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Short Communication

Confirmation of Rabies Infection by Mouse Inoculation Test and Reverse Transcriptase-Polymerase Chain Reaction in Suspected Samples of Cow and Mule

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ZAU performed the study and wrote the manuscript. AK designed the experiments and proofread the manuscript. AWM, NA, SA and MN performed the experiments.

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ABSTRACT

Rabies, a *Lyssavirus* infection of Rhabdoviridae family, is a potential neurotropic disorder affecting all mammals and humans. This infection spreads through biting of infected and/or carrier animals to healthy ones including humans. Incubation period of this infection is quite variable ranging from a few days which can last up to one year in few cases. This case report presents the diagnosis and screening of suspected rabies samples of cow and mule. Clear behavioral changes along with paralysis of tail and hind legs were noticed in the mice of both groups between 11-15 days post inoculation while all the mice were found dead between 15-18 days post inoculation in mouse inoculation test (MIT). Reverse transcriptase-polymerase chain reaction (RT-PCR) performed for both samples gave a product of 443-bp amplifying the highly conserved "N"-region gene of virus confirming the rabies infection in both cases.

Rabies, caused by *Lyssavirus* of Rhabdoviridae family, is a nervous disorder potentially affecting all mammals and humans (Blanton *et al*., 2006). Rabies virus through fast axonal transport spreads from site of peripheral inoculation or infection to central nervous system (CNS) at the rate of 12 to 100 mm in a day (Lycke and Tsiang, 1987; Tsiang *et al*., 1991). As far as incubation period of disease is concerned in humans and animals, distinct variability has been observed ranging from 20-90 days and even lasting up to 1 year in some cases. This variability and delay depends upon site of virus inoculation or viral entry and the distance of the site from CNS (Smith *et al*., 1991).

Nicotinic acetylcholine receptors are present at neuromuscular junction with whom rabies virus gets attached and enters the neurons as studies have shown that major site of entry in neurons is neuromuscular junction (Lentz *et al*., 1982; Lewis *et al*., 2000). In this disease severe neurologic signs can be observed which can ultimately lead to death along with few pathological alterations in CNS, hence it can be assumed that disease may occur due to neuronal dysfunction instead of neuronal cell death (Iwasaki and Tobita, 2002; Jackson, 2002). Moreover; this disease causes a decreased sodium and potassium ions channels hence preventing the infected neurons to flow action potentials properly leading to functional impairment (Jackson, 2003).

In one of the two cases being reported here, brain sample of a dead cow preserved in 50% glycerol was received from Sheikhupura district of Punjab province Pakistan with a history of nervous disorders like abnormal vocal sounds, excitement and restlessness, shaking of the head and striking the head with walls along with colic signs. The age of cow was approximately two and a half years and day before the death clear signs of paralysis and drooling of the saliva from mouth along with the tendency of self biting was noticed. The owner used to graze his animals in open fields and a lot of stray dogs were present in that village.

In the second case, a dead mule with an age of 14 months was brought in VRI, Lahore Cantt Pakistan from a village of Lahore District with froth coming out of the mouth for post-mortem and diagnosis of the cause of death. According to the history given by the owner, mule had a tendency to bite other animals with shaking of the head along with restlessness, excitement and colic signs. Drooling of the saliva from mouth was also observed.

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Post-mortem did not reveal any specific lesions. Brain was collected and preserved in 50% glycerol solution.

The study was designed to diagnose the rabies infection timely by using conventional and molecular techniques so that early treatment may be started to protect the affected animals.

Materials and methods

Samples of both cases were triturated separately in a tissue grinder. A 10% brain tissue suspension by weight was prepared in phosphate buffered saline (PBS) solution, for both cases separately. The triturate was then filtered using 0.45µm syringe filter in sterile conditions.

For mouse inoculation test (MIT), twenty (20) Swiss albino mice having an age of 14-21 days irrespective of their sex were selected and divided into two groups with ten mice in each group. Mouse inoculation test was performed as described by Koprowski (1996). Eight mice from group1 were injected intracerebrally, just in the middle of line between the lateral canthus of eye and ear opening, with 0.03ml of brain tissue suspension (10%) of cow. Similarly, eight mice from group 2 were injected intracerebrally with mule sample. Two mice of each group were kept as control. The guidelines of ethical use of animals were strictly followed (Dua, 2004).

All mice were given feed and water *ad libitum*. Mice were observed for 21 days and checked for any abnormality in fur, paralysis of legs and tail and deaths. Any mortality observed during first 24-28 h was considered as non-specific (due to any stress condition like bacterial infection *etc*.).

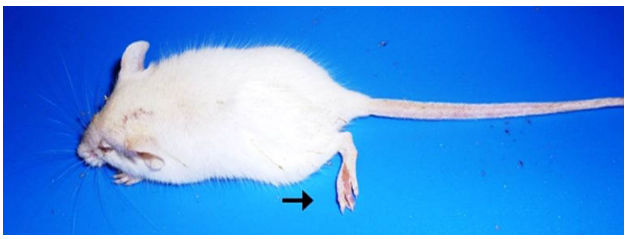

Fig. 1. Mouse from the group 1 inoculated with suspected cow sample (Case No. 1) at day 14 post-inoculation showing paralysis of tail and hind legs along with roughness of the fur.

The molecular diagnosis of the samples was done by using RT-PCR. RNA isolation was performed according to the method of Tordo *et al*. (1996) while RT-PCR was performed according to the method of Numan *et al*. (2011) with slight modifications. Highly conserved 'N' region of the gene was amplified using the primer pair 5′-TTTGAGACTGCTCCTTTTG-3′ (forward) and 5′-CCCATATAGCATCCTAC-3′ (reverse) amplifying a product of 443-bp.

Results

Figure 1 shows mouse from the group 1 injected with cow sample at day 14 post-inoculation while Figure 2 shows mouse from group 2 injected with mule sample at day 11 post-inoculation. The abnormalities in the behaviour of all the mice were observed 7 days after injection while after 10 days of injection, paralysis of the tail followed by paralysis of hind legs was observed. All the mice were found dead between 15-18 days post injection. Nervous disorders were clearly observed in the mice of both cases.

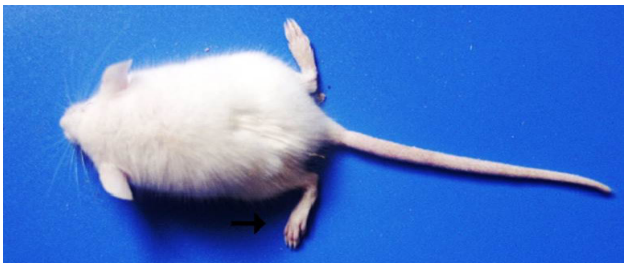

Fig. 2. Mouse from the group 2 inoculated with suspected mule sample (Case No. 2) at day 11 post-inoculation showing more pronounced paralysis of tail and hind legs.

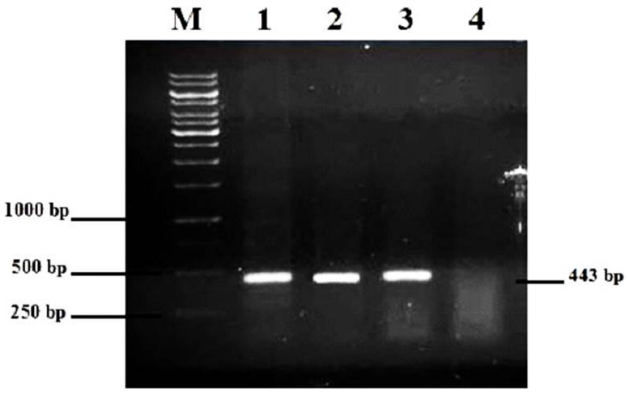

Fig. 3. RT-PCR results of the sample. M, ladder; 1, cow sample; 2, mule sample; 3, positive control; 4, negative control.

Figure 3 shows RT-PCR results of suspected samples, taken from UV-trans illuminator with band 1 as cow sample, 2 as mule sample, 3 as positive control and 4 as negative control. Both the samples were confirmed as rabies positive because amplification of 443-bp (conserved region) of N-region gene was done and amplicons of both samples gave product of 443-bp *i.e.* specific for rabies against this primer set.

Discussion

Rabies, a severe viral infection, occurs as a result of biting of infected or carrier animals especially dogs and bats (Durrani *et al*., 2017). As far as diagnosis of this infection at clinical level is concerned, in wild as well as domestic animals, no post-mortem lesion or clinical sign can be taken as pathognomonic regarding it due to which a laboratory diagnosis is always required to confirm the infection in a particular suspected case. Secondly, in equine species, differential diagnosis becomes difficult and different encephalitides such as equine encephalomyelitis, toxoplasmosis, leukoencephalomalacia and other diseases, cannot be completely ignored in this regard as found by Keane and Little (1987) and Peixoto *et al*. (2000).

Two suspected cases of rabies, one of cow and other of mule were received to rule out the cause of death. Change in the behavior and paralysis of hind legs and tail followed by death were peculiar signs of rabies in mouse inoculation test (MIT). MIT was preferred over other tests like fluorescent antibody staining technique (FAT) as it had been previously ruled out that FAT-negative samples might be MIT-positive as the strains might have the extended incubation period which could be maintained well in MIT as observed by Webster *et al*. (1976).

RT-PCR was conducted for the proper confirmation in which 443-bp product of highly conserved N-region gene was obtained by both samples confirming the rabies in both cow and mule samples under consideration. Numan *et al*. (2011) also performed RT-PCR in mule samples suspected for rabies gaining a final product of 443-bp.

Conclusion

Rabies is a highly zoonotic disease which can spread by the biting of the infected animals. Regarding its control, free range domestic animals can be protected through the adaptation of proper vaccination schedules while its control in wild animals is quite difficult. Secondly, in spite the ancient history of this infection, unavailability of proper screening facilities does not give any proper data regarding prevalence of this disease in Asia Pacific region. This case report uses two methods for confirmation which can prove effective in diagnosis of rabies due to less chances of getting false negative results so that early treatment may be started to get recovered from the disease.

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Statement of conflict of interest

It is declared that there is no conflict of interests regarding the publication of this article.

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