

Rabies Require Extra Caution in Endemic Areas

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Abstract

Rabies in man and animals has been discussed since centuries but still remains one of the neglected zoonotic diseases across the globe. Dogs being reservoir host, bite from an unknown dog and death of the bitten man or animal or of the dog that bit, within a span of ten days to three months, leave a suspicion of rabies especially in endemic areas like India. Though there are strict measures to control rabies by vaccination programmes, abundance of stray dogs makes it difficult to contain the disease. So, choosing the best diagnostic procedure is highly significant.

Keywords: Rabies; Diagnosis; Methods

Introduction

Rabies is a fatal disease of warm blooded animals widely reported from almost all parts of the world. Development of clinical rabies can be prevented by immediate and effective vaccination schedule. The clinical signs in rabid dogs include aggressive nervous dysfunction ranging from salivation, difficulty in swallowing, biting tendency, bellowing, frequent urination etc. There are also cases of dumb form of rabid animals showing depression, dysphagia, dyspepsia, dyspnea and slowly ends up in death. Since there are other diseases with similar neurological symptoms, confirmatory diagnosis of rabies is highly significant. Lack of curative treatment and dreadful behavior of the affected animals make it crucial to ascertain the occurrence of rabies in a suspected case to proceed for anti-rabies vaccination at the earliest. Studies conducted in the veterinary colleges in the state of Kerala, India have standardized the testing protocol in confirmatory diagnosis of rabies. Even in certain positive cases of rabies, it was difficult to trace out the negri bodies in the impression smear stained with Sellers stain. The gold standard test is the fluorescent antibody test (FAT) which requires expertise to prepare the impression from the brain gets stained with FITC conjugated nucleocapsid antibody in suitable buffer. This article explains the strategy adopted for diagnosis of suspected rabies cases and interpretation of the results.

Materials and Methods

The paper is based on a study conducted in 105 carcasses of dogs brought for post mortem examination of rabies diagnosis, over a period of two years in an urban area in the central part of Kerala. Standard protocols for PM examination were followed. The head was severed from the body at the atlanto-occipital joint. From the disarticulated head, the brain was removed from the skull as per the method described by Tierkel [1].

Pre-fixation examination of brain

The brain was examined for lesions in the meninges, blood vessels, surface corrugations, and ventricles, presence of parasitic cysts or

malacic foci. The intact brain was visually examined for size and symmetry of both cerebral hemispheres, for the evidence of any swelling, herniation, meningeal opacity, exudates, malformation, extra or intra medullary masses, focal or multifocal lesions ranging from infarcts to granuloma or metastatic tumors. Palpation of the whole brain was carried out gently for understanding the consistency of the brain.

Post fixation examination of brain

After the preliminary fixation in 10% buffered formalin for one week, the whole brain was examined and sectioned in transverse plane, 0.5 cm from rostral to caudal. Presence of any lesions like hemorrhage, edema causing swelling and displacement at the midline was observed. The grey matter and white matter were specifically examined for yellowish or brownish discoloration. The periventricular area was checked for thickening or nodular irregularity of the ependymal surface. A few cuts were then made on the cerebral hemisphere and preserved immediately in 1 to 2 liters of 10% neutral buffered formalin for minimum 14 days before processing for histopathology.

Collection of Brain Impression Smears

An incision was made on the posterior third of the hemisphere in fresh brain, externally about 1.5 cm from the midline through the grey matter and white matter until the third ventricle was reached. Small transverse sections of 2 mm to 3 mm thickness were cut from the hippocampus, cerebrum, cerebellum, medulla and spinal cord at the cranial region and placed with cut surface facing upward on a clean blotting paper. Impression smears prepared from cut sections were immediately stained with Sellers stain for detection of Negri bodies by Direct microscopical examination (DME) using Olympus BX41 microscope.

Direct Fluorescent Antibody Test (dFAT)

The air-dried impression smears were fixed in cold acetone for detection of rabies specific fluorescence with direct fluorescent antibody test, using Fluorescein isothiocyanate (FITC) conjugated

nucleocapsid antibody (Bio-Rad, France) on impression smears from different parts of brain as per the standard protocol for FAT [2].

The lyophilized adsorbed anti-rabies nucleocapsid Fluorescein isothiocyanate (FITC) conjugate (Bio-Rad, France) was reconstituted with 3 ml distilled water and centrifuged at 1500 rpm for 5 minutes for clarification. The slides with impression smears were fixed in chilled acetone at -2°C for minimum one hour. The area of impression was demarcated on the slide. The slides were then charged with diluted FITC conjugate and incubated at 37°C for one hour in dark humidification chamber. The excess antibody was removed by washing in PBS (pH-7.2) three times for 10 min. each. The slides were air dried and viewed under ultraviolet illuminated Fluorescent microscope using aqueous mountant (50% glycerol in PBS). The presence of apple green fluorescence was taken as positive signal. Negative and positive controls were run along with test slides during this process.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) for N Gene

The TRI-zol (Invitrogen) method was used for extraction of total RNA from brain tissue homogenate. The protocol recommended by the Reverse Transcription System (Cat # A3500, Promega, USA) was followed for cDNA synthesis. The primers for N gene were designed based on standard pasture virus strain sequence Acc. No. (NC 001542). The purified PCR product was confirmed by gel electrophoresis.

The specificity and sensitivity of the test were calculated (Gardner and Altman, 1989).

Specificity = $\frac{\text{No. of true negatives}}{\text{No. of true negatives} + \text{No. of false positives}} \times 100$

Sensitivity = $\frac{\text{No. of true positives}}{\text{No. of true positives} + \text{No. of false negatives}} \times 100$

Results

Direct microscopic examination (DME)

Seller's staining of brain impression smear for Negri bodies: Fourteen animals showed presence of varying sized round to oval shaped magenta colored intra cytoplasmic inclusion bodies in the perikaryon of nerve cell body and axons. The Negri bodies were also visible outside the cells in the neuropil. There was no visible difference in the size of Negri bodies observed in hippocampus, cerebrum, cerebellum, medulla and spinal cord. But the number varied significantly in these regions. Hippocampus and brain stem showed the maximum number of Negri bodies (Figure 1).

Direct fluorescent antibody test (dFAT): The brain impression smears from different regions like cerebrum, cerebellum and brain stem were used for direct FAT. Among the 105 suspected cases, 18 brain samples showed presence of rabies viral antigen with varying intensities of apple green fluorescence under ultraviolet illuminated fluorescent microscope. Hippocampus (Figure 2) and medulla showed the maximum intensity of viral antigen as scattered dusty bright apple green fluorescence. The intensity and distribution of the green fluorescence is given in Table 1.

Reverse transcriptase- polymerase chain reaction (RT-PCR)

Single step RT-PCR was carried out with twenty samples from rabies positive (n=9) and negative brain samples (n=11) from the brain stem.

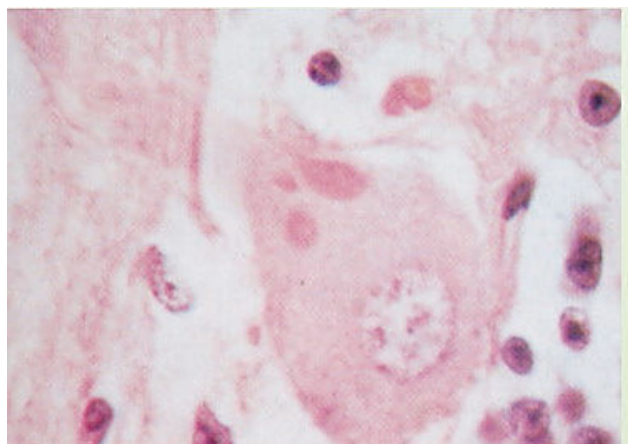


Figure 1: Eosinophilic intra cytoplasmic Negri bodies in the Purkinje neurons of cerebellum-sellers stain 100x.

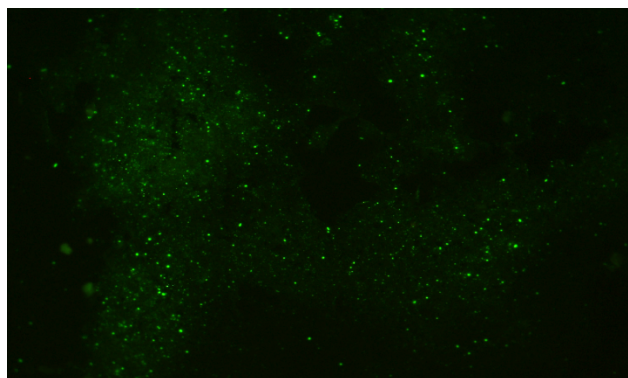


Figure 2: Star dust pattern of apple green fluorescence (Score 3) in the hippocampus-FITC 10x.

Tissue impression examined	Scoring of apple green fluorescence (0=no fluorescence; 1=2 to 3 fluorescent bodies; 2=4 to 5 and 3=many star dust pattern)			
	Score =0	Score =1	Score =2	Score =3
Cerebrum	4	7	7	0
Cerebellum	4	7	7	0
Hippocampus	0	5	9	4
Brain stem/ Medulla	0	4	9	5

Table 1: Distribution of nucleocapsid antigen detected by direct FAT in different tissues under low power (10X).

The rabies viral N gene was amplified in the nine FAT positive brain samples. Product size of 806 bp was confirmed in 1.5% agarose gel. Assuming that dFAT was 100% sensitive and specific, the test results with RT-PCR for the expression of N gene was evaluated. RT-PCR gave positive result with all the positive samples which showed positive fluorescence with FAT. The negative samples tested, failed to amplify the gene expression and the agarose gel did not show the bands in any of this negative sample.

Discussion

Though rabies seen in different species of wild and domestic animals, rabies cases reported from Kerala is mostly in dogs. Awareness among people has increased and so once a dog is found dead, the carcass is brought for rabies diagnosis. Dog bite cases and number of stray dogs grow parallel in the same direction and so diagnosis of rabies in animals is a routine practice in the two veterinary colleges.

Postmortem diagnosis of rabies based on microscopical demonstration of Negri bodies has been practiced since 1903, ever since Negri reported the presence of intracytoplasmic inclusion bodies in the nervous tissues of humans died of rabies [3]. In the present study, out of 105 suspected cases of rabies with or without a bite history, DME detected Negri bodies on impression smears from 14 brain samples (77.78%). Out of total 18 cases confirmed as rabies (by FAT&RT-PCR) four animals had history of salivation, dysphagia, roaming tendency and were bitten by other rabid dogs. But DME by Seller's staining could not reveal presence of Negri bodies in any of the brain regions in these four cases. This indicated that traditional method of Seller's staining for Negri body cannot be relied fully for a confirmatory diagnosis of rabies. The cases which showed Negri bodies in DME, gave positive result with FAT. Studies in other species of animals, especially cat, cattle and man had shown presence of inclusion bodies similar to Negri bodies [4].

The size, number and frequency of Negri bodies varied in different regions of the brain and also between individual animals. The rabies virus does not infect all parts of the brain equally and the chances of finding rabies antigen vary between the different regions of the brain [5]. Though hippocampus and medulla gave better results for finding Negri body or viral nucleocapsid antigen, it is desirable to check all the identifiable parts of brain for detection of Negri bodies by Sellers stain or viral antigen by FAT [6].

The quantitative analysis showed four dogs with zero score (score=0) for detection of Negri bodies with Seller's staining from all identifiable parts of brain, in spite of showing rabies symptoms. When the history was traced, it was found that, these animals were killed by local people due to their aggressive behavior and biting nature. Therefore, it is assumed that as the incubation period of rabies virus gets shortened, there will not be sufficient time for development of Negri bodies. Fekadu et al. stated that development of Negri bodies is related to the time length, a rabid victim stays alive after exhibiting the signs of rabies [7].

In DME, the intensity and frequency of Negri bodies seen in different regions of the brain were in the decreasing order from hippocampus, brain stem, cerebrum, cerebellum and spinal cord. Wiktor and Koprowski reported that, Negri bodies were detected in the impression smear only in 50% to 80% rabies confirmed cases [8]. The binding of neurotransmitters to the receptors are different at different locations of the brain. According to Dumrongphol et al. hippocampus

and brain stem were the most affected in terms of impaired specific binding to quinuclidinyl benzylate (QNB) and functional derangement of muscarinic acetyl choline receptors (mAChR), as compared to other regions [9].

The observations of Bingham and Vander Merwe for finding a reliable and specific region of the brain for immunofluorescent detection of viral antigens suggested that thalamus, medulla and pons gave the most abundant antigen with FAT and underlined the need of taking brain stem as the most reliable material for rabies diagnosis in human beings. It is better to include hippocampus, cerebrum, medulla and pons for rabies diagnosis [10-12].

The molecular diagnosis for detection of nucleocapsid antigen with RT-PCR is a time-consuming method not suitable for routine diagnostic purpose except in cases of liquefied brain (in extremely putrefied wild animal carcasses) samples and the result goes in line with the of dFAT in specificity and sensitivity [8]. Except in research works and rare highly demanding diagnostic dilemma, this method is not generally affordable. In an endemic region like Kerala, confirmation of rabies in suspected cases need to be carried out using a battery of tests available. Unless the exact cause of death is clearly traced out during PM examination followed by laboratory confirmation (by microbiology or histopathology) every suspected case of rabies in man or animal requires extra caution.

Conclusion

The gold standard for diagnosis of rabies is direct FAT, a test that could be completed in three to four hours. This requires specific anti rabies antibody tagged with fluorescent dye such as FITC and expertise in doing the FAT procedure. Negri body detection by sellers staining and DME is a quick method but sensitivity depends on incubation period, type and nature of brain sample. In an endemic region like Kerala, confirmation of rabies in suspected animals need to be carried out using a battery of tests available. Unless the exact cause of death is clearly traced out during PM examination followed by laboratory confirmation (by microbiology or histopathology) every suspected case of rabies in man or animal requires extra caution. The best strategy in rabies suspected case is to undertake detailed PM examination followed by FAT on Brain impression smears. This will help us to rule out other causes of death and bank on the result of FAT to confirm the presence of Lyssa viral Antigen.

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