



Astrocytic reaction in furious and paralytic forms of rabies with reference to GFAP expression in dog brain samples positive for rabies*

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Citation: Shruthi, A., Prasanna, K. S., Sachin and Ajith, J. G. 2022. Astrocytic reaction in furious and paralytic forms of rabies with reference to GFAP expression in rabies positive dog brain samples.

J. Vet. Anim. Sci. **53**(2): 195-200

DOI: <https://doi.org/10.51966/jvas.2022.53.2.195-200>

Received: 21.12.2020

Accepted: 12.10.2021

Published: 30.06.2022

Abstract

The aim of this study was to evaluate the astrocytic reaction in infected brains of dogs in that had been afflicted with the furious and paralytic form of rabies. Brain samples from 23 dogs that were positive for rabies were collected along with six brain samples that were negative for rabies. GFAP (Glial fibrillary acidic protein), specific astrocytic marker was used to differentially analyse the immunoreactivity of astrocytes to GFAP in specific brain regions in two the clinical forms of rabies viz, the furious and paralytic forms. The study showed the extent of astrogliosis in specific regions of brain in the two forms of rabies and also the intensity of GFAP expression. Region wise astrogliosis, was more diffuse in the brains of animals afflicted with the furious form of rabies but were more localized in the brains of animals with the paralytic form, specifically into the white matter of cerebellum.

Keywords: Rabies, Astrocytes, GFAP

Glia cells provide structural and functional support to the neuronal cells and account for 90 percent of the whole cells of CNS. Astrocytes are an integral component of the glial cell society in the CNS. They are multipolar, star shaped cells and are morphologically classified into protoplasmic cells observed in grey matter of the brain and fibrillary cells present in the white matter of the brain (Brat, 2018). Astrocytes are activated at any sort of CNS insult, either trauma or infection and respond to such situations through hypertrophy and/or hyperplasia. GFAP are the intermediate filament proteins that are commonly present in astrocytes acting as a specific marker of these cells and are helpful in delineating the pathological response of astrocytes at each stage of disease progression.

**Part of the MVSc. thesis of the first author submitted to Kerala Veterinary and Animal Sciences University, Pookode, Wayanad, Kerala.*

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Viral infections affecting the CNS are numerous and among these, the most important reviewed and researched one is rabies infection (Dhaka P and Vijay D, 2018). Both in humans and animals, the disease possesses a dreadful course with a fatal end (Krithiga *et al.*, 2019). Though the disease was known from time immemorial, the actual pathogenesis through which the disease attains its furious or paralytic form is still a mystery to scientific society. Time demands an appropriate measure to differentially analyse the roles played by each cell of the CNS, cellular proteins like cytokines and the immune mediatory cells in the pathogenesis and course progression of the disease. This study intends to look into the astrocytic response in accordance with the infection and variation in the response of the same in two different forms of rabies. Immunohistochemistry using GFAP and its expression in brain tissue is considered as a reliable parameter to evaluate the extent of astrocytic activation/response to the disease.

Materials and methods

The animal anamnesis was collected from the animal owner when the carcass suspected for rabies was brought for post-mortem examination. For diagnosis of rabies in suspected cases, the brain impression smear was carried out using d-FAT. Cases positive for rabies under study were classified into two forms based on the clinical signs exhibited by the animal (Tepsumethanon *et al.*, 2016). Brain samples collected were sliced based on the region required for the study and preserved in 10 per cent NBF.

Immunohistochemical analysis for Glial Fibrillary Acidic Protein positive astrocytes in the intended regions (*viz.*, cerebrum, cerebellum, hippocampus and brain stem) of brain samples positive for rabies and brain samples negative for rabies, that served as the control, (5µm thickness) were carried out using GFAP as the primary antibody (Vector Laboratories). The IHC procedure was performed using Super Sensitive TM Polymer-HRP IHC Detection Kit (QD420- YIKE) from Biogenex. Antigen retrieval solution used for the IHC was 0.1M Sodium citrate buffer (pH 6.0).

Formalin fixed paraffin embedded brain tissues were sectioned at 5µm thickness, collected on APES coated slides and heat fixed. The sections were deparaffinized by immersing the slides in xylene and then hydrated through descending grades of alcohol and finally treated in distilled water. Antigen retrieval was carried out by immersing these slides in sodium citrate buffer and incubating at 95°C for 20 minutes. After this, the sections were acted upon by the peroxide block and power block solutions for 3 minutes each. The sections were then incubated with 50µl of primary antibody at 4°C overnight. Later these sections are incubated with enhancing reagent available in the kit and then followed by the secondary antibody. After a through wash, DAB solution was added to the sections and incubated for 20 minutes. The sections were counterstained with Ehrlich's haematoxylin and treated with tap water (blueing) at room temperature. Slides were then treated with increasing grades of alcohol (50 per cent to 100 per cent) and cleared using xylene and mounted with cover slips using DPX mountant.

Immunohistochemical scoring

Grey matter and white matter of all the four regions of brain were examined under 40X magnification (high power) to evaluate the intensity of immunostaining and number of positive cells. Based on the intensity of staining, scores were assigned. The number of GFAP positive astrocytes per unit area was assessed by calculating the mean number of astrocytes counted in five random fields examined under high power magnification. Only the astrocytes with brown coloured cytoplasm and processes were considered GFAP positive (Machado and Alessi, 1997).

Statistical analysis

IBM SPSS Statistics software, version 21.0 was used for statistical analysis. One-way ANOVA was carried out followed by Duncan Multiple Range test (DMRT) for pair wise comparison in analysing the immunoreactivity for GFAP.

Results and discussion

In the present study samples of the

brain of dog carcasses with a clinical history of neurological abnormalities were screened for rabies using d-FAT. A total of 23 brain samples that were positive for rabies and 06 samples (control group) that were negative were collected for the study.

Immunoreactivity of GFAP in two forms of rabies

The semi quantitative analysis of

GFAP expression was carried out in two forms of rabies and immunoreactivity in different regions in furious and paralytic forms was recorded respectively (Fig. 1 and 2).

The intensity of immunoreactivity to GFAP was scored and differentially analysed in the two forms of rabies as shown in Table 1. In both forms of rabies, an intense expression of GFAP protein (63 per cent of paralytic cases and 75 per cent of furious form) was observed.

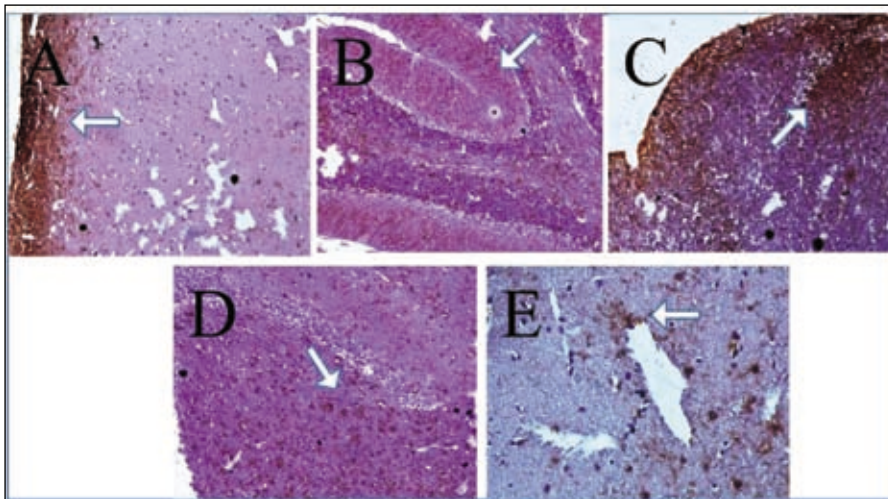


Fig. 1. GFAP reactivity in furious form of rabies (100X; DAB stained IHC sections counter stained by Heamatoxylin) A: Cerebrum- hypertrophy of astrocytes in glial limitans; B: Cerebellum- Astogliosis of white matter and extension of processes to the grey matter; C: Brainstem- astrogliosis; D: Hippocampus- astrocytosis near the dentate gyrus and E: Around the blood vessels in hippocampus(400X)

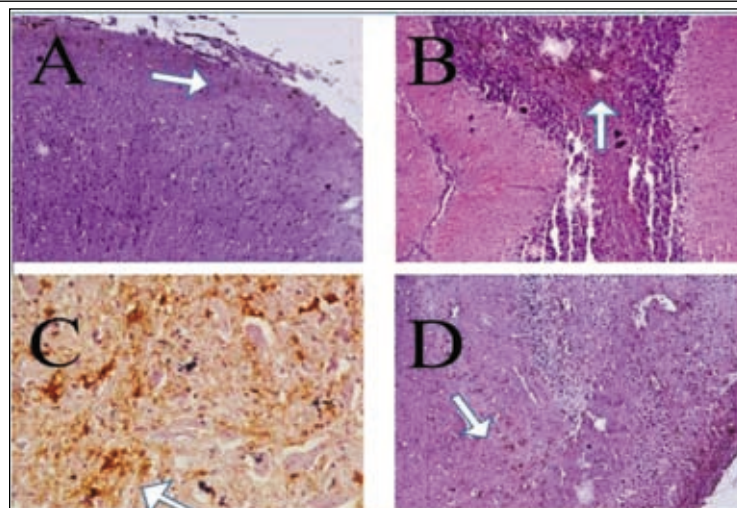


Fig. 2. GFAP reactivity in paralytic form of rabies (DAB stained IHC sections counter stained by Heamatoxylin) A: Cerebrum- mild hypertrophy of astrocytes in glial limitans (100X); B: Cerebellum- Moderated astogliosis of white matter and mild extension of processes to the grey matter (100X); C: Moderate astrogliosis of hippocampus (400X); D: Astrogliosis in the Brain stem (100X).

Table 1. Classification of cases according to GFAP reactivity

Intensity Score	Control		Paralytic Rabies Group		Furious Rabies Group		Overall	
	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent
+	3	50.0	0	0.0	1	8.3	4	13.8
++	3	50.0	4	36.4	2	16.7	9	31.0
+++	0	0.0	4	36.4	5	41.7	9	31.0
++++	0	0.0	3	27.3	4	33.3	7	24.2
Total	6	100.0	11	100.0	12	100.0	29	100.0

Table 2. Comparison of No. of positive cells per unit area in GFAP expression between groups

Period	CONTROL	PARALYTIC	FURIOUS	F-value (P-value)
Cerebrum GM	2.08 ± 0.33	2.27 ± 0.26	2.75 ± 0.26	1.471 ^{ns} (0.248)
Cerebrum WM	6.33 ± 0.99 ^b	5.09 ± 0.31 ^b	9.29 ± 0.92 ^a	9.03 ^{**} (0.001)
Hippocampus	5.25 ± 0.34 ^b	5.00 ± 0.40 ^b	6.92 ± 0.63 ^a	4.186 [*] (0.027)
Cerebellum GM	2.75 ± 0.54	3.86 ± 0.42	3.54 ± 0.48	1.075 ^{ns} (0.356)
Cerebellum WM	5.00 ± 0.29 ^b	8.55 ± 0.57 ^a	9.42 ± 0.80 ^a	8.532 ^{**} (0.001)
Brainstem	5.00 ± 0.90	7.96 ± 1.01	6.29 ± 0.68	2.409 ^{ns} (0.110)

Statistical analysis of the region wise GFAP positive cell density was carried out and the results were consolidated as shown in Table 2.

A remarkable increase in number and size of astrocytes positively marked for GFAP was observed in both forms of rabies. Statistically significant astrogliosis was noted in the white matter of the cerebellum in the case of the paralytic form ($p < 0.001$). While astrogliosis was seen diffusely in the white matter of both the cerebrum and cerebellum ($p < 0.001$) in the furious form of rabies, hypertrophy and significant astrocytosis in hippocampus ($p < 0.027$) were also observed. There was an evident cuffing of blood vessels with the astrocytic end feet processes in both forms of rabies which was completely absent in the control group.

There was prominent hyperplasia and hypertrophy of astrocytic cells in the white matter of the brain with the presence of elongated processes intensely positive for GFAP in both forms of rabies and this finding was endorsed by the observations of the study by Headley *et al.* (2001) in a dog affected with canine distemper. Machado and Alessi, (1997) reported a higher rate of astrocytosis in brain of cattle infected with rabies. Increase in the astrocytic cytoplasm and enlargement of processes were observed in both form of rabies but at different sites; the hippocampus specifically in furious

form and the cerebellum in the paralytic form. Zhan *et al.* (2017) reported the thickening and elongation of the astrocytic processes during the migration of astrocytes. Hypertrophy of the astroglial cells were specifically seen in the white matter of cerebellum in the paralytic form, and this was well evident in white matter of the brain as well as hippocampus in the case of the furious forms and this was a common observation at the areas of glial limitans in both forms of rabies. Kojima *et al.* (2009) observed a significant increase in expression of GFAP and morphological variations of astrocytes in the brain of animals infected by rabies.

The distribution of GFAP in the cerebellum of the control group of animals was seen mostly concentrated in the white matter at the tip of cerebellar folia; furious cases showed a wide distribution of GFAP processes throughout the white matter of the folia and the processes extended to the molecular layer of cerebellum as well. Headley *et al.* (2001) reported a higher amount of GFAP positive fibres in the grey matter of dogs infected with canine distemper.

The remarkable cuffing behaviour of astrocytic end feet processes was observed around the blood vessels in both forms of rabies but was however absent in control group. Astrocytes adopted a special mechanism during CNS infections such as neuronal

repair facilitation, regulation of synaptic functions and extension of astrocyte end feet processes to act as a protective barrier in guarding the blood brain barrier (Soung and Klein, 2018). Immunohistochemical studies of viral encephalitic diseases in the equine brain conducted by Delcambre *et al.* (2016) using GFAP protein revealed an abnormal increase in GFAP positive astrocytes in the brain parenchyma, near the blood vessels, at the glial limitans and around the areas of gliosis. Venugopal *et al.* (2013) reported fluctuations in the expression of proteins involved in signal transduction, energy pathways and cell growth and highlighted the invariable over expression of the astroglial marker GFAP, in encephalitis caused by rabies.

A significant increase in number of astrocytes was seen in the hippocampus of animals affected with the furious form of rabies. The dentate gyrus of the hippocampus had a remarkable increase in number and size of astrocytes in cases of the group of animals affected by the paralytic form of rabies and a similar observation in this regard was also made by Machado and Alessi (1997) who observed an increased astrocyte activity and higher GFAP immunoreactivity at the hippocampus and dentate gyrus region with large processes.

Highest immunoreactivity to GFAP was observed in 33.3 percent of cases of the furious form of rabies, while only 27.3 per cent of paralytic cases manifested the same level of intensity and this finding was comparable to that reported in the study by Headley *et al.* (2001) who suggested that acute encephalopathy expressed highest number of GFAP positive astrocytes in a given area which would become less as the disease progressed into a chronic phase.

Hol and Pekny (2015) conducted studies on the role of astrocytes and GFAP during CNS diseases and concluded that reactive gliosis was a beneficial phenomenon during a CNS insult due to the neuroprotective effect of this phenomenon ; the authors went on to suggest on possible opportunities for inventing novel treatment and prevention protocols using the same. Brat (2018) suggested that reactive astrocytosis was a

response to any insult to brain, wherein these cells proliferate through an increase in the size of the cell body or processes depending on the chronicity and severity of the injury or infection.

Conclusion

Astrocytes are key cells among the glial population that provide nutritional and structural support to the neurons. Immunostaining technique has enhanced the possibility of identification of astrocytes thus permitting an evaluation of the extent of any alterations. The results of this study report on significant hypertrophy and hyperplasia of astrocytes in brain samples from dogs affected with both forms of rabies. Region wise assessment indicated that astrogliosis was seen in a more diffuse form in brains of animals afflicted with the furious form of rabies but this was localized in brain samples of animals afflicted with the paralytic form of rabies, and this was specifically into the white matter of the cerebellum. Astrocytes in the brain samples of animals afflicted with the furious form of rabies had an intense expression to GFAP when compared to those afflicted with the paralytic form. There was extensive distribution of fibrous astrocytes with elongated fibres throughout the white matter of cerebrum and cerebellum. Also significant increase in the size of protoplasmic astrocytes and thickening of the astrocyte processes in the hippocampus was evident in samples from the furious form. The regions that showed astrogliosis were different and specific in the brains of dogs infected with rabies and manifesting two different forms of rabies, though neuronal alterations were not significant. The influence of these reactive astrocytes on the pathogenesis of the disease is still a potential area for investigation.

Acknowledgement

The authors duly acknowledge the College of Veterinary and Animal Sciences, Pookode and Department of Veterinary Pathology for the facilities provided.

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