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Original Full article

Comparative Analysis of Histopathological Alterations and Immunohistochemistry in Cattle for Diagnosis of Rabies

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Abstract

Comparative analysis of histopathological alterations and immunohistochemistry was performed on 15 brain samples of cow. In the present study, brain tissues were positive in 9 cases (60.0%), using polyclonal antiserum by immunohistochemistry and 7 cases (46.60%) were found positive for rabies by demonstration of Negri bodies. Hundred neurons per case were observed for Negri bodies; number of Negri bodies in positive neurons was counted and a comparison of IHC and histopathology was done. Number of neurons positive for rabies virus antigen per 100 neurons (900 neurons) by IHC were more (665) than H& E staining (344) and average number of Negri bodies per neuron detected by IHC were more (2.97**)** than histopathology (1.52**)**, therefore, IHC was found to be more sensitive than histopathology.

Key words: cattle, histopathology, immunohistochemistry, 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. 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. However, 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. Hence, there is a need for a better method of diagnosis of rabies using formalin-fixed paraffin-embedded tissues. Immunohistochemical testing can be performed on such samples. This technique improves diagnostic accuracy by promoting visualization of the distribution of the infectious disease agent in histological sections (1). It provides sufficient amplification of the antibody-antigen interaction to enable detection of antigens immunogenically altered by fixation. The present study was envisaged to explore the suitability of application of IHC for diagnosis of rabies in cow especially in comparison to the histopathology.

Material and Methods

Source of samples: A total of 15 cases of cows suspected for rabies were presented from July 2010 to May 2012 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 has been depicted in Table1. Brain samples were collected from cows suspected for rabies. The tissue samples were stored in 10% neutral buffered formalin solution.

Table 1. Details of animals from which tissue samples were collected

Sr. No.	Case No.	Age	Sex
1	RL 30/10	10 years	M
2	RL 39/10	4 years	F
3	RL 42/10	5 years	F
4	RL 43/10	6 years	F
5	RL 09/11	6 months	F
6	RL 21/11	1 month	F
7	RL 23/11	4.5 years	F
8	D-02	3 years	F
9	RL 38/11	6 months	F
10	RL 39/11	6 months	M
11	RL 41/11	2.5 years	F
12	RL 42/11	3 years	F
13	RL 49/11	5 months	F
14	RL 23/12	10 years	F
15	RL 26/12	6 years	F

M: male; F: female

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. Dehydration of samples was done through ascending grades of alcohol (70%, 80%, 90%, and absolute alcohol) followed by clearing with acetone and benzene. Tissues were embedded in paraffin wax (Leica Microsystem, Paraplast tissue embedding medium, 56^{o}C) for further processing and 4-5µ thick sections were cut. Paraffin sections were stained with routine Hematoxylin and Eosin technique (7) and slides were examined by BX61 Research Photomicrograph Microscope System of Olympus Corporation, USA.

Tissues were screened for the presence of neuronal necrosis, Negri bodies, satellitosis, gliosis, neuronophagia, edema, congestion, hemorrhage, perivascular cuffing and meningitis. Out of 100 neurons, number of neurons having Negri bodies, number of Negri bodies in infected cells were counted using Cell-A software of BX61 microscope to assess the percentage sensitivity of this approach.

Immunohistochemistry: 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 7.2-7.6) were used for immunohistochemical staining of brain tissue sections. Maximum dilution of antibody at which these samples revealed positive reaction was 1:1000.

Paraffin embedded tissues were sectioned at 4-5µm 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^{o} 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 (10). The dewaxing and rehydration of tissues sections were carried out by EZ-AR Common solution at 70^{0}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^{0}C for 10 minutes and at 98^{0}C for 5 minutes, respectively. The slides were cooled and brought to room temperature, washed with PBS buffer (pH 7.2-7.6) 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 7.2-7.6) 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 7.2-7.6) for 3 times, 3 minutes each subsequently. The tissue sections were incubated with secondary antibody ImmPRESS^{TM} 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 7.2-7.6) 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).

Results and Discussion

In the present study, brain tissues were positive in 9 cases (60.0%), using polyclonal antiserum by immunohistochemistry and 7 cases (46.60%) were found positive for rabies by demonstration of Negri bodies (Fig. 1, Table 2). Negri bodies appeared as single or multiple, eosinophilic intracytoplasmic inclusions within the Purkinje cells, as well as in axons and neurons of the hippocampus, which have been also reported earlier by many workers (6, 10, 12, 14). Immunohistochemistry provides sufficient amplification of the antibody-antigen interaction to enable detection of antigens immunogenically altered by fixation. 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 (Fig. 2), and in the neurons of the hippocampus, in the axons, in the neuropil. These findings were similar as reported by (3, 9). IHC technique was useful for early diagnosis of suspected cases, when conventional histopathological and immunofluorescent antibody techniques could not detect lesion or viral antigen as suggested by Sinchaisri et al. (13). However, freezing and prolonged formalin fixation was reported to be unsuitable for the detection of rabies virus antigen using IHC (2).

The amount of rabies viral antigen/number of Negri bodies detected in the present study with IHC were much more abundant than could be expected from the corresponding H & E stained sections which was same as already reported (4). Hundred neurons per case were observed for Negri bodies; number of Negri bodies in positive neurons was counted (Table 3) and a comparison of IHC and histopathology was done (Table 4). Number of neurons positive for rabies virus antigen per 100 neurons (900 neurons) by IHC were more (665) than H& E staining (344) and average number of Negri bodies per neuron detected by IHC were more (2.97**)** than histopathology (1.52**)** (Table 4), therefore, IHC was found to be more sensitive than histopathology. Similar finding have been indicated by some workers (5,8).

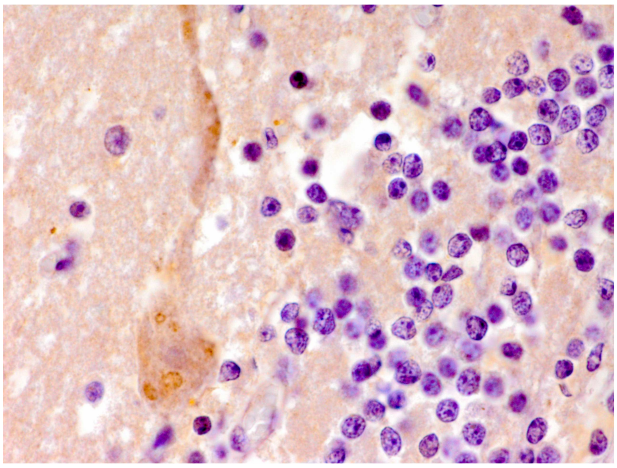

Figure 1. Intracytoplasmic granules in the cell body and dendrite of neuron. IHC 100x

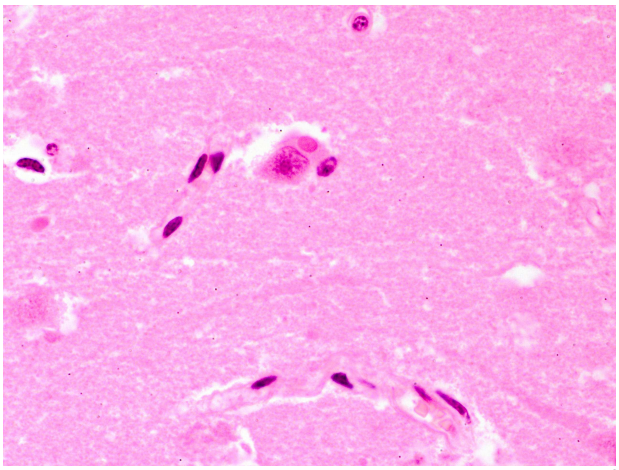

Figure 2. Intracytoplasmic Negri body in the neuron of cow. HE 100x

Although, immunohistochemistry is more sensitive than histopathology, however, the former technique also requires more sophisticated equipments and trained staff to conduct the test in contrast to more commonly available histopathology laboratories that can handle routine histopathological detection of rabies.

Further, in the present study, cows ranging from one month to ten years were found to be infected from rabies. As such, age of cattle did not have any bearing on the susceptibility and subsequent detection of rabies by immunohistochemical/histopathological approach in cattle.

Table 2. Comparison of IHC with histopathology for detection of rabies virus antigen

S. No.	Case no.	Species	Immunohistochemistry	Histopathology
1.	RL 30/10	Cow	+	+
2.	RL 39/10	Cow	+	+
3.	RL 42/10	Cow	+	+
4.	RL 43/10	Cow	-	-
5.	RL 09/11	Calf	+	+
6.	RL 21/11	Calf	+	+
7.	RL 23/11	Cow	+	-
8.	D-02	Cow	-	-
9.	RL 38/11	Calf	+	-
10.	RL 39/11	Calf	+	+
11.	RL 41/11	Cow	-	-
12.	RL 42/11	Cow	-	-
13.	RL 49/11	Calf	+	+
14.	RL 23/12	Cow	-	-
15.	RL 26/12	Cow	-	-
	% Test		9/15 (60%)	7/15 (46.60%)

+: positive; -: negative

Table 3. Histopathological and Immunohistochemical evaluation of brain tissues for number of Negri bodies

Sr. No.	Case No.	Species	No. of neurons positive for Negri bodies/100 neurons		No. of Negri bodies detected/100 neurons	
			HE	IHC	HE	IHC
1.	RL 30/10	Cow	42	91	53	663
2.	RL 39/10	Cow	58	83	103	177
3.	RL 42/10	Cow	21	89	34	101
4.	RL 9/11	Calf	51	90	74	224
5.	RL 21/11	Calf	49	93	63	441
6.	RL 23/11	Calf	0	78	0	110
7.	RL 38/11	Calf	0	11	0	21
8.	RL 39/11	Calf	55	70	92	102
9.	RL 49/11	Calf	58	60	105	138
Total			334	665	524	1977

HE: hematoxylin-eosin; IHC: immunohistochemistry

Table 4: Comparison of Histopathology and Immunohistochemistry

Parameter	Histopathology (HE)	Immunohistochemistry
Neurons having Negri bodies (n= 900)	344	665
%age of Neurons positive (Negri bodies)	38.22	73.88
Total number of Negri bodies detected	524	1977
Average number of Negri bodies per neuron	1.52	2.97

HE: hematoxylin-eosin

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The study relates to the naturally prevalent cases and, thus, does not include any experiments.

References

1. DEBORAH M., HAINES EG., CLARK. Enzyme immunohistochemical staining of formalin-fixed tissues for diagnosis in Veterinary Pathology. **Canadian Vet. J.**, 1991, 32, 295–302.
2. FAIZEE N, HAILAT NQ, ABABNEH MMK, HANANEH WM, MUHAIDAT A. Pathological, immunological and molecular diagnosis of rabies in clinically suspected animals of different species using four detection techniques in Jordan. **Transboundary and Emerg. Dis.**, 2011, 59, 154-64.
3. GUNAWARDENA GSPDES., BLAKEMORE WF. Immunohistochemical Detection of Rabies Virus Antigen in the Brainstem and Spinal Cord of Rabid Dogs in Sri Lanka. **Proc. Peradeniya Univ. Res. Sessions**, Sri Lanka., 2007, 12, 168.
4. HAMIR AN., MOSER G., RUPPRECHT CE. Morphologic and immunoperoxidase study of neurologic lesions in naturally acquired rabies of raccoons. **J. Vet. Diag. Invest.**, 1992, 4, 369-73.
5. LEMBO T., NIEZGODA M., VELASCO-VILLA A., CLEAVELAND S., ERNEST E., RUPPRECHT C E. Evaluation of a direct, rapid immunohistochemical test for rabies diagnosis. **Emerg. Infec. Dis.**, 2006, 12, 310-13.
6. LIMA KC., MEGID J., SILVA AV., CORTEZ A. The heminested RT-PCR for the study of rabies virus pathogenesis. **J. Virol. Methods.**, 1987, 124, 79-85.
7. LUNA LG., **Manual of Histological Staining Methods of the Armed Forces Institute of Pathology**. 3rd ed., The Blakiston Divison, New York: McGraw-Hill, 1968
8. NUOVO GJ., DEFARIA DL., CHANONA-VILCHI JG., ZHANG Y. Molecular detection of rabies encephalitis and correlation with cytokine expression. **Modern Pathol.**, 2005, 18, 62–67.
9. PEDROSO PMO., COLODEL EM., PESCADOR CA., ARRUDA LP., DRIEMEIER D. Clinical and pathological aspects in cattle affected by rabies with special reference to the rabies antigen mapping by immunohistochemistry. **Pesq. Vet. Bras.**, 2009, 29, 899-04.
10. PEDROSO PMO., PESCADOR CA., BANDARRA PM., RAYMUNDO DL., BORBA MR., WOUTERS F., BEZERRA-JUNIOR PS., DRIEMEIER D. Standardization of immunohistochemistry technique for detection of rabies virus in formalin-fixed and paraffin-embedded tissue samples from central nervous system of cattle. **Pesq. Vet. Bras.**, 2008, 28, 627-32.
11. PERRIN P., SUREAU P. A collaborative study of an experimental kit for rapid Rabies enzyme immunodiagnosis. **Bull. WHO.**, 1987, 65, 489-93.
12. RISSI DR., FIGHERA RA., IRIGOYEN LF., KOMMERS GD., BARROS CSL. Occurrence of rabies in sheep in Rio Grande doSul, Brazil. **Pesq. Vet. Bras.**, 2008, 28, 495-500.
13. SINCHAISRI TA., NAGATA T., YOSHIKAWA Y., KAI C., YAMANOUCHI K. Immunohistochemical and histopathological study of experimental rabies infection in mice. **J. Vet. Med. Sci.**, 1992, 54, 409-16.
14. SRINIVASAN A., BURTON EC., KUEHNERT MJ., RUPPRECHT C., SUTKER WL., KSIAZEK TG., PADDOCK CD., GUARNER J., SHIEH WJ., GOLDSMITH C., HANLON CA., ZORETIC J., FISCHBACH B., NIEZGODA M., ORCIARI L., SANCHEZ EQ., LIKOS A., KLINTMALM GB., CARDO D., LEDUC J., CHAMBERLAND ME., JERNIGAN DB., ZAKI, SR. Transmission of rabies virus from an organ donor to four transplant recipients. **N. Eng. J. Med.**, 2005, 352, 1103-11.

