Journal of Infection and Molecular Biology 2 (3): 39 – 42
http://dx.doi.org/10.14737/jimb.2307–5465/2.3.39.42

Short Communication

Surveillance of Insectivorous Bat Population for Rabies Virus in Pakistan

Farzana Shaheen[1], Masood Rabbani[4], Zafar ul Ahsan Qureshi[2], Mahmood ul Hassan[3], Arfan Ahmad[4], Fareeha Akhtar[4], Suliman Qadir Afridi[1], Hassaan bin Aslam[5], Mehboob Akbar[6], Tasra Bibi[1], Nadia Mukhtar[1*]

[1]Quality Operations Laboratory, University of Veterinary and Animal Sciences, Lahore, Pakistan; [2]Veterinary Research Institute, Lahore Cantt, Pakistan; [3]University of Agriculture Faisalabad, Pakistan; [4]University Diagnostic Laboratory, University of Veterinary and Animal Sciences, Lahore, Pakistan; [5]Department of Microbiology, University of Veterinary and Animal Sciences, Lahore, Pakistan; [6]Poultry Production Rajanpur, Livestock and Dairy Development Department, Punjab
*Corresponding author: nadiamukhtar84@yahoo.com

ARTICLE HISTORY

Received: 2014–03–29
Revised: 2014–05–12
Accepted: 2014–05–14

Key Words: Lyssavirus, Surveillance, Bats, iFAT, MIT, RT–PCR

ABSTRACT

Rabies is an ancient zoonotic disease of mammals caused by a RNA virus within *Rhabdoviridae* family. Rabies virus surveillance in bats was performed in Pakistan during 2009 and 2010 through indirect fluorescence test (iFAT), Mice inoculation test (MIT) and reverse transcriptase–polymerase chain reaction (RT-PCR) techniques. One hundred oropharyngeal swabs and brain tissues of different bat species i.e; *Taphozous nudiventris*, *Scotophilus heathii*, *Scotoecus pellidus*, *Pipistrellus pipistrellus and Scotophilus kohlil* were collected from five areas named Lahore, Pattoki, Sheikhupura, Bahawalpur and Bahawalnagar of Punjab province, Pakistan. All the techniques were validated by suspected brain samples of dog, cow, mule, and known positive control. In the study, all insectivorous bats species were found negative to rabies virus.

ARTICLE CITATION: Shaheen F, Rabbani M, Qureshi ZA, Hassan M, Ahmad A, Akhtar F, Afridi SQ, Aslam H, Akbar M, Bibi T, Mukhtar N (2014). Surveillance of insectivorous bat population for rabies virus in Pakistan. J. Inf. Mol. Biol. 2 (3): 39 – 42.

Rabies is an ancient zoonotic disease of mammals, caused by an envelope, negative sense RNA virus– a member of the Lyssavirus genus within Rhabdoviridae family (Warrell and Warrell, 2004). Rabies virus infects domestic and wild animals and is present in the saliva of the carrier animal (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 "dead–end" hosts, meaning that there is no subsequent human–to–human transmission (Stephen G. Baum, 2008).

Rabies 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 (Anonymous, 2004). Bats belonging to order Chiroptera have about 1100 species (Harris et al., 2006). On the basis of food habits, bats are divided into two groups fruit eaters (frugivorous) and insect eater (insectivorous). It is reported that bats are also natural reservoirs of the emerging and re–emerging infectious diseases (Omatsu et al., 2007). Bats are responsible for the aerial cycle of rabies virus which is transmitted among vampire, insectivorous, and frugivorous bats and from bats to wild animals. Humans can be accidentally infected by the bite of any of the above cycles (Barbosa et al., 2008). Bats are equally susceptible to rabies as carnivores. Basic aspects of rabies pathogenesis to and from the CNS of bat are the same as for the other animals. Physiologically active, minimum and maximum incubation periods of rabies in the Chiroptera are believed to be not different than those observed in the Carnivora. Both paralytic and furious rabies have been found in bats. With the exception of Antarctica, the distribution of rabies virus in bats is global in nature. Bats are the most prominent direct source of human rabies in parts of North America, Western Europe and Australia, especially where the disease in carnivores has been controlled (Niezgoda et al., 2002). Fifty species of bats representing 23 genera and 8 families are reported to exist in Pakistan. (Horacek et al., 2000)

Among the rabies cases in animals and human beings, the exact source often could not be ascertained. In Pakistan no research has been taken whether the bat is carrier of rabies virus or not. Positive cases of rabies due to bat bite have been found in other countries (Favi et al., 2002). There is the need of the time to rule out bats as a source of rabies transmission. The present study has been conducted know the current status of Rabies virus circulating in the bat populations in Pakistan.

A total of 100 samples of oropharyngeal swabs (n = 50) and brain tissues (n = 50) were collected from bats. Both 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 Lab, University of Veterinary and Animal Sciences, Lahore for further processing. Beside the bats samples; 6 preserved brain samples (2 each of dog, cow and mule from Veterinary research Institute, Lahore (VRI) and one known positive (Rabid dog brain from VRI) and negative (Chicken brain) were also included in the study.

The MIT was performed as recommended by Koprowski et al., (1996). Briefly the oropharyngeal swabs

were thoroughly squeezed in the microfuge tube containing glycerol buffer. The suspension was semi–purified using refrigerated centrifuge machine (1000 x g for 30 min at 4°C) to remove the debris. The supernatant was harvested and filtered through syringe filter of 0.2µm porosity (Sartorius, USA) and collected in aliquots for MIT. For brain samples, 20 per cent homogenate 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/mL. 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 about 20–25 days old (weighing 12–15gms) free from any possible infection were procured from Veterinary Research Institute (VRI), Ghazi Road, Lahore. MIT was performed at Veterinary Research Institute (VRI), Lahore, briefly; 0.03 mL of each inoculums 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.

During 5 – 28 days post–inoculation, brain samples from all inoculated mice were removed that were at a clinical score of 2–3. 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 samples including oropharyngeal swabs and brain tissues of bats, cow, dog and mules, inoculated mice along with known positive and negative tissues were tested through iFAT as recommended by Kang et al. (2007). 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. Smears of swab samples were also prepared on slides. 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.

Total RNA from oropharyngeal swabs and 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 David et al., (2002). cDNA was synthesized by using Revert Aid first Strand Synthesis Kit (Fermentas, EU) followed by RT–PCR as recommended by David et al., (2002). Briefly 5µL, of each template DNA was mixed to reaction mix containing 5µL of PCR buffer (10x), 1µL of dNTPs (10 mM), 3µl of $MgCl_2$, 34µL of Double Distilled H_20 and 1µL of each of forward and reverse primers. After preparing the reaction mix, PCR reaction was completed by 45 cycles after initial denature 94°C for 4 minute followed by denaturation 94°C for 45 second, annealing at 45°C 90 second and, extension at 72 °C for 2 minute. The RT–PCR products were examined by gel electrophoresis as recommended by David et al. (2002).

Figure 1: Captured bat in mist net used in this study

None of the bat species (*Taphozous nudiventris*, *Scotophilus heathii*, *Scotoecus pellidus*, *Pipistrellus pipistrellus* and *Scotophilus kohlil*), were tested positive for rabies virus through mouse inoculation test (MIT), indirect fluorescent antibody technique (iFAT) and reverse transcriptase polymerase chain reaction (RT–PCR). However, mice inoculated with positive control tissue and brain tissues originated from rabies suspected dogs, cows, and mules showed typical signs of rabies like ruffled fur, slow / circular movements, trembling, lameness, paralysis, convulsions, prostration, permanent recumbence, lack of appetite and death at terminal stage on 5th, 6th, 7th, and 8th day of inoculation. While negative control group of mice remained healthy and active till end of the fourth weeks. The results of MIT were also validated by iFAT and RT–PCR as no immune–fluorescence was observed under UV light of fluorescent microscope in slides prepared from bat samples and negative control, whereas, the positive control and suspected dog, cow and mule samples showed prominent focal areas of apple green color fluorescence. Through RT–PCR, the samples tested positive by MIT and IFAT remained positive. Specific bands of 879bp were obtained by the amplification of intergenic region using specific set of primers.

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 (Salahuddin, 2001). Bats are the important reservoirs for the Lyssavirus. According to the recent observations and the virus variants associated with Chiroptera bat order may occasionally spill over into other mammals (WHO, 2005). Bats have been associated with a number of emerging zoonotic viral diseases. The significance of bats as reservoirs of such emerging infectious diseases (EIDs) has been increasingly appreciated (Calicher Ch et al., 2006).

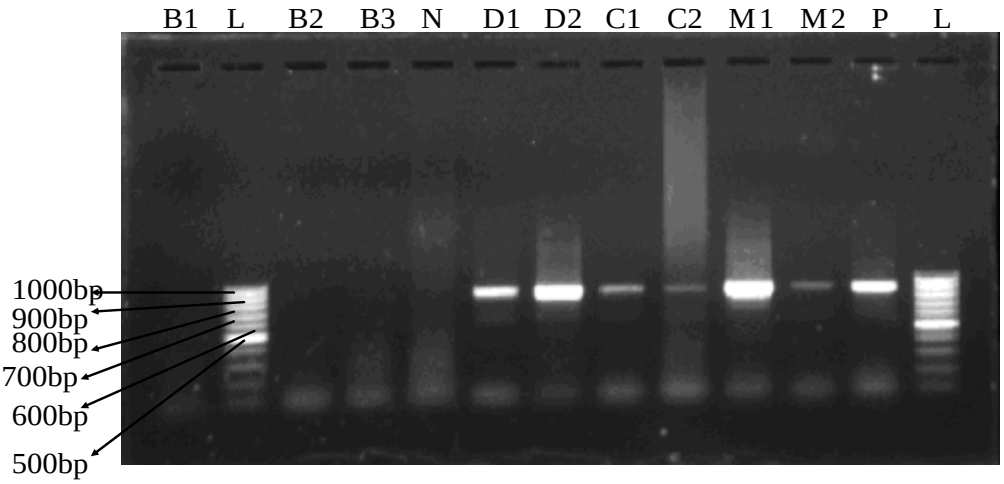

Figure 2: RT–PCR of six positive brain samples including positive and negative controls; Lane 1: bat brain sample; Lane 2: Ladder; Lane 3–4: Bat brain sample: Lane 5: negative control; Lane 6–7: dog brain sample brain; Lane 8–9: cow brain sample; Lane 10–11: mule brain sample; Lane 12 positive control; Lane 13: Ladder, indicating 900bp of conserved "pseudogene" region

Indirect fluorescent antibody technique (iFAT), mouse inoculation test (MIT) and reverse transcriptase polymerase chain reaction (RT–PCR) have already been established to detect rabies virus antigen from bat (Whitfield et al., 2001; Woldehiwet, 2005; McElhinney et al., 2008) All the three techniques applied in this study were validated using known positive, negative controls as well as 6 rabies suspected samples. All suspected samples along with known positive control were tested positive by all the three assays. After validation of techniques, bats samples were processed to detect rabies virus. None of the samples originated from *Taphozous nudiventris*, *Scotophilus heathii*, *Scotoecus pellidus*, *Pipistrellus pipistrellus, and Scotophilus kohli* species of bats was tested positive by all three techniques. This result is in concordance with the findings of Kuzmin et al. (2006) who declared same species of bats free from rabies virus using FAT and MIT. In another study, brain tissues of *Mormoops megalophylla*, *Myotis californicus*, *M. ciliolabrum*, *M. thysanodes*, *M. velifer*, *M. yumanensis*, *Eptesicus fuscus*, *Pipistrellus hesperus*, *P. subflavus*, *Plecotus townsendii*, *Antrozous pallidus and Tadarida brasiliensis* species of bats were also found negative for rabies virus in USA (Megalid et al., 2010). However some researchers got positive results in bats (Gordon et al., 2001; Arai et al., 2003; Scheffer et al., 2007) that might be due to testing of small sample size.

In this study, only insectivorous bats were tested, whereas frugivorous bats that have been reported to play role in dissemination of rabies virus remained were untested. 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

Though none of the bats studied found to carry the rabies virus, a comprehensive surveillance is required to be done in future studies particularly northern and southern regions where the dogs, cats, wolves, jackals, foxes, mongoose and bats can thrive more. On similar analogy bats populations in other provinces of Pakistan may be screened for rabies virus.

REFERENCES

Anonymous (2004). Effective surveillance of rabies imperative the humanitarian news and analysis service of the UN office for the coordination of humanitarian affairs. IRIN (News Report) Islamabad, Pakistan. Nov. 22 (http://www.irinnews.org/report).

Arai TY, Kuzmin IV, Kameoka Y, Botvinkin AD (2003). New Lyssavirus genotype from the lesser mouse–eared bat (*Myotis blythi*) in Kyrghyzstan. Emerg. Infec. Dis. 9: 333 – 337.

Baum SG (2008). Zoonosis with friends like this, who needs enemies. Trans Am. Clin. Climatol. Assoc. 119: 39 – 52.

Barbosa TFS, Daniele BAM, Elizabeth STR, Livia MNC, Rita M, Armando SP, Antonio CRV, Marcelo V, Alberto LB, Reynaldo JSL, Pedro FCV, Marcio RTN (2008). Molecular epidemiology of rabies virus isolated from different sources during a bat-transmitted human outbreak occurring in Augusto Correa municipality, Brazilian Amazon. Virol. 370 (2), 228 – 236.

Calisher CH, Childs JE, Field HE, Holmes KV, Schountz T (2006). Bats: Important reservoir hosts of emerging viruses. Clin Microbiol Rev. 19: 531 – 545.

David D, Yakobson B, Rotenberg D, Dveres N, Davidson I, Stram Y (2002). Rabies virus detection by RT–PCR in decomposed naturally infected brains. Vet. Microbiol. 87(2): 111 – 118

Favi M, Mattos CC, Yung V, Chala E, Lopez LR, Mattos CA (2002). First Case of Human Rabies in Chile Caused by an Insectivorous Bat Virus Variant. Emerg. Infec. Dis. 8 (1):79 – 81

Gordon SLL, Smith JS (2001). Survey of bat populations from Mexico and Paraguay for rabies. J. Wildlife Dis. 37(3): 582 – 593.

Harris SL, Brookes SM, Jonesa G, Hutson AM, Racey PA, Aegerter J, Smith GC, McElhinney LM, Fooks AR (2006). European bat Lyssaviruses: distribution, prevalence and implications for conservation. Biol Cons. 13: 93 – 210.

Horacek I, Hanak V, Gaisler J (2000). Bats of the Palearctic Region: A Taxonomic and Biogeographic Review. Proceedings of the VIIIth EBRS, 1: 11 – 157.

Kang B, Ohb J, Leea CS, Park BK, Park YN, Hong KS, Lee KG, Cho BK, Songa DS (2007). Evaluation of a rapid immunodiagnostic test kit for rabies virus. J. Virological Meth. 145: 30 – 36

Koprowski H (1996). The Mouse Inoculation Test. *In:* Laboratory Techniques in Rabies by F.X. Meslin., M.M. Kaplan and H. Koprowski. 4th ed .World Health Organization (WHO). Geneva.80–86.

Kuzmin VI, Niezgoda M, Carroll DS, Keeler N, Hossain MJ, Breiman RF, Ksiazek TG, Rupprecht CE (2006). Lyssavirus surveillance in bats in Bangladesh. Emerg Infec Dis, 12(3): 486 – 488.

McElhinney LM, Fooks AR, Radford AD (2008). Diagnostic tools for the detection of rabies virus. Zoonosis. 18(3): 224 – 229.

Megalid A, Yannic G, Zahno ML, Brgger D, Bertoni G, Christe P, Zanoni R (2010). Surveillance for European bat lyssavirus in Swiss bats. Archiv. Virol. (10):1655 – 1662.

Niezgoda M, Hanlon CA, Rupprecht CE (2002). Animal Rabies: *In.* Rabies by A.C. Jackson and W.H. Wunner. ed: San Diego. Calif., Academic Press, Elsevier Science. 199 – 200.

Omatsu T, Watanabe S, Akashi H, Yoshikawa Y (2007). Biological characters of bats in relation to natural reservoir of emerging viruses. Comp. Immunol. Microbiol. Infec. Dis. 30: 357 – 374.

Salahuddin N (2001). In Pakistan sheep brain vaccine or nothing a moral dilemma. In: Rabies control in Asia by B. Dudet and F.X. Meslin. John Libbey Eurotext, Paris.66.

Stephen G. Baum (2008). Zoonoses-with friends like this, who needs enemies? Trans Am Clin Climatol Assoc. 119: 39–52.

Scheffer CK, Carrieri ML, Albas A, Santos HCPD, Kotait I, Ito FH (2007). Rabies virus in naturally infected bats in the State of Sao Paulo, Southeastern Brazil. Rev Saude Publica, 41(3): 1 – 8.

Warrell MJ, Warrell DA (2004). Rabies and other lyssavirus diseases. Seminar. Lancet. 363: 959 – 969.

Whitfield GS, Fekadu M, Shaddock JH, Niezgoda M, Warner CK, Messenger SL (2001). A comparative study of the fluorescent antibody test for rabies diagnosis in fresh and formalin–fixed brain tissue specimens. J. Virol. Methods. 95: 145 – 151.

WHO Expert Committee on Rabies. First Report. Geneva World Health Organization (2005). (WHO) Technical Report Series, No. 931.

Woldehiwet Z (2005). Clinical laboratory advances in the detection of rabies virus. Clin. Chimica. Acta. 35: 49 – 63.