory, Department of Veterinary Pathology, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana from various parts of Punjab. The data regarding age, sex, history of bite, date of bite, source of bite and clinical signs was acquired as per the questionnaire prepared for the purpose from owners of the animals.

Clinical samples for detection of rabies

Brain samples were collected from dogs suspected for rabies. Three pieces of each tissue sample were stored at 20°C, in 50% glycerol saline solution, and in 10% neutral buffered formalin solution respectively.

Detection of rabies virus antigen in fresh tissue impression smear using fluorescein-labeled antibody (Direct-FAT)

The direct FAT is employed as diagnostic technique because of its sensitivity, accuracy and speed as recommended by World Health Organization (Meslin *et al*., 1996). A pair of thin impression smears from each of brain tissue were prepared in grease-free labelled glass slides 1 cm in diameter, about 1.5 cm from each end, from either fresh tissue or preserved tissues kept at 20°C in deep freeze.

Control positive slides from known rabies case and control negative from normal uninfected and unvaccinated animal were prepared along with the test smear. Impression smears were air dried for 30 minutes at room temperature. Smears were fixed by immersing in coupling jars containing cold acetone in a deep freeze at 20°C for overnight. Acetone was drained off and impression smear slides were air dried at room temperature for 20 minutes. Lyophilized, anti-rabies nucleocapsid Fluorescein isothiocyanate (FITC) conjugate acquired from Bio-radMarnes-La-Coquette, France was reconstituted with 3 ml of distilled water as recommended by the manufacturer and centrifuged at 1500 rpm for 5 minutes for clarification. The clarified conjugate (0.1 ml) was added on the duplicate impression smears on every slide for each tissue samples and on positive and negative control slides. Then, smears were covered with cover slips and slides incubated at 37°C for 30 minutes by placing in a humidified chamber. Slides were washed twice in 0.01 M phosphate buffered saline (PBS) pH 7.5 for 5 minutes each. Thereafter, slides were air-dried and mounted in 90% buffered glycerol (pH 8.5). Slides were examined using an $AHBT_3$ - RFC reflected light fluorescence attachment (Olympus, Japan).

Detection of rabies virus antigen in formalin-fixed tissues using fluorescein-labeled antibody (Indirect-FAT)

The FAT of formalin-fixed tissues was performed as described by 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 minutes 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 (BioGenex Laboratories Inc., San Ramon, California, USA) at different time and temperature combinations-2 cycles-95°C for 10 minutes and at 98°C for 5 minutes, respectively; Thereafter, slides were washed with PBS washing buffer (pH 7.2 to 7.6) for 3 times, 5 minutes each; and allowed to dry at room temperature.

Then Fluorescein isothiocyanate (FITC) conjugate (0.1 ml) was added on every paraffin embedded tissue sections. After this slides were incubated at 37°C for 60 minutes by placing in a humidified chamber. Then slides were washed with PBS washing buffer (pH 7.2 to 7.6) for 2 times 5 minutes each, then twice more in deionized water at room temperature. The sections were air dried and cover glasses were applied using aqueous mounting media Fluoromount^{TM} (SIGMA-ALDRICH, Saint Louis, Missouri, USA). The slides were examined using a fluorescent microscope (Nikon, 800*i*, Japan).

Immunohistochemistry (IHC)

Antirabies polyclonal antisera (Rabbit) available in the Rabies Research-cum-Diagnostic Laboratory of the department of GADVASU; Ludhiana was used as primary antibody for immunohistochemical studies. Different dilutions of 1:50, 1:100, 1:500, 1:1000, and 1:2000 of polyclonal antisera in PBS (pH 7.2 to 7.6) were used for immunohistochemical staining of brain tissues sections. Maximum dilution of antibody at which these samples revealed positive reaction was 1:1000.

Paraffin embedded tissues were sectioned at 4 to 5µm thickness and mounted on Superfrost/ Plus, positively charged microscopic slides (Fisher Scientific, USA). The slides were then placed in hot air oven to melt the paraffin at 60°C for 30 minutes and stored till further use. Advanced SS^{TM} two step polymer Horseradish Peroxidase (HRPO) Immunohistochemical detection system (BioGenex Laboratories Inc., San Ramon, California, USA) was used for staining of paraffin embedded tissue sections as per recommendation of the manufacturer with some modifications Pedroso *et al.* (2008). The dewaxing and rehydration of tissues sections were carried out by EZ-AR Common solution at 70°C for 10 minutes in microwave oven.

Antigen retrieval

Antigen retrieval was done in EZ-AR^{TM} 3 in EZ-Retriever^{R} System V.2.1 (BioGenex Laboratories Inc., San Ramon, California, USA) at different time and temperature combinations-2 cycles-95°C for 10 minutes and at 98°C for 5 minutes, respectively. The slides were cooled and brought to room temperature, washed with PBS buffer (pH 7.2 to 7.6) for 3 times for 3 minutes each. The endogenous peroxidase activity was blocked by incubating slides with a solution of 3% H_2O_2 in methanol for 25 minutes at room temperature in humidified chamber. Slides were washed with PBS buffer (pH 7.2 to 7.6) for 3 times 3 minutes each and sections were encircled with hydrophobic pen (Pap pen). Non-specific protein binding was blocked using power block solution (BioGenex Laboratories Inc., San Ramon, California, USA) for 15 minutes in moist chamber.

Slides were incubated with primary

polyclonal rabbit anti-rabies antibody (1:500 and 1:1000 dilution in PBS 1% BSA) for one and half hour in humidified chamber at room temperature. For each staining a negative control was run on sister section in which primary antibody was replaced by PBS. Slides were washed with PBS washing buffer (pH 7.2 to 7.6) for 3 times, 3 minutes each subsequently.

The tissue sections were incubated with secondary antibody ImmPRESS™ UNIVERSAL REAGENT Anti-mouse/Rabbit Ig (Vector Laboratories Inc., Burlingame, U.S.A.) for 30 minutes at room temperature in humidified chamber. Slides were washed with PBS washing buffer (pH 7.2 to 7.6) for 3 times, 3 minutes each. Substrate 3, 3 '-diaminobenzidine (DAB) solution, freshly prepared by mixing a drop of ImmPACT™ DAB chromogen with 1 ml of ImmPACT™ DAB buffer (Vector Laboratories Inc., Burlingame, U.S.A) and 5 µl hydrogen peroxide. The antigen-antibody-peroxidase reaction was visualized by adding substrate 3, 3 '-diaminobenzidine (DAB) solution on sections for 1 to 2 minutes. Sections were washed in tap water for 5 minutes to stop the antigen-antibody-peroxidase reaction. Slides were counterstained with Gill's haematoxylin (Merck, Germany) for 30 seconds and washed with running tap water for 5 minutes. Finally the sections were dehydrated in ascending grades of alcohol (70%, 80%, 90%, and absolute alcohol) and cleared in xylene for 2 minutes and mounted with DPX. Slides were examined under microscope (BX 61, Olympus Corporation, Japan).

Histopathology

All nervous tissues samples, viz. cerebellum, cerebrum, hippocampus, pons, medulla oblongata from dead animals were collected in 10% neutral buffered formalin solution. 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 5 µ thickness and stained by haematoxylin and eosin (H and E) method (Luna, 1968). Slides were examined by by BX61 Research Photomicrograph Microscope System of Olympus Corporation, USA.

Sensitivity comparison with direct-FAT

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

$$\text{Sensitivity} = \frac{\text{True positive}}{(\text{True positive} + \text{false negative})} \times 100$$

RESULTS AND DISCUSSION

Clinical signs in rabid buffaloes

In case of rabid buffaloes, anorexia was found in 94.44% (17/18) cases, followed by behavioral change and pressing of head against hard objects in 55.55% (10/18) cases, difficulty in feed intake and hyper-salivation and bellowing in 50% (9/18) cases (Figure1 and 2). Whereas, paralysis and fever in 38.88% (7/18). However, frequent micturition in 27.77% (5/18) cases. Non-recognizing owner 22.22% (4/18) and pica were found in 11.11% (2/18) cases, respectively (Table1). Similar symptoms have been reported by (Salem *et al*., 1995; Rissi *et al*., 2008; Pedroso *et al*., 2009).

Direct FAT

Out of 28 cases, 17 cases (60.71%) were

diagnosed positive for the presence of rabies viral antigen (Table2). Characteristic apple green immunofluorescence was observed intra-cytoplasmic in neurons as well as in form of diffused fluorescence in the brain tissue smears (Figure 3). FAT is sensitive, specific, and easy to perform, serves as standard diagnostic procedure and is the preferred test for rabies diagnosis (Smith, 1999; Whitfield *et al*., 2001).

Indirect FAT

Out of 28 cases, 18 (64.28%) were found positive for rabies virus antigen (Table 2) and revealed 100% sensitivity in comparison to direct-FAT on fresh tissue smears (Table 3). 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 (Figure 5 and 6). Similar finding have been reported by Swoveland and Johnson (1979), Johnson *et al.*(1980), Reid *et al.*(1983), Umoh *et al.*(1984), Bourhy and Sureau (1990). Detection of viral antigen was almost same in tissues stored in formalin for short and long period of time. Johnson *et al.* (1980) suggested FAT on formalin fixed tissue as complementary to standard diagnostic techniques.

Immunohistochemistry

Brain tissues were positive in 18 out of 28 cases (64.28%) (Table 2), using polyclonal antiserum by immunohistochemistry and it revealed 100% sensitivity in comparison to direct-FAT (Table 4). No positive reaction was observed with monoclonal antibody. The controls were negative and free of endogenous peroxidase (Figure 4). 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 (Figure 5and 6). These findings were similar as reported by (Gunawardena and Blakemore, 2007; Pedroso *et al*., 2009).

Histopathology

Out of 28 cases, 17 cases (60.71%) were

Table 1. Clinical signs in rabid buffaloes (Total positive cases=18).

Symptom	No. of animals	Percentage
Off feed	17	94.44
Hyper-salivation	9	50
Fever	7	38.88
Not recognizing owner	4	22.22
Circling/Head pressing	10	55.55
Difficulty in standing/paralysis	7	38.88
Difficult intake of food	9	50
Frequent micturition	5	27.77
Bellowing	9	50
Pica	2	11.11
Behavioral change	10	55.55

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

S. no.	Case no.	Direct-FAT	Indirect-FAT	IHC	Histopathology
1	RL 25/10	+	+	+	+
2	RL 26/10	-	-	-	-
3	RL 33/10	+	+	+	+
4	RL 36/10	+	+	+	+
5	RL 38/10	-	-	-	-
6	RL 41/10	+	+	+	+
7	RL 02/11	+	+	+	+
8	RL 04/11	+	+	+	+
9	RL 10/11	-	-	-	-
10	RL 17/11	+	+	+	+
11	RL 25/11	-	-	-	-
12	RL 28/11	+	+	+	+
13	RL 31/11	+	+	+	+
14	RL 33/11	+	+	+	+
15	RL 36/11	-	-	-	-
16	RL 37/11	+	+	+	+
17	RL 03/12	-	-	-	-
18	RL 04/12	-	-	-	-
19	RL 10/12	+	+	+	+
20	RL 11/12	-	-	-	-
21	RL 14/12	+	+	+	+
22	RL 16/12	+	+	+	+
23	RL 17/12	+	+	+	+
24	RL 18/12	-	-	-	-
25	RL 19/12	+	+	+	+
26	RL 24/12	+	+	+	-
27	RL 25/12	+	+	+	+
28	RL 27/12	-	-	-	-
	% Test positive	64.28% (18/28)	64.28% (18/28)	64.28% (18/28)	60.71% (17/28)

Table 3. Sensitivity of Indirect-FAT in comparison to Direct-FAT on brain tissue smears.

Test	Direct-FAT (Positive)	Direct-FAT (Negative)	Total
Indirect-FAT (Positive)	18	0	18
Indirect-FAT (Negative)	0	10	10
Total	18	10	28

$$\text{Sensitivity of IHC for brain sample} = \frac{\text{True positive}}{\text{(True positive + False negative)}} \times 100$$

$$= 18/18+ 0 \times 100 = 1800/18 = 100\%$$

Table 4. Sensitivity of Immunohistochemistry (IHC) on Brain samples in comparison to FAT on fresh brain tissue smears.

Test	Direct-FAT (Positive)	Direct-FAT (Negative)	Total
IHC on brain (Positive)	18	0	18
IHC on brain (Negative)	0	10	10
Total	18	10	28

$$\text{Sensitivity of IHC for brain sample} = \frac{\text{True positive}}{\text{(True positive + False negative)}} \times 100$$

$$= 18/18+ 0 \times 100 = 1800/18 = 100\%$$

Table 5. Sensitivity of Histopathology on Brain samples in comparison to FAT on fresh brain tissue smears.

Test	Direct-FAT (Positive)	Direct-FAT (Negative)	Total
Histopathology (positive)	17	0	17
Histopathology (negative)	1	10	11
Total	18	10	28

$$\text{Sensitivity of IHC for brain sample} = \frac{\text{True positive}}{\text{(True positive + False negative)}} \times 100$$

$$= 17/17+ 1 \times 100 = 1700/18 = 94.4\%$$

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

Sr. No.	Case No.	No. of neurons positive for Negri bodies/100 neurons		No. of Negri bodies detected/100 neurons	
		H &E	IHC	H & E	IHC
1	RL 25/10	66	87	172	408
2	RL 33/10	46	95	125	381
3	RL 36/10	43	88	69	325
4	RL 41/10	77	80	114	230
5	RL 2/11	59	78	103	164
6	RL 4/11	69	83	119	233
7	RL 17/11	6	33	7	52
8	RL 28/11	14	90	18	217
9	RL 31/11	76	98	282	580
10	RL 33/11	87	100	152	495
11	RL 37/11	39	94	50	190
12	RL 10/12	80	96	146	426
13	RL 14/12	69	89	174	210
14	RL 16/12	41	72	45	141
15	RL 17/12	67	91	87	211
16	RL 19/12	75	86	150	219
17	RL 25/12	95	79	65	178
Total		1009	1341	1878	4044

Table 7. Comparison of histopathology and immunohistochemistry.

Parameter	Histopathology (H& E)	IHC
Neurons having Negri bodies (n=1700)	1009	1341
%age of Neurons positive (Negri bodies)	59.35	78.88
Total number of Negri bodies detected	1878	4044
Average number of Negri bodies per neuron	1.8	3.01

Figure 1. Rabies suspected buffalo exhibiting head pressing and paralysis.

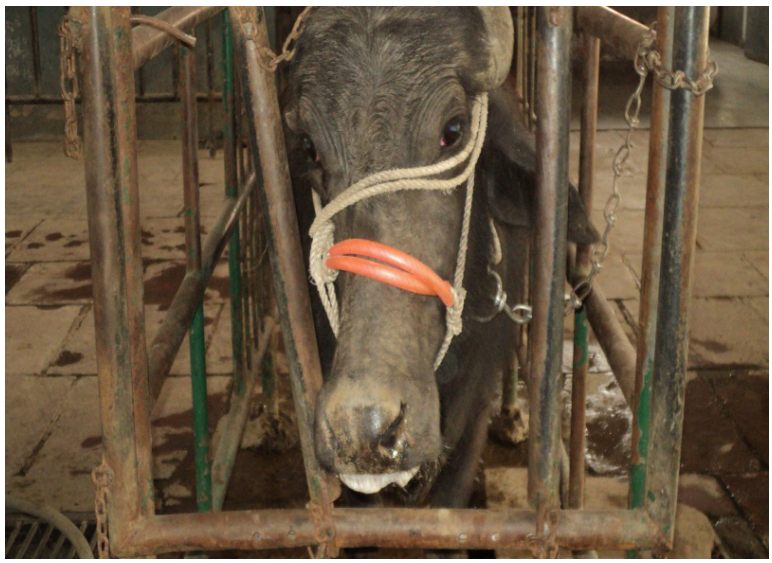

Figure 2. Rabies suspected buffalo exhibiting hypersalivation.

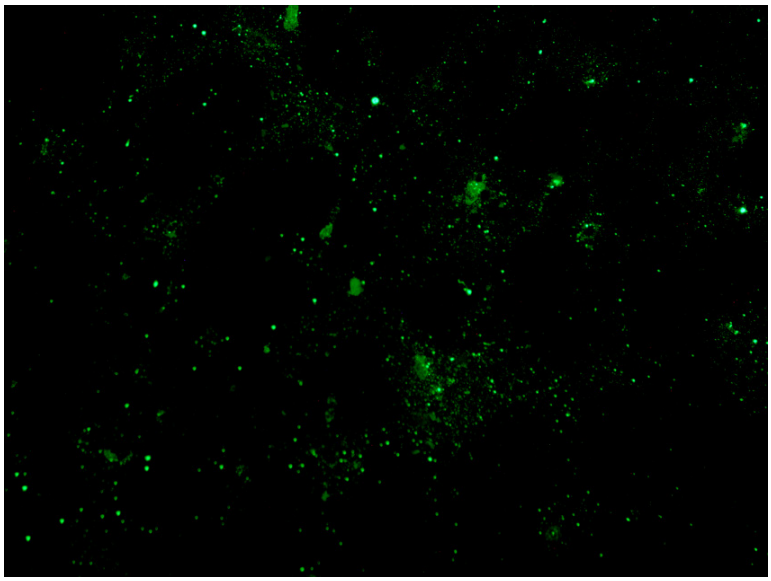

Figure 3. Impression smear drawn from hippocampus of a rabid buffalo showing apple green fluorescence in neurons. Direct FAT X 165.

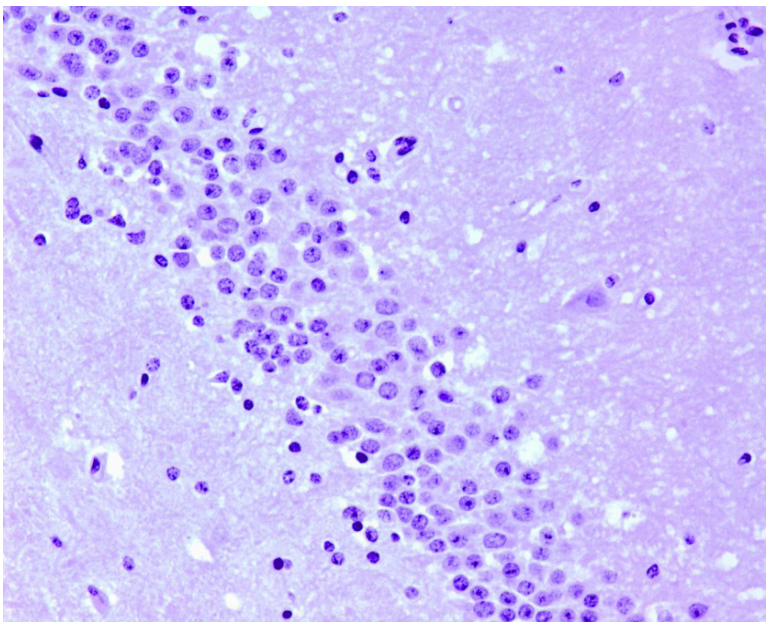

Figure 4. Negative control of IHC-Section of cerebellum showing absence of reaction. IHC-One step polymer HRPO Technique - Original magnification x 400X.

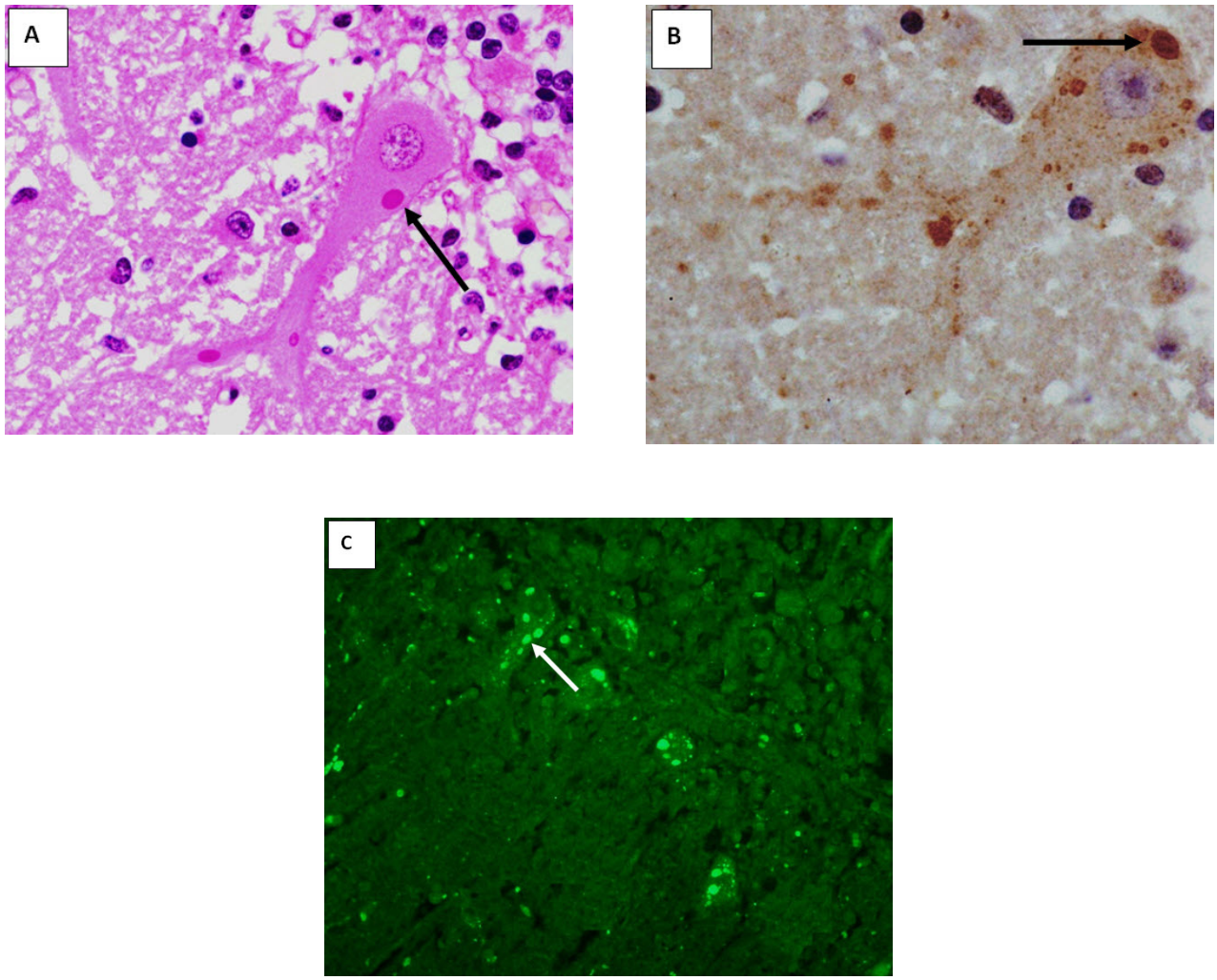

Figure 5. Section of hippocampus of rabid buffalo showing few Negri bodies with H&E stain- Original magnification x 1000X (A), corresponding IHC stained section - Original magnification x 1000X (B) showing brown coloured Negri bodies (arrow) and corresponding FAT stained section- Original magnification x 400X (C) showing more clearly green coloured Negri bodies (arrow).

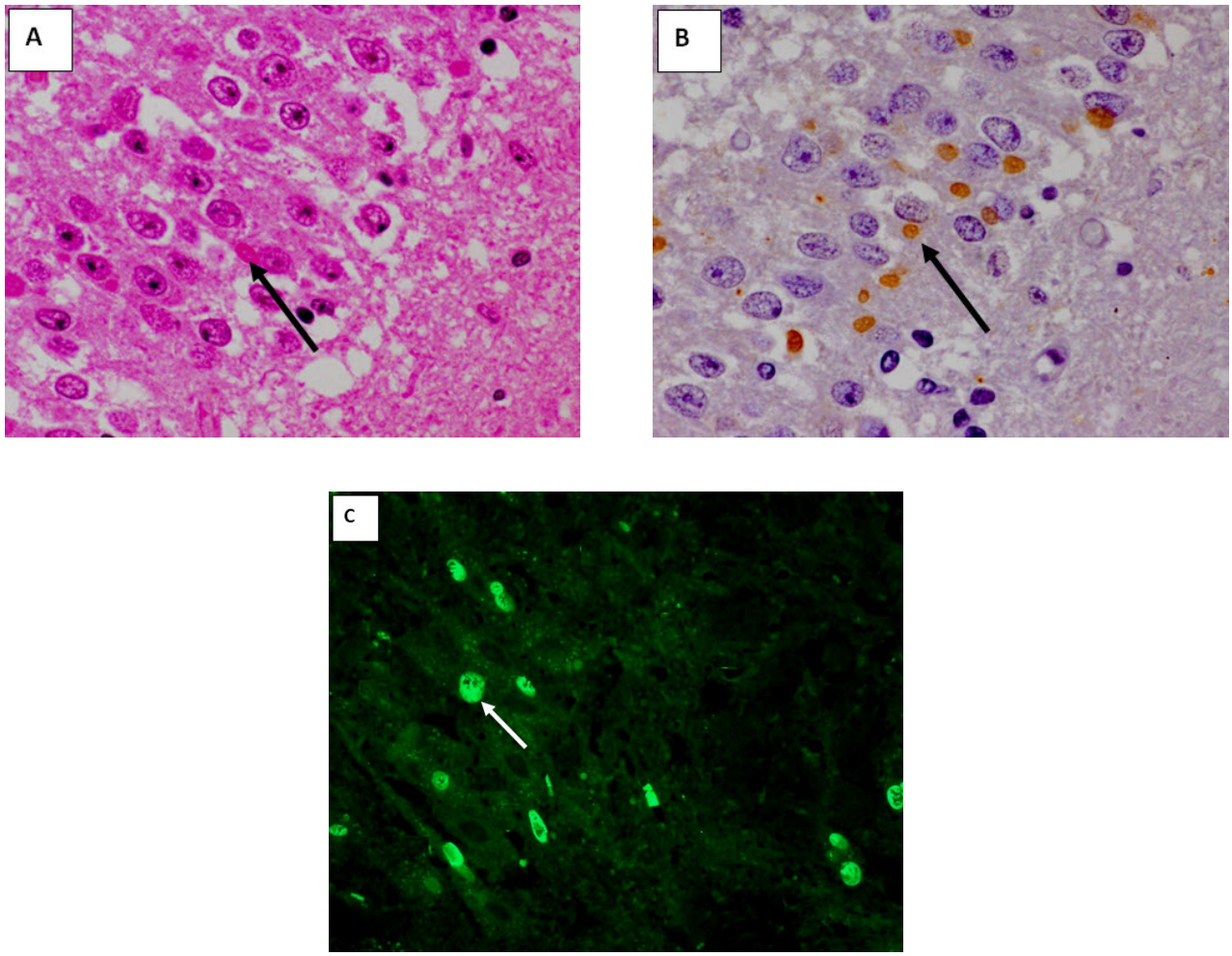

Figure 6. Section of hippocampus of rabid buffalo showing few Negri bodies with H&E stain- Original magnification x 1000X (A), corresponding IHC stained section - Original magnification x 1000X (B) showing brown coloured Negri bodies (arrow) and corresponding FAT stained section- Original magnification x 400X (C) showing more clearly green coloured Negri bodies (arrow).

found positive for rabies by demonstration of negri bodies (Table 2) and thus, histopathology revealed 94.4% sensitivity in comparison to direct-FAT (Table 5). Negri bodies appeared as single or multiple, eosinophilic intracytoplasmic inclusions within the Purkinje neurons**,** in the axons and in the neurons of the hippocampus (Figure 5 and 6). Which has been also reported by (Gonzalez and Stephano, 1984; Salem *et al*., 1995; Lima *et al*., 2005; Srinivasan *et al*., 2005; Pedroso *et al*., 2008 and Rissi *et al*., 2008).

Comparison of immunohistochemistry and histopathology

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. 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 78.88% neurons were positive for negri bodies and 59.35% with H and E. It can be concluded that IHC established many more virus infected cells than H and E stained sections which is same as reported by Feiden *et al.* (1985).

Average number of negri bodies detected per neuron by IHC was 3.01 which were greater than H and E stained brain sections (1.8). The amount of antigen detected with IHC was much more abundant than histopathological findings (Figure 5 and 6) which are reported by several workers (Hamir *et al*., 1992; Martinez-Burnes, 1997; Jogai *et al*., 2001 and Suja *et al*., 2004). Palmer *et al.* (1985) reported that rabies antigen with IHC was apparent in 62% of the brain area in which inclusion bodies were not found in the corresponding H and E sections. In present study a comparison of polyclonal and monoclonal antibodies employed in detection of rabies antigen in formalin fixed paraffin embedded tissue sections using IHC revealed polyclonal antibody to be highly efficacious.

As compared to IHC specificity of FAT on formalin fixed 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. Thus, it can be concluded that 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.

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REFERENCES

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

1230.

Bourhy, H. and P. Sureau. 1990. Laboratory methods for rabies diagnosis. *Collection of Institute Louis Pasteur*, Paris, France, p. 154-197.

Deborah, M., E.G. Haines and Clark. 1991. Enzyme immunohistochemical staining of formalin-fixed tissues for diagnosis in Veterinary Pathology. *Canadian Vet. J.*, **32**(5): 295-302.

Feiden, W., U. Feiden, L. Gerhard, V. Reinhardt and A. Wandeler. 1985. Rabies encephalitis: Immunohistochemical investigations. *Clin. Neuropathol.*, **4**(4): 156-164.

Gonzalez, C. and H.A. Stephano. 1984. Histopathology of the central nervous system in rabid dogs. *Vet. Mexico*, **15**(1): 39-52.

Gunawardena, G.S.P. de. S. and W.F. Blakemore. 2007. Immunohistochemical Detection of Rabies Virus Antigen in the Brainstem and Spinal Cord of Rabid Dogs in Sri Lanka. *Proceedings of the Peradeniya University Research Sessions, Sri Lanka*, **12**(1): 168.

Hamir, A.N., G. Moser and C.E. Rupprecht. 1992. Morphologic and immunoperoxidase study of neurologic lesions in naturally acquired rabies of raccoons. *Journal of Veterinary Diagnostic and Investigation*, **4**(1): 369-373.

Jogai, S., B.D. Radotra and A.K. Banerjee. 2001. Immunohistochemical study of human rabies. *Neuropathology*, **20**(3): 197-203.

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

Lima, K.C., J. Megid, A.V. Silva and A. Cortez. 2005. The heminested RT-PCR for the study of rabies virus pathogenesis. *J. Virol. Methods*, **124**(1-2): 79-85.

Martinez-Burnes, J., A. Lopez, J. Medellin, D. Haines, E. Loza and M. Martinez. 1997. An outbreak of vampire bat-transmitted rabies in cattle in northeastern Mexico. *Canadian Vet. J.*, **38**: 175-177.

Palmer, D.G., P. Ossent, M.M. Suter and E. Ferrari. 1985. Demonstration of rabies viral antigen in paraffin tissue sections: Comparison of the immunofluorescence technique with the unlabeled antibody enzyme method. *Am. J. Vet. Res*., **46**(1): 283-286.

Pedroso, P.M.O., E.M. Colodel, C.A. Pescador, L.P. Arrudo and D. Driemeier. 2009. Clinical and pathological aspects in cattle affected by rabies with special reference to the rabies antigen mapping by immunohistochemistry. *Pesquisa Vet. Brasil.*, **29**(11): 899-904.

Pedroso, P.M.O., C.A. Pescador, P.M. Bandarra, D.L. Raymundo, M.R. Borba, F. Wouters, P.S. Bezerra-Junior and D. Driemeier. 2008. Standardization of immunohistochemistry technique for detection of rabies virus in formalin-fixed and paraffin-embedded tissue samples from central nervous system of cattle. *Pesquisa Vet. Brasil.*, **28**(12): 627-632.

Perrin, P. and P. Sureau. 1987. A collaborative study of an experimental kit for rapid Rabies enzyme immunodiagnosis. *B. World Health Ogan.*, **65**(4): 489-493.

Reid, F.L., N.H. Hall, J.S. Smith and G.M. Baer. 1983. Increased immunofluorescent staining of rabies-infected, formalin-fixed brain tissue after pepsin and trypsin digestion. *J. Clin Microbiol*., **18**: 968-971.

Rissi, D.R., R.A. Fighera, L.F. Irigoyen, G.D.

Kommers and S.L.B. Claudio. 2008. Occurrence of rabies in sheep in Rio Grande doSul, Brazil. *Pesquisa Vet. Brasil.*, **28**(10): 495-500.

Salem, S.A.H., E.I. Mashed and F.K. Hamoda. 1995. Pathological and epidemiological studies on bovines in Kauobia Governorate. *Proceeding 3th Science Congress Egypt Science Cattle Diseases*, **2**: 303-311.

Smith, J. 1999. Rabies virus, p. 1099-1106. *In* Murray, P.R., E.J. Baron, M.A. Pfaller, F.C. Tenover and R.H. Yolken (eds.) *Manual of Medical Microbiology*, 7th ed. American Society for Microbiology, Washington, DC, USA.

Srinivasan, A., E.C. Burton, M.J. Kuehnert, C. Rupprecht, W.L. Sutker, T.G. Ksiazek, C.D. Paddock, J. Guarner, W.J. Shieh, C. Goldsmith, C.A. Hanlon, J. Zoretic, B. Fischbach, M. Niezgoda, W.H. El, L. Orciari, E.Q. Sanchez, A. Likos, G.B. Klintmalm, D. Cardo, J. LeDuc, M.E. Chamberland, D.B. Jernigan and S.R. Zaki. 2005. Transmission of rabies virus from an organ donor to four transplant recipients. *The New England Journal of Medicine*, **352**: 1103-1111.

Suja, M.S., A. Mahadevan, C. Sundaram, J. Mani, B.C. Sagar, T. Hemachudha, S. Wacharapluesadee, S.N. Madhusudana and S.K. Shankar. 2004. Rabies encephalitis following fox bite, histological and immuno-histochemical evaluation of lesions caused by virus. *Clin. Neuropathol.*, **23**(6): 271-276.

Swoveland, P.T. and K.P. Johnson. 1979. Enhancement of fluorescent antibody staining of viral antigens in formalin fixed tissues by trypsin digestion. *The Journal of Infectious Diseases*, **140**: 758-764.

Umoh, J.U., C.D. Ezeokoli and A.E. Okoh. 1984. 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. *J. Hyg.-Camb.,* **94**: 129-134.

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

Whitfield, C.G., M. Fekadu, J.H. Shaddock, M. Niezgoda, C.K. Warner and S.L. Messenger. 2001. The rabies working group. A comparative study of the fluorescent antibody test for rabies diagnosis in fresh and formalin-fixed brain tissue specimens. *J. Virol. Methods*, **95**: 145-151.