



## Surveillance for Probable Detection of Rabies Virus in Wild and Domestic Animals

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### Abstract

Rabies (lyssavirus) virus is an avertable viral disease caused by the rabid animals to the warm blooded animals. Presence and prevalence of rabies virus threatens population hygiene since spread cases often knock out in villages and cause substantial losses to human populations. Lyssavirus surveillance in wild animals including bats, dogs, cattle and mules was performed. Twenty Brain samples from the suspected wild animals were processed by means of the fluorescent antibody test (FAT) and mouse inoculation test (MIT). The brain samples were retested through reverse transcriptase polymerase chain reaction (RT-PCR). In the study, suspected brain samples of the dogs, cattle and mules were found positive while all the insectivorous bats representing *Taphozous nudiventris*, *Scotophilus heathii*, *Scotoecus pellidus*, *Pipistrellus pipistrellus* and *Scotophilus kohlil* species were tested negative by all the three assays.

**Keywords:** Lyssavirus, Surveillance, Bats, FAT, MIT, RT-PCR.

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### Introduction

Rabies is an ancient zoonotic disease of mammals, caused by an enveloped, negative sense RNA virus, a member of the Lyssavirus genus within Rhabdoviridae family [22]. Rabies virus infects domestic and wild animals and saliva of the carrier animal contains this virus (dogs, cats, skunks, foxes, raccoons, coyote, bats, groundhogs, farm livestock including cows, sheep, goats, horses and pigs etc). The virus spreads by bite of infected animals. Humans are recognized as dead end host for rabies virus [4].

Rabies is the 11<sup>th</sup> most common cause of death in the reportable infectious diseases [18]. It kills large number of animals and humans in many countries every year [3]. The disease remains a significant public health concern due to its persistence in a variety of terrestrial and aerial wildlife hosts [11]. Many diagnostic techniques have been developed for the identification of rabies virus. FAT for detection of rabies antigen is a sensitive method for diagnosing the disease in animals and humans [23]. The sensitivity and specificity of FAT have been compared with those of histologic examination and MIT [20]. Compared with FAT and MIT, RT-PCR to identify the RNA of rabies virus is more accurate for the diagnosis [8]. Bats are considered as the natural hosts. However, dogs are the main source of infection in humans. It is estimated that 55,000 deaths per year worldwide are due to rabies

infection with about 56% of which occur in Asia and 44% in Africa [9].

Pakistan is also facing a threatening situation regarding rabies and it is a non reportable disease in Pakistan and its incidence is grossly under reported. So it is needed to develop an effective surveillance network to assess the magnitude of the disease in Pakistan [1]. Pakistan is a rabies endemic country and 50,000 cases of the dog bite have been estimated per year in some government hospitals treating as many as 150 cases per day. Although, no accurate figures are available probably 5000 persons die from rabies each year [15]. Only in Karachi, the estimated population having rabies is 9 per million. Every day, there are about 25-30 new cases of dog-bites treated by the doctors at civil hospital Karachi, one of the biggest public hospitals in city [17].

Among the rabies cases in animals and human beings, the exact source often could not be ascertained. Unfortunately, as for the bat as a source for transmission of rabies among the animals, no research work has been conducted in Pakistan. There is need of the time to rule out bats as a source of rabies transmission. Keeping in view the above facts the present study has been conducted for probable surveillance to identify the rabies virus in wild animals as an emerging infectious disease of human and animals and to know the current status of rabies virus circulating in the bat populations in Pakistan.

## Materials and Methods

A total of 20 suspected samples (five for each) origination from brain tissues were collected from bats, dogs, cattle and mules. All the samples were transferred to sterile pre-labeled tubes containing 1.5 mL of glycerol buffer (1:1 aqueous sol of glycerol; pH 8.2) and transported on ice pack to the University Diagnostic Laboratory (UDL), University of Veterinary & Animal Sciences (UVAS), Lahore, Pakistan, for further processing. All the collected samples were subjected to MIT, FAT and RT-PCR for confirmation of Rabies virus. MIT was performed as recommended [10]. Briefly, 20% homogenate of brain samples was prepared by grinding the brain tissue in glass tissue grinder (SGA, 527, USA) in a sterile phosphate buffer saline (PBS, pH 7.4) containing 2mg streptomycin and 500 IU penicillin. The suspension was centrifuged at refrigeration temperature (4°C; at 200 xg) for 5 minutes and the supernatant was collected in aliquots. Albino Swiss mouse with age of between 20-25 days (weighting 12-15 gms), free from any infection were procured from Veterinary Research Institute (VRI), Ghazi Road, Lahore, Pakistan. MIT was performed at Veterinary Research Institute (VRI), Lahore, Pakistan. Briefly, 0.03 mL of each inoculum was injected between eye and ear over the eye orbit at symphysis of frontal temporal and occipital bone (0.1 - 0.2 cm deep) to each mouse using one ml tuberculin syringe (26 gauge and 1.5 cm long needle) and placed in a clean pre-labeled mice cage. All mice were observed for four weeks with daily observation for the first five days, and then twice daily from day six to 28. The findings were recorded on a clinical score sheet that included ruffled fur (score 1), slow/circular movements (score 2), trembling, shaking movements or lameness (score 3), paralysis / convulsions (score 4), prostration / permanently recumbent (score 5). If there was no sign of disease, a score of '0' was given. Mice that died within first five days after inoculation were considered as non-specific deaths, and not due to rabies infection. However, there brain samples were later on confirmed negative through indirect fluorescent antibody technique.

During 5-28 days post-inoculation, brain samples from all inoculated mice were removed. These samples were tested for presence of any intra-cytoplasmic inclusion bodies through indirect FAT. If no mice succumbed to rabies infection after 28 days, the sample was deemed to be negative. After completion of 28 days, the mice were killed by using chloroform for collection of brain. All the brain tissues samples of bats, cow, dog and mules along with known positive and negative tissues were tested through FAT as recommended by [6]. The

brain tissue was transferred to a sterile Petri dish and a thin section of the brain was cut out on to a clean poly-L lysine coated glass slide and pressed slightly against the cut surface of the section to make an impression smear for FAT. A test impression was recorded as positive when brilliant apple-green fluorescence areas were observed against dark background, whereas, no such color or fluorescence was taken as negative.

RT-PCR was used as gold standard for confirmation rabies virus and to validate the results of MIT and FAT. Total RNA from brain tissues of bats, cow, dog and mules, inoculated mice along with known positive and negative tissues were extracted using TRIZOL method as recommended by [5], and using KIT method (High Pure RNA extraction, Roche) as recommended by [6]. The cDNA was synthesized by using Revert Aid first Strand Synthesis Kit (Fermentas, EU) followed by RT-PCR as recommended by [19]. Briefly 5µl, of each template DNA was mixed to reaction mix containing 5µl of PCR buffer (10x), 1µl of dNTPs (10 mM), 3µl of MgCl<sub>2</sub>, 34µl of DD.H<sub>2</sub>O and 1µl of each of forward and reverse primers (**Table 1**). After preparing the reaction mix, PCR reaction was completed by 45 cycles after initial denature 94°C for 4 min followed by denaturation 94°C for 45 sec, annealing at 45C 90 sec and, extension at 72°C for 2 min. The RT-PCR products were examined by gel electrophoresis as recommended by [5].

**Table 1: Nucleotide sequence of primers used for PCR**

Primer	Viral locus	Nucleotide Sequence	length
P-F	G-L	GACTTGGGTCTCCCTAACTGGGG	879 bps
P-R		CAAAGGAGAGTTGAGATTGTAGTC	

## Results

Identification of rabies viral antigen by FAT was successfully evaluated in 15 brain samples out of 20 samples tested. In addition, clinical signs characteristic of rabies like ruffled fur, slow / circular movements, trembling, lameness, paralysis/convulsions, prostration, permanent recumbency, lack of appetite and death at terminal stage on 5<sup>th</sup>, 6<sup>th</sup>, 7<sup>th</sup>, and 8<sup>th</sup> day of inoculation were observed in the mice when these samples were applied to MIT (**Figure 1**). The results of MIT were confirmed again by FAT, indicating that all the 15 samples were positive for rabies by either FAT or MIT. The results obtained from RT-PCR with the G/L (G and L intergenic region of the virus genome) primer set correlated completely with those from either FAT or MIT, where all 15 samples yielded a single band of the expected size (879bp) for the G-L

intergenic region targeting with these primers (Figure 2).

While none of the sample from bat species (*Taphozous nudiventris*, *Scotophilus heathii*, *Scotoecus pellidus*, *Pipistrellus pipistrellus* and *Scotophilus kohli*), was found positive for rabies virus through FAT, MIT and RT-PCR.

Figure 1 (A & B): Mice inoculation test (Mice showing rabies signs)

A



B



Figure 2: Results for RT-PCR assay

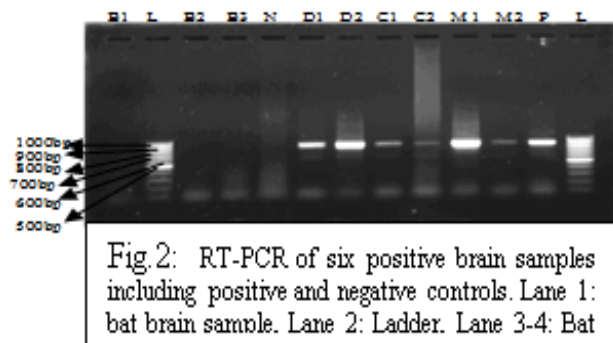


Fig. 2: RT-PCR of six positive brain samples including positive and negative controls. Lane 1: bat brain sample. Lane 2: Ladder. Lane 3-4: Bat

## Discussion

Out of 20 brain samples, used in the present study, a total of 15 samples yielded positive results in the FAT, MIT and RT-PCR for the rabies diagnosis. While the 5 brain samples collected from insectivorous bat failed to yield the positive results under the same conditions.

In another study conducted by Shabbir et al, rabies virus in mule brain tissue samples was also diagnosed

by FAT, MIT and RT-PCR and positive results were found. These findings are strongly comparable with our results [28]. In another study, the G-L intergenic region was amplified and they also obtained band size of 879bp was [20,29]. FAT, MIT and RT-PCR were also used by other researchers to detect rabies virus antigen from bat [13,24,26].

None of the samples originated from *Taphozous nudiventris*, *Scotophilus heathii*, *Scotoecus pellidus*, *Pipistrellus pipistrellus*, and *Scotophilus kohli* species of bats were tested positive. Our results are in concordance with the findings of Kuzmin et al, who declared same species of bats free from rabies virus using FAT and MIT [12]. In another study brain tissues of bats were also found negative for rabies virus [27, 14]. However some researchers got positive results in bats [2,27,18,17].

According to a study by the National Rabies Control Program, Pakistan, Punjab, Sindh and Khyber Pakhtunkhwa as well as few districts i.e. Naseerabad, Jafferabad, and Pishin in Baluchistan are categorized as the high risk areas for rabies [17]. In this study, due to lack of proper sampling facilities, only insectivorous bats were possible to test, where as frugivorous bats that have been reported to play role in dissemination of rabies virus remained untested. Therefore testing of frugivorous bats may lead to otherwise results. It is pertinent to mention here that according to our information, this is first time study of this type in Pakistan that depicted the status of rabies virus in bats.

## Conclusion

Rabies is a viral disease that can be spread by domestic and wild animals. The complete surveillance work is required to be done in future studies in the remaining potential areas of Punjab province including its northern and southern regions where the dogs, cats, wolves, jackals, foxes, mongoose and bats thrive more. On similar analogy bats populations in other provinces of Pakistan may be screened for rabies virus. A comprehensive surveillance network and public awareness campaign are needed.

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