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Immunohistochemical localisation of Glutamate ammonia ligase (GLUL) in cerebrum in different clinical forms of canine rabies#

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Abstract

Rabies, one of the earliest known diseases reported in the history of mankind, is a fatal acute viral disease of the central nervous system. Despite numerous studies on etiology of rabies, its exact mechanism of neuropathogenesis remains unidentified. Glutamate ammonia ligase (GLUL) is a protein present in brain which play an important role in the pathogenesis of many neurological conditions. GLUL was identified to be over expressed in brain of human rabies. Cerebral cortex is the part of brain primarily responsible for coordination of movements and behaviour in animals and humans. Hence the work was designed to study the immunohistochemical localisation of GLUL in cerebrum in rabid carcasses in order to elucidate its role in the pathology of this deadly viral infection. The carcasses of 219 rabies suspected dogs that were brought to the Department of Veterinary Pathology, CVAS, Mannuthy, for necropsy between January 2021 and August 2022 formed the materials for the study. One hundred and thirty-three cases were found positive for rabies using the gold standard test for rabies - Direct Fluorescent Antibody Test (dFAT) which was further confirmed by polymerase chain reaction targeting N gene with amplicon size 533 bp. Among these 133 confirmed cases, 30 selected samples (frontal, occipital, temporal, parietal lobes of cerebrum) were further processed for histopathological and immunohistochemical (IHC) studies. The IHC signals of

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GLUL obtained were compared with the clinical form of rabies. Immunohistochemical staining revealed localisation of GLUL in glial cells with different staining intensity in encephalitic and paralytic form of rabies. The study concluded that the IHC signals for GLUL were stronger for furious form of rabies and weak for dumb form of the disease.

Keywords: *Rabies, cerebrum, immunofluorescence, immunohistochemistry, GLUL*

Since the dawn of human civilisation, rabies has been one of the deadliest zoonotic diseases with high public health concern. According to the WHO Expert Consultation on Rabies Report (2018), India accounts for 59.9 per cent of rabies deaths and approximately 35 per cent of human rabies deaths worldwide. Kerala has seen a significant increase in rabies deaths due to dog bites over the last five years, with unvaccinated dogs being the primary source of disease transmission. Even though canine rabies is on the rise, neuropathogenesis remains unknown.

Glutamate ammonia ligase (GLUL) is a protein present in brain which is primarily involved in monitoring neuronal synaptic plasticity by regulation of glutamate- glutamine cycle. By altering synaptic plasticity and thereby causing excitotoxic and neurotoxic changes, GLUL play an important role in the pathogenesis of many neurological conditions. This protein has been found to be overexpressed in brain in various clinical forms of rabies in a proteomic analysis conducted by Venugopal *et al*. (2013). Glutamate ammonia ligase is a glial cell-specific metabolic enzyme that maintains the glutamate-glutamine cycle by removing excess glutamate from extra synaptic junctions and maintains normal brain function *via* neurotransmission. Cerebral cortex is the part of brain that is primarily responsible for coordination of movements and behaviour in animals and humans as well as for maintaining neuronal synaptic plasticity *via* various glial cell enzymes. Changes in GLUL levels in the cerebral cortex cause excitotoxic and neurotoxic effects in a variety of degenerative conditions *via* irregulated synaptic plasticity. Decreased levels of GLUL in astrocytes could lead to increased brain ammonia which might induce seizures (Eid *et al*., 2019). This study is aimed to examine the GLUL localisation in cerebral cortex and its comparison in various clinical forms of rabies

Materials and methods

The study was conducted at the Department of Veterinary Pathology, College of Veterinary and Animal Sciences, Mannuthy, Kerala, India. The brain samples of dogs (219 numbers) for the study were collected from the carcasses brought for rabies diagnosis to the Department of Veterinary Pathology, College of Veterinary and Animal Sciences, Mannuthy. Gross lesions observed in the brain were recorded.

Impression smears from the brainstem were collected for direct fluorescent antibody test (dFAT) from all the carcasses presented for rabies diagnosis. Tissue samples from the brainstem for reverse transcriptase polymerase chain reaction (RT-PCR) were collected in RNAlater® from all the rabies positive carcasses. The whole brain was collected in 10 per cent neutral buffered formalin (NBF) from 30 positive cases (where intact brain could be obtained with no or minimal putrefactive changes) for histopathology and immunohistochemistry.

Direct fluorescent antibody test (dFAT) was performed on the samples to diagnose rabies and positive samples were taken for further studies. The impression smear preparations were fixed in chilled acetone at -20°C in a coplin jar. The slides were air-dried before being incubated with 100µL of lyophilised adsorbed antirabies nucleocapsid fluorescein isothiocyanate conjugate (Biorad India Ltd.) from prediluted aliquots (3mL of triple distilled water to powdered conjugate and stored at 8°C). The slides were then incubated at 37°C for 30 min in a humid chamber before being washed twice with phosphate- buffered saline (PBS) for 1- 2 min each. The slides were air dried and mounted with mounting buffered glycerol before being examined with an immunofluorescent microscope (Carl Zeiss AXIO) at 20x and 40X objectives. Particles emitting bright apple green fluorescence were considered positive for rabies virus antigen and graded according to the protocol recommended

by Rupprecht *et al*. (2018) as follows:

1+: Inclusions in <10 per cent of microscopic fields

2+: Inclusions in 10-50 per cent of microscopic fields

3+: Inclusions in most of the fields

4+: Inclusions in all microscopic fields

For RT-PCR, RNA was isolated using Qiagen RNA isolation kit and converted to cDNA using cDNA synthesis TAKARA kit. The amplification of 533 bp region of nucleoprotein (N) gene of rabies virus was carried out as per the protocol of Tordo *et al*. (1986), using oligonucleotide primers (RVNF- 5′ACT GAT GTA GAA GGG AAT TG-3′; RVNR – 5′-GAA CGG AAG TGG ATG AAA TA-3′) sequences synthesised and supplied by M/s. Eurofins Genomics India Pvt limited. The amplicons of nucleoprotein (N) gene obtained by PCR were electrophoresed in two per cent agarose gel containing 0.5 µg/mL ethidium bromide in 1X Tris-Acetate-EDTA (TAE) buffer. The gels were visualised and documented in Gel DocTM EZ Imager; BIO-RAD software version 4.1 available with the automatic gel documentation system.

For histopathological evaluation whole brain was fixed in 10 per cent neutral buffered formalin and processed using the standard paraffin embedding method. Serial sections were cut at 6-micron thickness with a rotary microtome and stained with the standard haematoxylin and eosin procedure. The sections were examined using a Zeiss light microscope, and lesions were recorded (Suvarna *et al*., 2018). Histopathological lesions were studied according to Tangchai and Vejjajiva (1971).

For immunohistochemistry, adhesive coating of the slides was done using 3- aminopropyltriethoxysilane (APTES). Deparaffinisation and dehydration of the sections were done by immersing them in two changes of xylene for 20 min each, followed by hydration in descending grades of alcohol (sequentially in 100% two changes, 90%, 80%, 70%, 60%, and 50%) for one minute each, and treatment in two changes of distilled water for two minutes each. Antigen retrieval was done with EDTA solution which was preheated at 45°C for 10 minutes. The copling jar containing slides was closed loosely and incubated in a water bath at 95°C for 20 minutes. After the antigen retrieval step, hydrogen peroxide blocking reagent was added and incubated for 30 min followed by 1X Tris buffered saline (TBST) buffer wash. Non-specific antibody binding was reduced by a protein blocking step, incubated for 30 minutes followed by 1X TBST buffer wash. A 1:200 concentration of prediluted GLUL rabbit monoclonal antibody (GLUL # 27857S; Cell signalling technology, USA) was added to the section and incubated overnight at 4°C. The next day, a buffer wash was done. Rabbit super enhancer was added and incubated for 30 min followed by 1X TBST buffer wash. The secondary antibody (mouse anti-rabbit HRP conjugate) was added and incubated for 30 min before being washed with 1X TBST. DAB substrate solution (3 in 100l) was applied to the sections and incubated at room temperature for 5 - 10 min (depending on colour development), followed by a 1X TBST wash. Counter staining was done with Harry's Haematoxylin for 5 minutes. Sections were dehydrated, then cleared with xylene and mounted with DPX before being examined under a microscope.

Based on the intensity of staining and the number of cells stained, qualitative and quantitative scores were assigned to the samples respectively. The qualitative & quantitative scores were summed up to give combined score.

Grading based on intensity of staining

Weak cytoplasmic staining- 1

Moderate cytoplasmic staining- 2

Strong cytoplasmic staining- 3

Very strong cytoplasmic staining- 4

Grading based on quantity of staining

No GLUL +ve immunostaining cells = 0

<25% cells staining = 1

25-50% cells staining = 2

50-70% cells staining = 3

>70% cells staining = 4

Combined score based on qualitative and quantitative grading

+ = no or weak immunostaining = score 0-2

++ = moderate immunostaining = score 3-5

+++ = strong immunostaining = score 6-8 (Vakkala *et al*., 1999).

Statistical analysis

Association of immuno-fluorescence score and form of rabies, association of GLUL grade and form of rabies was done by using Fisher's Exact test.

Results and discussion

Out of the 219 carcasses brought for rabies diagnosis, a total of 133 were found to be positive for rabies by the gold standard test for rabies – dFAT (Fig. 1). The samples were further confirmed positive by employing RT-PCR targeting 533bp of N gene (Fig. 2).

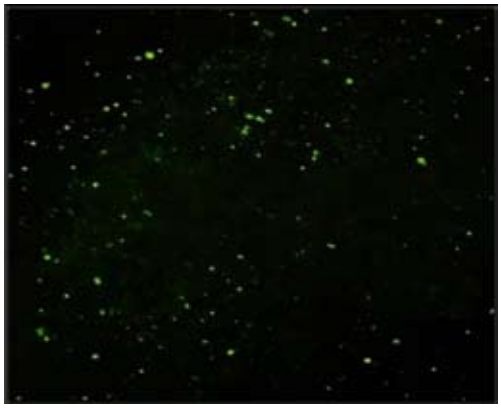

Fig. 1. Cerebrum- Apple green fluorescence - inclusions over 10-50% of the field - Grade 2+ (dFAT x 100)

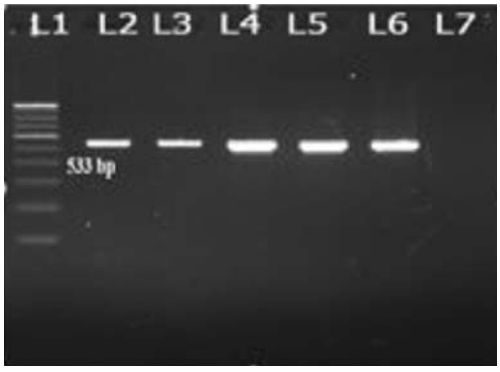

Fig. 2. (L1- 100bp DNA ladder, L2- positive control, L3- L6- positive samples, L7- negative control).

Immunohistochemical study was conducted on 30 canine brain samples. On necropsy, gross lesions such as mild to severe congestion, oedematous brain, meningeal thickening (Murphy, 1977) and hydrocephalus were noticed randomly in ten cases. Blood clots were found in the cerebral cortical region and cranial cavity of animals that had been beaten to death, which was consistent with the findings of Divya (2013).

Histopathological studies revealed vascular and neuronal changes including cerebral congestion (Fig. 3), perivascular edema and perineuronal edema consistent with findings of Prasanna (2012) and Ahmad *et al*. (2016). Oligodendrocytes with prominent perinuclear haloes with the typical "fried egg'' appearance was also observed (Fig. 4), which were similar to the findings of Garman (2011). Residual microglial nodules were noticed in some areas consistent with the observations of Hsu *et al*. (2006). Microglial cell proliferation (gliosis) was evidently noticed in the grey matter and white matter of cerebrum in almost all the cases.

To determine the location of GLUL, immunohistochemical studies were performed on 30 samples - 23 furious and 7 dumb forms. Immunohistochemical studies revealed diffused strong immunopositivity in the astrocytes and oligodendroglia cells of molecular layer, external, inner granular layer

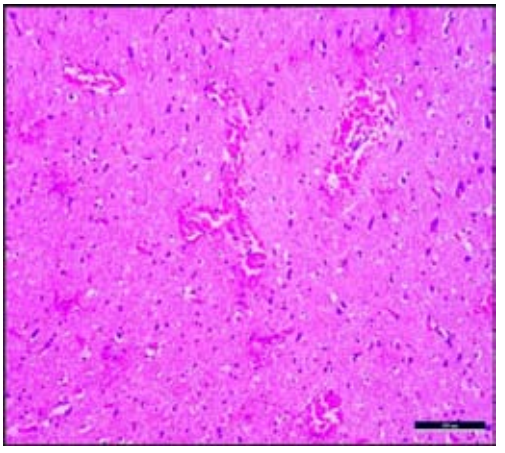

Fig. 3. Cerebrum- mild congestion in the external granular layer (H&E x 200)

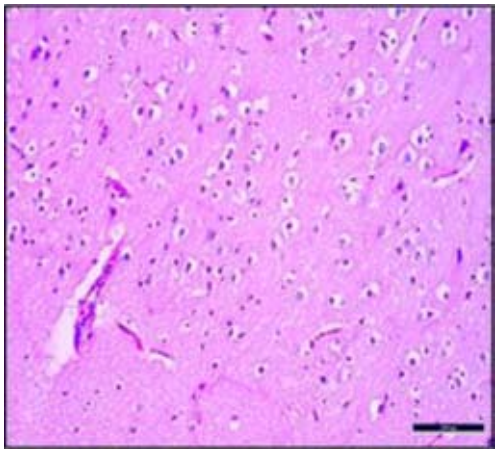

Fig. 4. Cerebrum- Oligodendrocytes with prominent perinuclear haloes (H&E x 200)

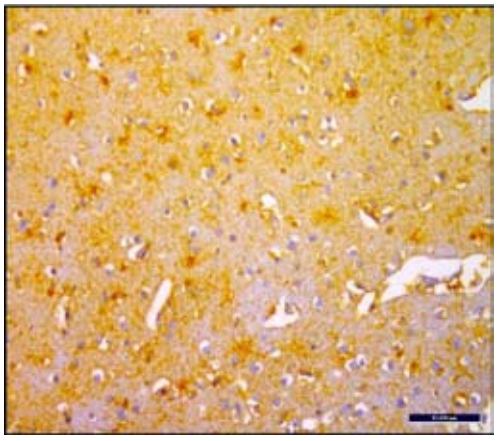

Fig. 6. Cerebrum - moderate immunopositivity in the glial cells of granular layer (IHC x 200)

(Fig. 5) and moderate immunopositivity in the microglial cells and astrocyte end feet process of multiform layer of cerebrum (Fig. 6). The results of this localization were consistent with those of Norenberg and Martinez-Hernandez (1979) and Hallermayer *et al*. (1981) who studied localisation of GLUL in rat brain.

Animals with furious form of rabies were found to exhibit stronger immunopositivity for GLUL. Glutamate being an excitatory neurotransmitter (Platt, 2007), its increased level will lead to phase of excitation which could lead to manifestation as furious form in rabies. Strong immunopositive signals of GLUL noticed in furious form of rabies in the present study substantiates this fact. Strong immunopositive signals in case of animals with furious form which were traumatised were consistent with the findings of Petito *et al*. (1992) who found increased immunoreactivity of GLUL in traumatic conditions of brain. Similar immunostaining pattern of GLUL was described by McCormick *et al*. (1990) in an intracranial tumour condition. Shruthi *et al.* (2021) who performed studies on localisation of glial fibrillary acidic protein (GFAP) also obtained comparable results in glial cells.

The immunopositivity of GLUL in the glial cells of molecular layer, external and internal granular layers was low in the dumb form of rabies (Fig. 7). When the level of GLUL decreases, there will be increased level of glutamate and decreased level of glutamine. This increased level of glutamate initially causes

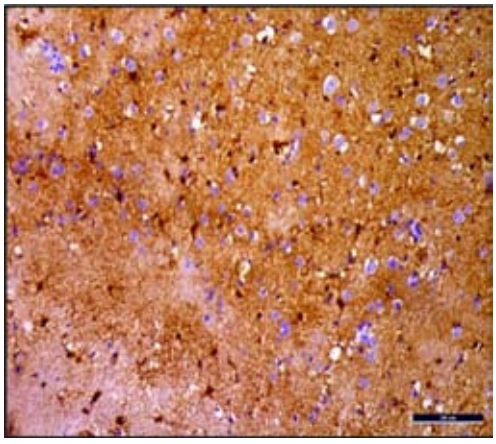

Fig. 5. Cerebrum - astrocytes of molecular layer showing strong immunopositivity (IHC x 200)

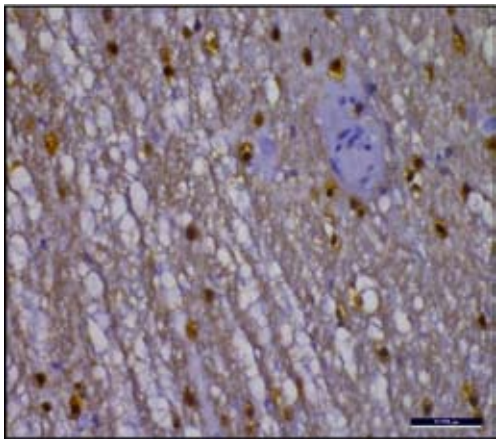

Fig. 7. Cerebrum- weak immunopositivity in the glial cells of white matter (IHC x 400)

Table 1. Association of GLUL grade with form of rabies in cerebrum

GLUL grade	Forms of rabies				Total	
	Encephalitic		Paralytic			
	No	Per cent	No	Per cent	No	Per cent
Weak	6	27.3	5	62.5	11	36.7
Moderate	10	45.5	2	25.0	12	40.0
Strong	6	27.3	1	12.5	7	23.3
Total	22	100	8	100	30	100
Fisher's exact test P-value < 0.001**						

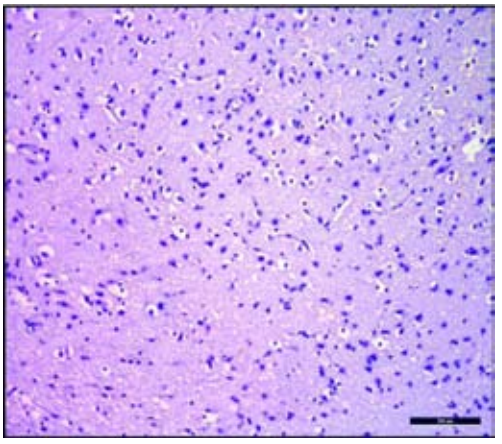

Fig. 8. Cerebrum- negative control (IHC x 400)

excitotoxicity of neurons (severe excitation) (Yeh *et al*., 2013) and finally leads to exhaustion of neurons resulting in paralysis (Sayin and Rutecki, 2003). The weak immunopositive signals of GLUL noticed in dumb form of rabies in the study explains this fact.

Statistical analysis was done to determine the relationship between GLUL grade and form of rabies using the Fisher exact test. It was found that the observations are significant at 1% level (Table 1).

Conclusion

Immunohistochemical studies were performed on brain tissues of 30 canine rabies cases regarding the protein GLUL. It was observed that GLUL was found in glial cells in various layers of cerebrum. The encephalitic form of rabies exhibited strong immunopositivity, compared to the dumb form which showed weak immunopositivity. This study provides an information on localisation of GLUL in brain of rabies affected dogs leading to neuronal impairment in furious and dumb form of rabies. Further research is needed to determine the precise role of GLUL in rabies so that it can be used as a biomarker in future.

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