RESEARCH ARTICLE Indian Journal of Animal Research, Volume 56 Issue 5: 601-606 (May 2022)

Occurrence of Rabies in Post Exposure Vaccinated Cattle with Dog Bite Injury above the Neck Region

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10.18805/IJAR.B-4825

ABSTRACT

Background: Investigated the fatality in bovines infected with rabies virus through dog bite, that have undergone the post bite therapy. The site of dog bite close to the brain was an important factor and reason behind the fatality.

Methods: The findings from cattle infected naturally with Rabies virus and vaccinated with post bite shots of anti-rabies vaccine were considered for the present study. The rabies cases *i.e.*, 20 cases (out of total 58 cases reported) in bovines, from Bidar, Karnataka and Maharashtra border area, reported in last three years and bovines with site of dog bite specifically above neck region were considered. All the clinical samples were tested with direct fluorescent antibody test and reverse transcriptase polymerase chain reaction, amplifying the glycoprotein, G gene of rabies virus.

Result: In the diseased cattle, with history of dog bite above neck region, the average incubation period observed was 15 days in case of calves and 20 days in case of adults. The occurrence of clinical signs was seen in cattle undergoing the post bite vaccination. Fatality rate recorded was 100 per cent, despite having treated with post bite anti-rabies vaccine alone, without use of rabies antisera as a passive way of treating successfully in such cases. Rabies post bite vaccination is not protective, if the site of dog bite is above the neck region.

Key words: Cattle, Direct fluorescent antibody test, Post exposure vaccination, Rabies, Reverse transcriptase polymerase chain reaction.

INTRODUCTION

Rabies is a devastating and fatal zoonotic disease of human and animals (Jackson *et al*., 2007). Rabies is acute encephalitis caused by rabies virus (Mahadevan *et al*., 2016). Rabies virus is prototype species of the genus *Lyssa virus,* family *Rhabdoviridae*. Once clinical signs of Rabies set in 100 per cent fatality is seen in animals as well as in humans, with few exceptions in humans, in the history of rabies (Willoughby *et al*., 2004; Madhusudana *et al*., 2002; Alvarez *et al*., 1994; Porras *et al*., 1976; Hattwick *et al*., 1972). Report of survival once clinical signs are manifested in animals, is not on record.

Rabies is endemic in developing countries of Africa and Asia. Globally canine rabies causes approximately 70,000 human deaths (Bedeković *et al*., 2018) and 8.6 billion USD economic losses annually (Hampson *et al*., 2015). It is endemic in India, with an estimated annual 20,000 human deaths (one-third of the global rabies burden). India accounts for the most deaths in Asia (59.9% of human rabies deaths) and globally (36% of human rabies deaths) (WHO 2018).

Information about the incidence of rabies in animal populations is not available in most of the developing countries. In cattle, no such specific numbers of deaths are placed on record, due to poor reporting, lack of awareness, surveillance and recording system. Rabies incidence in animals, particularly in cattle, is much higher than suspected and reported (Gill *et al*., 2019).

Dog bite is the main source of rabies virus in human and cattle. Though post-exposure prophylaxis (PEP) (Vaccination on 0, 3, 7, 14 and 28 days) is the only proven approach in preventing rabies deaths is however, not effective to the tune of cent per cent.

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How to cite this article: Bhosale, A.V., Awati, B.I., Mallinath, K.C., Isloor, S., Ravindra, B.G., Lunavat, G., Kavitha, K. and Kharate, A.G. (2022). Occurrence of Rabies in Post Exposure Vaccinated Cattle with Dog Bite Injury above the Neck Region. Indian Journal of Animal Research. 56(5): 601-606. DOI: 10.18805/IJAR.B-4825.

Submitted: 08-11-2021 **Accepted:** 22-12-2021 **Online:** 29-01-2022

Rapid transfer of Rabies virus to the central nervous system (CNS) through the peripheral nervous system (PNS) is the important step in Rabies virus pathogenesis (Salinas *et al*. 2010). At the site of dog bite, after entry of virus into the infected cell, neurons (PNS), Rabies virus particles are mistaken for cargo. PNS uses trafficking components, allowing virus particles to undergo axonal transport. The virus

uses neurotrophin transport machinery to reach the CNS. Rabies virus travels faster and is more directed when transported with p75NTR. (Gluska *et al*., 2014).

The aim of present study was to determine the pattern of animal bite and onset of clinical signs of the rabies in cattle, with history of dog bite above neck region (*i.e.,* close to brain) and to discuss the significance of the observations made to prevent further losses in term of fatality in the exposed cattle.

MATERIALS AND METHODS

Collection of clinical samples

Brain tissue was collected from dead animals after post mortem examination using Skull open method. From live animals, saliva was collected (at hourly interval and pooled) intermittently from clinically suspected cases. Clinical specimens were preferred from the cases showing clinical signs of rabies with detailed history of dog bite above the neck region and post bite vaccination, in cattle. A total of 58 samples were collected in a span of three years from the area around Bidar district in Karnataka and Maharashtra border area (Fig 1). Twenty samples with known history of dog bite above the neck region and post exposure vaccination were selected for the present investigation. The samples were stored at -86°C until further processed.

Direct fluorescent antibody test (DFAT)

All the samples were subjected to Direct Fluorescent Antibody Test for confirmation of rabies. The test was done as per the standard protocol given by OIE. The test was carried out at OIE Twinned Rabies Diagnostic Laboratory, Dept. of Veterinary Microbiology, Veterinary College, Hebbal, KVAFSU, Bangalore.

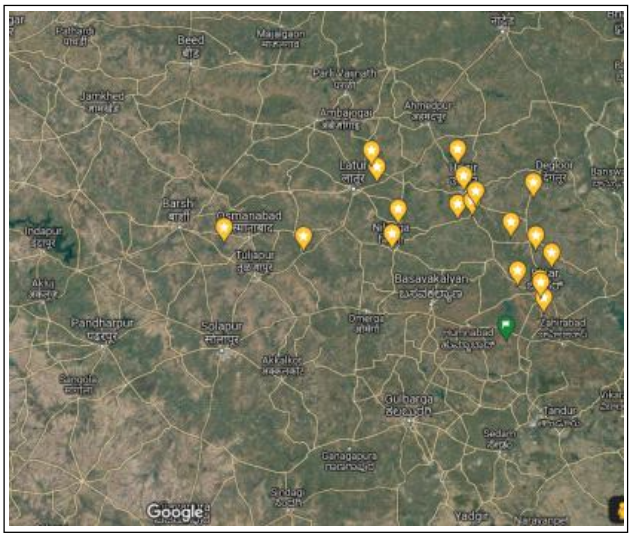

Fig 1: Map of the area depicting geographic location of the sample collection, in and around Bidar and some part of Maharashtra, where incidences were recorded during the study period of three years 2018-2021.

In brief, for each of the samples, three impressions smears were made one each for the anti-rabies nucleoprotein IgG- FITC conjugate (Millipore-Light Diagnostics, Rabies DFA III) and negative control FITC conjugate (Millipore- Light Diagnostics). The brain sample of healthy cattle was also included in the test as a negative control. Rabies CVS strain infected brain sample of mice was used as positive control. The brain impression smears on taken on slides were blotted using paper towels in order to remove excess of moisture and the blood stains. Then were initially air dried for 5 min. before fixing in high grade chilled acetone (80 % v/v) for an hour at -20°C. The fixed impressions were air dried to ensure the removal of acetone traces and stained using 1:100 dilution of the above said FITC conjugates by incubating in a humid chamber at 37°C for 60 min. The impressions were washed with 1x PBS for 5 min. and the wash step was repeated twice to remove excess stain. The stained impressions were observed under a fluorescent microscope. Presence of typical granular intra-cytoplasmic apple green fluorescence of aggregated nucleocapsid was used as a criterion in declaring positive result, whereas absence of green fluorescence as test negative for the samples. Positive and negative samples were further subjected to RT-PCR for molecular detection of rabies virus.

Viral RNA extraction

RNA extractions were performed using triturated brain samples in phosphate buffer saline, as starting material. RNA was extracted using Trizol (Life Technologies, Rockville, MD).

In brief, following, manufacturer's instructions (Chomczynski *et al*., 1987) with slight modifications, for RNA extraction, 0.75 ml of Trizol reagent per 0.25 ml of brain tissue triturated along with PBS was used. Lysate was pippeted up and down several times to homogenize and incubated for 5 minutes at room temperature. Followed by centrifugation and aqueous phase was collected. Addition of Trizol reagent again, in the same proportion was done as per the manufacturers protocol. All the sample and a control of nuclease-free water were processed to yield a total volume of 100 ul of total RNA for each sample.

The total RNA of each sample was quantified using Nano-spectrophotometer with ultraviolet light absorbance at 260 nm. The ratio of optical density at wavelengths of 260 nm and 280 nm was used to assess the purity of the RNA.

Primer used for reverse transcriptase polymerase chain reaction (RT-PCR)

Published primer for G gene of Rabies virus was used in the present study. Primer sequences used in the present study are as follows:

Target gene	Primer	Sequence	Amplicon size (bp)	Reference
Glycoprotein gene	Forward	TAA TCC CAG AGA TGC AAT CA	406	Gupta *et al*., 2005
	Reverse	CCT CAG AGT CTG GTC TCA CC		

Reverse transcriptase polymerase chain reaction

One-Step RT-PCR protocol (Superscript Platinum III one step RT-PCR) required 60 min at 50°C for cDNA synthesis by Reverse Transcription, followed by 4 min at 94°C for inactivation of reverse transcriptase and *Taq* polymerase activation, 35 cycles of 94°C for 45 s, primer annealing at 53°C for 45s, 72°C for 1 min, followed by a final elongation at 72°C for 5 min.

PCR products were visualized on a 1.5% TAE agarose gel containing 0.5 ug/ml ethidium bromide. One-Step RT-PCR protocol resulted in a 406 bp product from the glycoprotein gene *i.e.,* G gene of rabies virus.

RESULTS AND DISCUSSION

Out of 58 samples, the cases with known history of dog bite above neck region and vaccination history, 20 clinical samples subjected to direct fluorescence antibody test were found positive.

Observations recorded from dog bite to onset of clinical signs of rabies

The rabies cases under investigation were observed carefully and recorded the site of dog bite, incubation period and the onset of symptoms. The cases were treated under the supervision or by the veterinarians in village clinics and or at the farm of the handler of cattle. The anti-rabies vaccination schedule *i.e.,* 0,3,7,14 and 28 day was adopted and followed for all the cattle under study.

In cattle, very commonly recorded site of dog bite was at the muzzle, near nostrils, base of horn, base of ear, dorsolateral aspect of muzzle (Fig 2). Entry of virus through dog bite close to the brain, facilitates rapid entry of virus in to the brain.

In adult cattle including cow, bullock, buffalo after completion of 0, 3, 7 and 14th day post bite vaccination, onset of clinical signs were recorded on 20th day from the day of dog bite. And within 48-72 hours after onset of clinical

Fig 2: Site of rabid dog bite injury in Jersey calf.

Table 1: The sampling details with special reference to site of dog bite and occurrence of first clinical sign after dog bite.

Sample ID/strain	Species	Age	Sex	Location	Date of Sample collection	Site of dog bite	Occurrence of first clinical sign
BASWA K1	Buffalo	8 Y	Female	Kamthana, Bidar, Karnataka	22-02-2019	Muzzle	20th day
AB K2	Buffalo	3.5 M	Male	Belur, Bidar, Karnataka	18-02-2020	Dorsolateral aspect of muzzle	20th day
AB K3	Buffalo	6 Y	Female	Bidar, Karnataka	04-08-2020	Muzzle	20th day
AB K4	Bullock	8Y	Male	Khanapur, Bidar, Karnataka	28-09-2020	Muzzle	20th day
AB M5	Buffalo	6 Y	Female	Ujani,Maharashtra	21-10-2020	Ear	20th day
AB M7	Buffalo	7 Y	Female	Udgir,Maharashtra	06-11-2020	Muzzle	20th day
AB M9	Buffalo	8 Y	Female	Belsakharga, Maharashtra	08-11-2020	Muzzle	20th day
AB M10	Cow calf	3 M	Female	Kharola, Maharashtra	19-11-2020	Base of horn	15th day
AB M13	Buffalo	4 Y	Female	Bhatambra Ashta Mod, Maharashtra	10-12-2020	Muzzle	20th day
GL K14	Buffalo	18 M	Female	Shrimandal, Bidar, Karnataka	10-12-2020	Muzzle	20th day
AB K15	Bullock	4 Y	Male	Ghodepalli, Bidar, Karnataka	12-12-2020	Dorsal aspect of muzzle	20th day
AB K16	Buffalo	30 M	Female	Shrimandal, Bidar, Karnataka	14-12-2020	Muzzle	20th day
AB K17	Buffalo	20 M	Female	Nirna,Bidar, Karnataka	18-12-2020	Muzzle	20th day
AB M18	Buffalo	8 Y	Female	Shirol, Nilanga, Maharashtra	01-01-2021	Muzzle	20th day
AB M19	Buffalo	5 Y	Female	Nitur, Nilanga Maharashtra	05-01-2021	Muzzle	20th day
AB K20	Cow heifer jersey	1 Y	Female	Hippalgaon, Karnataka	14-01-2021	Dorsolateral region of muzzle	15th day
SPY M21	Cow calf	2 M	Male	Gurnal, Deoni,Maharashtra	15-01-2021	Muzzle	15th day
AB K23	Bullock	7 Y	Male	Karvat	27-01-2021	Base of horn	20th day
AB M26	Buffalo	5 Y	Female	Jamb, Maharashtra	09-03-2021	Muzzle	20th day
AB M38	Buffalo	6 M	Female	Kudali, Hanegaon, Maharashtra	23-04-2021	Base of left horn	20th day

Y- Year; M- Month.

signs (as detailed below), death of animal was recorded Table 1.

In calves under study, after completing of 0, 3 and 7 days post bite vaccination schedule, onset of clinical signs were recorded *i.e.,* on 15th day from the day of dog bite, was reported by the farmers. And within 48-72 hours after onset of clinical signs, death of animal was recorded.

Similar findings were recorded by Hudson *et al*., 1996, LojkiÄ *et al.,* 2013. In experimentally infected cattle, the average incubation period recorded by Hudson *et al*., 1996 was 15.1 days and the average morbidity period was 3.7 days. Of those, the naive cattle had significantly shorter incubation and morbidity periods than the test-vaccinated cattle.

The incubation period of rabies depends on the location and severity of the wound and the amount of virus introduced and is highly variable in a wide range of host species. For cattle, it varies from 20 to 165 days (Hudson *et al*., 1996), but in this present study, the dog bite above the neck region was considered, in which 15 days in calves and 20 days incubation period in case of adult cattle was recorded. These findings are in concurrence with findings of Hudson *et al*., but the incubation period in calves recorded in this study differs, *i.e.,* 15 days.

Clinical signs recorded in cattle

The first clinical sign observed was anorexia (100%), hyperaesthesia, behavioural change followed by increase in aggressiveness (90%), micturition (70%), head pressing or hitting inanimate objects (70%), bellowing (60%), salivation (90%) were the prominent and common signs seen. Bellowing was very commonly seen in buffaloes (100%).

The most obvious symptoms recorded by Barnard *et al*., 1979, were salivation (92%), bellowing (69%), aggressiveness (47%), paresis or paralysis (30%) and straining (12%). Common clinical signs included excessive salivation (100%), behavioural change (100%), muzzle tremors (80%), vocalization (bellowing; 70%), aggression, hyperaesthesia and/or hyper excitability (70%) and pharyngeal paresis/paralysis (60%). The furious form of rabies was seen in 70% of the cattle was recorded by Hudson *et al*., 1996. The present study findings were in accordance with the findings of Barnard *et al*., 1979, Hudson *et al*. 1996, except the bellowing was observed very commonly in 100 per cent buffaloes.

Direct florescent antibody test (DFA)

All the 20 clinical samples subjected to Direct fluorescent Antibody test were found positive for rabies virus (Fig 3).

The use of fluorescent antibodies for diagnosing rabies as the standard and reliable test was placed on record by Asil'eva *et al*., 1967; Nikolaenko *et al*., 1967; Tarabrina *et al*., 1968; Prabhu *et al*., 2018 and recommended by OIE. The present study findings are in concurrence with findings mentioned above. But in DFAT weakly positive fluorescence results in 03 samples were attributed to improper storage of the brain tissue, due to purification of the lipid rich brain tissue might have resulted into weakly positive results with pale background instead of reddish background (DFAT using 0.0125% Evan's blue) with direct fluorescent test, which was further confirmed by Reverse Transcriptase Polymerase chain reaction.

Amplification of G gene of rabies virus by RT-PCR

The application of Reverse Transcriptase Polymerase chain reaction to the samples under study amplified glycoprotein gene *i.e*., G gene, the product size was 406 base pairs (Fig 4). The amplified product was sequenced and BLAST was done using NCBI gene bank. The sequences revealed 99 per cent identity.

Reverse transcriptase polymerase chain reaction amplification of Glycoprotein gene was used as a molecular diagnostic tool for detection of Rabies Lyssavirus and to study the variation in amino acid sequence of glycoprotein genes by Gupta *et al*., (2005), Pharande *et al*., (2021). Similar findings were recorded in the present study.

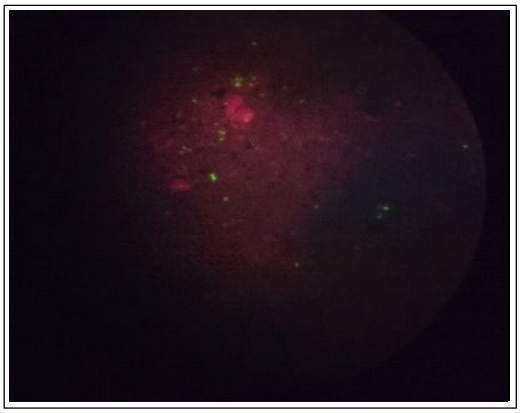

Fig 3: Direct florescent antibody test (DFA) showing fluorescence of Rabies virus inclusions Magnification 40X.

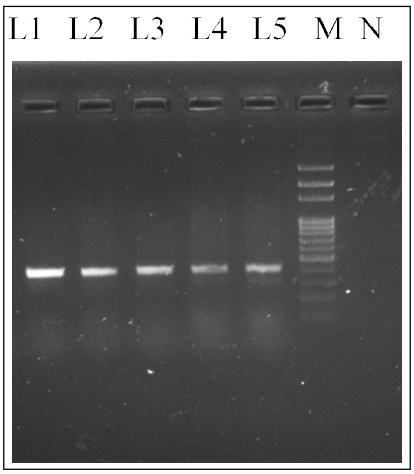

Fig 4: Amplification of G gene of Rabies virus by RT-PCR, 406 base pair amplicon size and. Lane 12,3,4 - Clinical Samples, Lane 5- Known standard CVS strain of Rabies virus, M-100 base pair marker and N-Negative control.

Most of rabies deaths occur among those who delayed, did not receive, or complete rabies PEP. Important observation to put on record is that the dog bite above the neck region in cattle, even after giving the post exposure vaccination, turned to be fatal.

The neutralizing antibody test using serum, after post bite immunization in domestic camels and cattle revealed the cut-off value of rabies RVNA titre as 0.50 IU/mL, is defined as the minimum antibody level affording complete protection (Liu *et al*., 2016).

Use of Rabies antisera, is the recommendations given by OIE and WHO for the post exposure prophylaxis in exposed animals, is generally not practised in the treatment of large ruminants or bovines. Being, economically not viable, the dose of immune sera recommended. Use of antisera at least at the local site of dog bite injury immediately after dog bite reduces the fatality to a great extent.

Further investigation requires the detailed study on antibody titres in post bite cases of rabies in relation to the present post bite vaccination schedule in cattle and the efficacy especially in bites above the neck region, where the incubation period is very short ranging from 15 days to 20 days. The study should also include the use of Anti-rabies serum at the dog bite site along with recommended anti-rabies post exposure regime.

CONCLUSION

The occurrence of clinical signs was seen in cattle undergoing the post bite vaccination. Fatality rate recorded was 100 per cent in case of cattle with site of dog bite above the neck especially at the muzzle and base of the neck, despite of having treated with post bite anti-rabies vaccine alone, without use of rabies antisera as a passive way of treating successfully in such cases. Rabies post bite vaccination is not protective, if the site of dog bite is above the neck region *i.e.*, muzzle, base of horn, ear, dorso-lateral aspect of muzzle in cattle. Further study with antibody titre assessment of naturally infected cattle, immediately after dog bite and after post bite shots, antibody titres should be pursued.

The treatment of dog bite cases in large ruminants should include the recommendations given by OIE and WHO regarding the use of Rabies antisera at the site of dog bite, if the dog bite is close to brain *i.e.,* above the neck region can save the large population of exposed animals.

Conflict of interest

The authors declare that they have no conflict of interests.

Ethical Issues

Ethical clearance was obtained from the institutional ethical committee. Permission to carry out the study was granted by following the set guidelines by OIE and WHO for handling infected material of rabies suspected and confirmed cases.

ACKNOWLEDGEMENT

I would like to thank Dr. Srikrishna Isloor, Incharge, OIE Twinned Rabies Diagnostic Reference Laboratory, Department of Veterinary Microbiology, Veterinary College, Hebbal, KVAFSU, Bangalore for his assistance in identifying specimens and making available to us facilities for rabies diagnosis and guidance.

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