RABIES: A PATHOMORPHOLOGICAL AND IMMUNOHISTO-CHEMICAL EVALUATION IN ANIMALS

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Abstract: To study the pathomorphological changes and to evaluate immunohistochemical test in diagnosis of rabies, 114 brain samples from different animals like dog, cattle, horse and cat suspected for rabies were collected at post mortem. The brain samples were subjected for Negri body demonstration by Seller's staining technique, fluorescent antibody technique, immunohistochemistry and histopathology. Immunohistochemistry and fluorescent antibody techniques detected more number (67.54 %) of rabies cases than Seller' staining technique (56.114%). Histopathological examination demonstrated Negri bodies only in 50 per cent of rabies positive cases. The immnohistochemistry appears to be more sensitive and equivalent to FAT in significance and could be used as routine alternative diagnostic technique.

Key words: Rabies

INTRODUCTION

Rabies is an infectious zoonotic disease caused by highly neurotropic negative sense single strandarded (ss) Lyssa virus, which belongs to Family Rhabdoviridae. Every year 55,000 people die all over the world because of rabies [1,2], of which approximately 35,000 are from Asia and about 25,000 from India alone [3].

Rabies diagnosis in domestic and wild animals is severely constrained. Conventional diagnostic tests like Seller's staining technique and histopathological examination of formalin fixed tissues detect Negri bodies only in 50 to 60 per cent of suspected cases. FAT is a gold standard and validated diagnostic test that confirm the presence of rabies virus in brain tissue. FAT is performed on fresh brain tissues and is hazardous due to possible risk of contamination of environment with rabies virus. Hence there is need for a better method for diagnosis of rabies virus using formalin fixed paraffin-embedded tissues. Immunohistochemistry is a test performed on formalin fixed histological sections. The advantage of IHC over other tests is that, the pathologic changes in the brain are clearly observable and formalin fixation of brain preserves the tissue architecture and allows histological evaluation to formulate a differential diagnosis.

MATERIALS AND METHODS

Sample collection: In the present study, a total of one hundred fourteen rabies suspected brain samples were collected from different species of animals like, bovine, dog, horse and cat submitted to Department of Veterinary Pathology, Veterinary College, Bangalore, for a period of ten months from October

2011 to July 2012. The brain areas viz., hippocampus, cerebrum, cerebellum and brain stem were separated and stored in 50% glycerol saline, 10% NBF and in defreezer at -80°C until further use.

Microscopic Examination for Negri Bodies (**MEN**) by Seller's staining: For Negri body demonstration by Seller's staining technique touch impression smears were prepared from hippocampus and cerebellum at postmortem. The smears were stained by Seller's stain and observed for Negri bodies [4].

Histopathology: After fixation in 10% neutral buffer formalin for 48 hours, the tissues were processed by paraffin embedding technique and 4μ thick sections were cut. The sections were stained with hematoxylin and eosin [5] and observed for Negri bodies and other pathological changes.

Fluorescent antibody technique (FAT): FAT was performed as per WHO recommended procedure [6]. Touch impression smears were prepared from suspected brain samples on labeled duplicate slides. The smears were air dried for five minutes and fixed in chilled acetone for 2 hour. Then the smears were stained with 1:40 anti-rabies virus nucleoprotein polyclonal FITC antibody (Chemicon USA, Cat No. F 5009) and kept for incubation at 37°C for 30 min in a humid chamber. The slides were washed in phosphate buffer saline (PBS) and mounted in 90 per cent buffered glycerol. The slides were examined under fluorescent microscope using 40X objective. Positive and negative smears made from infected and normal mouse brains were used as controls. Brain smears showing apple green fluorescent particles of varying size were considered as positive.

Immunohistochemistry: The paraffin embedded tissues were cut at 4µm thickness and sections were mounted on 3-aminopropyltriethoxy-silane coated slides and dried at 37°C for three hours. The slides were then placed in hot air oven at 60°C for 30 minutes. The sections were deparaffinized using xylene and rehydrated using descending grades of ethanol. The sections were covered with 3 per cent H_2O_2 in methanol to block endogenous peroxidase.

Antigen retrieval was carried out by immersing tissue sections in coupling jar containing Trypsin 1:250 (Pig pancreas origin) and was incubated in a humid chamber at 37°C for 60 minutes. Sections were

allowed to cool down to room temperature for 30 minutes. Later they were washed in two changes of TPBS.

Slides were incubated in a humidity chamber at room temperature with biotinylated anti-rabies monoclonal antibody (mAb) for 60 minutes (CDC Atlanta, cocktail of mouse anti-rabies mAb store at 4°C, ready-touse) then washed in TPBS twice. The slides were incubated with streptavidin-peroxidase complex in a humidity chamber at room temperature for 60 minutes (Kirkegaard & Perry Laboratories Inc., USA) and dip, rinsed in TPBS. Then the slides were incubated with peroxidase substrate, amino-ethylcarbizole (AEC) (Sigma-Aldrich Corp, St Louis, MO, USA) for 10 minutes in a humid chamber - the working dilution was prepared just prior to use. Sections were washed in distilled water for 5 min and counterstained with Gill's haematoxylin (1:2) for 30 sec and washed in running tap water for 5 min. Finally the slides were mounted with water-soluble mounting medium and examined under microscope for red inclusions and particles against blue background.

RESULTS AND DISCUSSION

In the present study a total of one hundred and fourteen rabies suspected brain samples were collected upon postmortem examination of different species of animals (Table. 1) presented to Department of Veterinary Pathology, Veterinary College, Bangalore, for a period of ten months. The samples were subjected for different diagnostic tests like microscopic examination for Negri bodies by Seller's staining, Fluorescent antibody technique (FAT), Histopathology and Immunohistchemistry (IHC) for the diagnosis of rabies. Out of one hundred fourteen suspected cases of rabies, seventy seven cases were found positive for rabies with an overall incidence of 67.54 per cent (Table 2).

Seller's staining: Seller's staining technique detected Negri bodies only in 64 cases out of 114 rabies suspected cases with an incidence of 56.14 percent. Negri bodies were intracytoplasmic, round, oval or elongated with halo around, maganta pink colored with basophilic internal granules and occurred singly or multiple in number, size varied from very small to large (Fig. 1), some occurred also extra cellularly in the brain matrix. The Negri bodies were small to moderate sized in dog, cat and horse and large sized in cattle [7-11].





Fig. 1: Brain impression smear from rabid cattle showing large Negri bodies. Seller's staining X 1000 ; **Fig. 2:** Brain Impression smear from rabid dog showing apple green fluorescencein neuron by FAT. FAT X 400; **Fig. 3:** Photomicrograph to show large intra cytoplasmic Negri bodies with inner bodies in a rabid cattle. H & E 1000 ; **Fig. 4:** Photomicrograph to show Satelittosis in brain stem from a rabid dog. H & E 200; **Fig. 5:** Microscopical picture of dog brain showing perivascular cuffing with mononuclear cells. H & E 400; ; **Fig. 6:** Section of bovine cerebellum showing immunostaining for N protein of rabies virus. IHC X 1000

Fable	1:	Rabies	suspected	samples	from	different	species
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Sl.No	Animals	Number of animals presented for diagnosis of rabies	
1	Dog	98	
2	Bovine	07	
3	Horse	05	
4	Cat	04	
Total number of animals		114	

Table 2: Comparative evaluation of diagnostic tests

	Number of samples positive by different diagnostic tests						
Species	S eller's staining	Histopathology	FAT	IHC			
Dog	56	56	67	67			
Bovine	06	06	06	06			
Horse	01	01	03	03			
Cat	01	01	01	01			
	64 (56.14 %)	64 (56.14%)	77 (67.54%)	77 (67.54%			

Histopathology: Out of 77 positive cases of different animal species subjected for histopathological studies, Negri bodies were observed in sixty four cases (56.14%) of the brain samples. The presence of Negri bodies is variable in histopathological examination and hence histopathology is neither as sensitive nor as specific as other tests in diagnosis of rabies. The presence of Negri bodies is considered as pathognomonic of rabies but in the present study Negri bodies were most consistently found in cattle rabies cases than in dogs and the inclusions were observed more in cerebellum followed by hippocampus in cattle and in hippocampus followed by cerebellum in dogs. In cattle the number of Negri bodies were higher in infected Purkinje cells and also the size was larger compared to other species of animals examined (Fig. 3). Similar observations have also been made by several workers in different animals [11-14].

The histological alterations in brain included vascular changes, degeneration and necrotic changes. The various vascular changes observed were congestion of blood vessels, hemorrhages and perivascular oedema and the congestions were characterized by dilatation of blood vessels with accumulation of red blood vessels in the lumen. The congestion and haemorrhages were more appreciable in the brain stem, hippocampus, cerebellum and cerebrum in the order of severity. Perivascular oedema was observed in all the segments of brain examined which was characterized by increase in the space of Virchow Robin with presence of faintly pink colored material. The perivascular oedema was more prominent in cerebrum, around congested blood vessels.

The neuronal degeneration was characterized by shrinkage of neurons in pyramidal cell layer of cerebrum and purkinje cells of cerebellum with a halo space around. The cells appeared darkly stained with eosin and referred to as dark eosinophilic degenerating cells. The nucleus was condensed, fragmented or karyolysed and some of the neurons appeared coagulated with central chromatolysis, characterized by swelling, pale cytoplasm with devoid of Nissl substance and nucleus.

The inflammatory changes included nonsuppurative encephalitis and meningitis and it was characterized by congestion of blood vessels, haemorrhages and oedema with infiltration of inflammatory cells. perivascular cuffing (Fig. 5), glial reaction, satellitosis (Fig. 4) and neuronophagia.

The microscopic changes, like neuronal degeneration, necrosis of neuronal cells, inflammatory changes in rabies, are also recorded by several investigators [11-14]. However, compared to other viral encephalitis, the lesions in brain in rabies are less dramatic.

Fluorescent antibody technique (FAT): In the present study FAT detected rabies N antigen in 77 out of 114 rabies suspected cases. The rabies positive reaction was characterized by bright apple green fluorescent particles or clumps of varying size either scattered or within the neurons (Fig.2) as also observed by other investigators [15,16]. FAT is gold standard for the diagnosis for rabies, developed by Goldwasser and Kissling [17] in 1957. FAT is most sensitive, fast and reliable test for diagnosis of rabies. However, the sensitivity of FAT has been shown to decrease with extensive deterioration of the brain tissue [18]. Hence, other supplementary rapid and sensitive diagnostic test for rabies like RT-PCR and IHC are required when a questionable FAT result is obtained in order to arrive at a definite diagnosis. Hence in the present study, immunohistochemistry was performed on tissue sections.

Immunohistchemistry (IHC): Immunohistochemistry is a sensitive diagnostic technique which demonstrates antigen in fixed paraffin embedded tissue sections. In the present study IHC detected rabies viral antigen in 77 cases out of 114 suspected

cases. In cattle, the distribution of viral antigen was found either in granular form or in the form of inclusion bodies or both was observed extensively in cerebellum, brain stem followed by hippocampus and cerebrum similar to those observed by earlier workers [13,19]. In case of dogs the location of rabies antigen was comparatively higher in hippocampus followed by brain stem and cerebellum in the order of immunoreactivity. The IHC staining also detected rabies antigen in a small neurons, especially the cells of granular layer of cerebellum and in a few astrocytes which are usually missed in routine histopathological techniques.

Immunohistochemistry is most sensitive in rabies diagnosis as routine histopathology fails to detect Negri bodies in all the cases of rabies. IHC yields positive results in all the cases of rabies, and the sensitivity of IHC in rabies diagnosis in comparable to that of gold standard FAT [13,19-23]. IHC is even more sensitive in early diagnosis of suspected cases when traditional histopathology and FAT technique fail to detect antigen or lesions [19,24].

CONCLUSION

IHC is reliable and enhance probability of accurate diagnosis over routine Seller's staining and histopathology. In addition IHC could be used as an alternative to FAT which has similar specificity and also is very useful when fresh samples are not available for FAT and absence of fluorescent microscopy.

Abbreviations used: FAT = Fluorescent antibody technique, IHC = Immunohistochemistry, NBF =Neutral buffer formalin, MEN = Microscopic Examination for Negri Bodies, PBS = Phosphate buffer saline, TPBS =Tween phosphate buffer saline, RT-PCR = Reverse transcriptase polymerase chain reaction.

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