Asian Journal of Pharmaceutical and Health Sciences
www.ajphs.com

Ante-mortem detection of rabies from saliva, skin and hair follicles

A. Kaw[1], C. K. Singh[1]*, Ramneek[2], N. K. Sood[1], B. S. Sandhu[1], D. Deka[2], S. Awahan[1]

Department of Veterinary Pathology[1] and Department of Animal Biotechnology[2], GADVASU, Ludhiana-141004, India

ARTICLE HISTORY

Received: 30.08.2012

Accepted: 03.10.2012

Available online: 10.11.2012

Keywords:

Hair follicles, Rabies, saliva, skin

***Corresponding author:**

Email : rabiesck@gmail.com
Tel : +91-9888466676

ABSTRACT

Rabies is an infectious zoonotic disease, which is fatal once symptoms appear. Thus, early detection of this dreaded disease is of paramount importance. So, the present study was undertaken to apply two molecular approaches viz. nested RT-PCR and SYBR Green real time PCR for ante-mortem detection of rabies from non neural, non-invasive samples like saliva, skin and hair follicles. A total of 12 rabies suspected live animals were presented over a time of eight months to Veterinary Clinics, GADVASU, Ludhiana India. Out of 12 cases, rabies viral RNA could be detected in 36.3% (4/11), 33.3% (4/12) and 25% (3/12) skin biopsy, hair follicle and saliva samples respectively. By real time PCR, viral RNA could be detected in 45.5% (5/11) skin biopsy samples, 41.6% (5/12) hair follicle samples and 41.6% (5/12) saliva samples. The results were in full concordance with the immunofluorescence results of brain tissues of these animals after death. Therefore, sensitivity of 57.1%, 50% and 37.5% was obtained with nested RT-PCR on skin biopsy, hair follicles and saliva samples respectively. A sensitivity of 71.4%, 62.5% and 62.5% was observed with real time PCR on skin biopsy, hair follicles and saliva samples respectively. Thus, real time PCR is more sensitive than nested RT-PCR for ante-mortem detection of rabies.

INTRODUCTION

Rabies is a viral, fatal, zoonotic disease which is worldwide in distribution, with the exception of a few areas and countries that have been free from it primarily due to their geographical isolation. The annual number of deaths worldwide caused by rabies is estimated to be 55,000 and about 10 million people receive post-exposure treatments each year after being exposed to rabies-suspect animals [1]. It is caused by single stranded negative sense RNA virus belonging to the genus *Lyssa virus* of the family *Rhabdoviridae* [2]. The appearance of overt disease is usually preceded by a prodromal period in which there are a number of non-specific symptoms of malaise [3]. It is invariably fatal, once symptoms appear. Rabies is transmitted from animal to animal or animal to humans through bites scratches on skin or licks on the mucosal surface [4]. The absence of definite cure and certainty of death makes this disease more dreadful than any other known disease. Therefore, early detection of this disease is needed, to decrease the number of humans and animals which are at risk due to exposure to the infected animal. The rapid identification of suspect rabies infection is essential to allow specific control strategies [5].

Fluorescent antibody technique is one of the most accurate and reliable laboratory test available for diagnosis of rabies. But application of this approach is possible only post mortem i.e. after death; however, with the advent of molecular approaches, it is now possible to detect rabies ante-mortem i.e. before death with the reverse transcription-polymerase chain reaction (RT-PCR). PCR has become a valuable tool being faster than MIT and more sensitive and specific than the FAT. It can also be used for rapid epidemiological analysis [5]. Since rabies virus is secreted and excreted in all secretions and excretions of rabid animals, the molecular approaches can be employed for reliable intravitam diagnosis. Ante-mortem detection of rabies by molecular techniques based on detecting virus or viral RNA has been reported in body fluids of live animals such as saliva [6] and CSF [7]. The rabies virus is also present in nerve cells surrounding the base of hair follicles [8]. The ante-mortem diagnosis of rabies can be established from skin samples collected from living animals [9]. Therefore, the present study was envisaged with the objectives of applying nested RT-PCR and SYBR Green real time PCR for ante-mortem detection of rabies on samples like saliva, skin biopsy and hair follicles.

MATERIAL AND METHOD

Clinical samples

The samples were collected from the 12 rabies suspected animals presented to the Clinics, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, India. Most of these animals were presented at the clinics 3-4 days after onset of clinical symptoms. Soon after the clinical diagnosis was made, the saliva samples were collected directly or with sterile syringe. The skin samples were collected with 3mm skin biopsy punch from nape of neck and hair follicles were extracted from area around lips and muzzle. 15-20 hair follicles were extracted approximately. All the samples were stored at -20ºC until further processing. All the samples were also obtained from 2 healthy animals, as negative controls for the molecular assays. Lyophilized anti-rabies vaccine was used as positive control.

Extraction of RNA and cDNA synthesis

Total RNA in the saliva, skin and hair follicle samples, positive and negative controls was extracted using Qiazol (Qiagen, USA) according to the manufacturer's instructions. The RNA was subjected to cDNA synthesis using a primer RabN1 (Table 1) and subjected to 65ºC for 10 min, followed by 37 ºC for 15 min, chilled on ice and briefly spun down. Reverse transcriptase (Qiagen, USA) mix was prepared and subjected to conditions 37˚C for 2 h, 95˚C for 5 min and chilling on ice for 5 min in a thermal cycler (Eppendorf). This cDNA was used for amplification in both the nested and the real time PCR assays.

Nested PCR assay

The procedure used for the nested RT-PCR was that used earlier [10,11] with minor modifications. Briefly, 10 µl of cDNA was subjected to a first round amplification using RabN1 and RabN5 primers (30 pmol/µL) (Table 1), dNTP's and Taq polymerase for 95˚C for 2 min followed by 35 cycles of 95˚C for 1 min, 55˚C for 1 min, 72˚C for 1 min 30 s and a final extension step at 72˚C for 5 min. For the second round, 5 µL of first round PCR product was used and subjected to initial denaturation at 95ºC for 2 mins, followed by 35 cycles of 95 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min and a final extension step at 72 °C for 5 min.

SYBR Green real time PCR assay

The real time assay was carried out in 25 µL PCR mixture volume consisting of 12.5 µL of SYBR Green master mix (Qiagen, USA) with 1 µL of primers O1 and R6 (3 pmol/ µl) (Table 1) and 5 µL of the cDNA prepared using RabN1 primer. Amplification was carried out at 55˚C for 2 min, 95˚C for 10 min, followed by 40 cycles in two steps: 95˚C for 15 s, 60˚C for 1 min. Amplification, data acquisition and analysis were carried out by using ABI 7500 instrument and ABI prism SDS software which determines the cycle threshold (*C*t) that represents the number of cycles in which the fluorescence intensity is significantly above the background fluorescence.

RESULTS AND DISCUSSION

Clinical details of animals

Amongst the 12 animals that were included in this study 4 were buffaloes, 4 cows and 4 dogs. All cases presented happened to be female animals. Mean average age of cattle was 2.7 years (range 7 months 3 years) and that of dogs was 5.87 months (range 2½ months 10 months).

Nested RT-PCR

Amplification with primers RabN1 and RabN5 yielded a 1477 bp first round product while amplification with RabNF and RabNR yielded a 762 bp second round product. Out of 12 cases, rabies viral RNA could be detected in 36.3% (4/11), 33.3% (4/12) and 25% (3/12) skin biopsy, hair follicle and saliva samples with a sensitivity of 57.1%, 50% and 37.5% respectively.

SYBR Green real time PCR

For amplification in real time PCR oligonucleotides O1 and R6 (Table 1) were used and the PCR product was 135 bp in length. The cycle threshold (*C*t) of the positive control was at the 26th cycle while most of the clinical samples had *C*t values ranging from 26 to 29 cycles. In order to determine whether they were signals obtained from genuine PCR products or spurious signals, melting curve analysis was used. Sharp peak was noted at 78˚C for the positive control as well as all samples that were positive on or before the 29th cycle. The samples that yielded a positive result beyond the 35th cycle showed diffuse shallow peaks obtained over 7075˚C temperature range, represent primer dimer. By real time PCR, viral RNA could be detected in 45.5% (5/11) skin biopsy samples, 41.6% (5/12) hair follicle samples and 41.6% (5/12) saliva samples and a sensitivity of 71.4%, 62.5% and 62.5% was obtained with real time PCR on skin biopsy, hair follicles and saliva samples respectively. These results were in concordance

Table 1: Details of primers used in the study

Primer name	Nucleotide sequence 5'-3'	Position	Sense
RabN1	GCTCTAGAACACCTCTACAATGGATGCCGACAA	59–84	+
RabN5	GGATTGAC(AG)AAGATCTTGCTCAT	1514–1536	-
RabNF	TTGT(AG)GA(TC)CAATATGAGTACAA	135–156	+
RabNR	CCGGCTCAAACATTCTTCTTA	876–896	-
O1	CTACAATGGATGCCGAC	66–82	+
R6	CCTAGAGTTATACAGGGCT	201–183	-

with the immunofluorescence assay on brain tissue obtained after death of these animals.

Most of the conventional techniques used for postmortem analysis of the brain are of limited value to support the intravitam diagnosis of rabies [12-14]. Conventional diagnostic methods such as the FAT cannot be applied with clinical samples such as skin and hair follicle for laboratory confirmation. RT-PCR has been used successfully for detecting the viral nucleic acid of rabies virus in ante mortem specimens. Molecular detection by RT-PCR technique has the highest sensitivity, but it requires standardization and very stringent quality control in order to avoid false results. Saliva and neck skin biopsy samples are the most widely studied specimens for attempting antemortem diagnosis [6, 15].

The aim of the present study was to establish a rapid and sensitive molecular diagnostic method for ante mortem diagnosis of rabies. Accordingly, we evaluated two molecular techniques nested RT-PCR and SYBR Green Real time PCR, for the detection of rabies viral RNA in animal saliva samples, skin biopsy and extracted hair follicles. To avoid major mismatches due to rabies virus genetic diversity, we designed oligonucleotides (Table 1) that recognize specific and highly conserved sequences on the N protein [11]. None of the samples from healthy controls were positive in either the nested or the real time PCR indicating that the primers were specific to rabies virus.

We were able to detect rabies virus RNA in 5/11skin samples that we tested. Conventional RT-PCR has been reported to be a reliable test for ante mortem diagnosis in two other separate case reports by other workers [15, 16]. They observed that detection of rabies specific antigen in skin biopsies from nape of the neck and hand generated a positive result with RT-PCR. The probability of successful antemortem detection of rabies virus using neck skin samples is high. Skin samples from 51 patients were tested by heminested RT-PCR and presence of rabies virus nucleic acid in 43 cases was confirmed with a sensitivity >98% and specificity as 100%. RT-PCR assay was recommended along with direct immunofluorescence test on skin biopsy specimens as a simple testing protocol for intravitam diagnosis of rabies. Conventional RT-PCR has been reported to be a reliable test for ante mortem diagnosis in two other separate case reports. They observed that detection of rabies specific antigen in skin biopsies from nape of the neck and hand generated a positive result with RT-PCR. The probability of successful antemortem detection of rabies virus using neck skin samples is high [17].

A very less number of workers have worked on diagnosis of rabies from hair follicles in human beings and there is no such report in animals till now. The presence of rabies RNA was demonstrated in the ends of the hair follicles in human patients [18]. Fifty hair samples were extracted from one patient instead of excising skin with hair follicles from the nape of the neck, obtained 4 days after onset of symptoms. A sensitivity of 50% was observed for hair follicles obtained from 26 patients during a study conducted on 56 patients from 1998-2009 [19]. Nucleic acid sequence-based amplification for detection of rabies virus RNA in extracted hair follicles in 21 of 23 confirmed rabies patients on the first day of hospitalization [20].

Saliva samples from 28 patients by conventional RT-PCR confirmed the presence of rabies virus nucleic acid in five cases [6]. Higher sensitivity (75%) was obtained by using SYBR Green real time PCR than conventional RT-PCR (37%) for ante mortem diagnosis of rabies using saliva samples [11]. 13 of the 15 dogs (87%) had saliva samples that were positive for rabies RNA [7]. Second best results were obtained after skin biopsy, by saliva samples and observed sensitivity of 63.2% in group 1 and 70.2% in group 2. These sensitivities are in the upper limit of the range (10%- 70%) [9].

Rabies virus RNA was detected in 5/12 saliva samples that we tested. In the present study, most of the samples were collected 3-4 days after manifestation of clinical symptoms. Perhaps the diagnosis could have been confirmed by nested RT-PCR in the remaining animals had a second and third sample been collected later in the course of the disease and tested with RT-PCR. The presence of virus in saliva is the most important factor for transmission of disease. Rabies virus may be excreted in the saliva of dog before clinical signs appear and may lead to infection of an unexpecting and untreated bite victim [21]. The particular time of salivary virus excretion before sickness is crucial. Typically, rabid dogs shed virus concomitant with illness or a few days before [22]. In naturally infected dogs the rate at which virus is present in the salivary glands ranges from 75-100% [16].

CONCLUSION

Real time PCR is the most sensitive molecular approach for ante-mortem detection of rabies. The molecular detection of rabies in skin is the most sensitive ante-mortem diagnostic approach. Molecular detection in hair follicles is the most feasible approach for mass epidemiological survey of rabies.

ACKNOWLEDGMENTS

Authors are grateful to Dr. A S Nanda, Director of Research, Guru Angad Dev Veterinary and Animal Science University for providing the necessary facilities. The work was conducted in the Government of Punjab sponsored research scheme entitled “Development of Research-cum-Diagnostic laboratory for Rabies”.

The study relates to the naturally prevalent cases and, thus, does not include any experiments.

REFERENCES

1. World Health Organization WHO Expert Consultation on Rabies, First report: WHO technical report series. Geneva, 2004. No. 931
2. Tordo Poch N O, Ermine A, Keith G, Rougeon F. Walking along the rabies genome: is the large G-L intergenic region a remnant gene. Proceedings National Acad. Sci. USA. 1986:83:3914-3918.
3. Woldehiwet Z. Clinical laboratory advances in the detection of rabies virus. Clinica Chimica Acta. 2005: 351(1-2):49-63.
4. de Mattos C A, de Mattos C C, Rupprecht C E. Rhabdoviruses. Fields Virology. Lippincott Williams & Wilkins: Philadelphia; 2001, p.1245 1277
5. Heaton P R, McElhinney L M, Bowen-Davies J H A, Lowings J P. Detection and identification of Rabies and Rabies related viruses using Rapid-Cycle PCR. J. Virol. Meth. 1999: 81(1-2):63-69.
6. Crepin P, Audry L, Rotivel Y, Gacoin A, Caroff C, Bourhy H. Intravitam diagnosis of human rabies by PCR using saliva and cerebrospinal fluid. J. Clin. Microbiol. 1998:36: 11171121.

7. Saengseesom W, Mitmoonpitak C, Kasempimolporn S, Sitprija V. Real - time PCR analysis of dog cerebrospinal fluid and saliva samples for ante-mortem diagnosis of rabies. Southeast Asian J. Trop. Med. Pub. Heal. 2007: 38(1):53-57.

8. Madhusudana S N, Sukumaran S M. Antemortem diagnosis and prevention of human rabies. Ann. Indian Acad. Neurol. 2008:11(1):312.

9. Dacheux L, Reynes J M, Buchy P, Sivuth O, Diop B M, Rousset D, Rathat C, Jolly N, Dufourcq J B, Nareth C, Diop S, Iehle C, Rajerison R, Sadorge C, Bourhy H. A reliable diagnosis of human rabies based on analysis of skin biopsy specimens. Clin. Infect. Dis. 2008: 47:1410 1417.

10. Nadin-Davis SA. Polymerase chain reaction protocols for rabies virus discrimination. J. Virol. Meth. 1998:75:1-8.

11. Nagaraj T, Joel P V, Desai A, Kamat A, Madhusudana S N, Ravi V. Ante mortem diagnosis of human rabies using saliva samples: Comparison of real time and conventional RT-PCR techniques. J. Clin. Virol. 2006:36:1723.

12. Bourhy H, Sureau P. Laboratory methods for rabies diagnosis. Institut Pasteur: Paris; 1991.

13. Hemachudha T, Phanuphak P, Sriwanthana B, Manutsathit S, Phanthumchinda K, Siriprasomsup W, Ukachoke C, Rasameechan S, Kaoroptham S. Immunologic study of human encephalitic and paralytic rabies. Am. J. Med. 1988: 84:673677.

14. Warrel M J, Warrel DA. Rhabdovirus infections of humans. Chapman & Hall Medical: London; 1995. p. 343383

15. Smith J, McElhinney L, Parsons G, Brink N, Doherty T, Agranoff D, Miranda M E, Fooks A R. Case report: rapid ante mortem diagnosis of a human case of rabies imported into the UK from the Philippines. J. Med. Virol. 2003:69:150 155.

16. Fooks A R, McElhinney L M, Pounder D J, Finnegan C J, Mansfield K, Johnson N, Brookes S M, Parsons G, White K, McIntyre P G, Nathwani D. Case report: isolation of a European bat Lyssavirus type 2a from a fatal human case of rabies encephalitis. J. Med. Virol. 2003:71:281-289.

17. Macedo C I, Carnieli J P, Brandao P E. Diagnosis of human rabies cases by polymerase chain reaction of neck-skin samples. Brazilian J. Infect. Dis. 2006:10(5):341-345.

18. Hemachudha T, Wacharapluesadee S. All antemortem diagnosis of human rabies. Clin Infect Dis. 2004: 39:10851086.

19. Wacharapluesadee S, Hemachudha T. Ante- and post-mortem diagnosis of rabies using nucleic acid-amplification tests. Expert Rev. Mol. Diag. 2010:10(2):1-12.

20. Wacharapluesadee S, Hemachudha T. Nucleic-acid sequence based amplification in the rapid diagnosis of rabies. Lancet. 2001:358:892893.

21. Fekadu M, Shaddock JH, Bear GM. Excretion of rabies virus in the saliva of dogs. J. Infect. Dis. 1982:145:715-719.

22. Fekadu M, Shaddock JH. Peripheral distribution of virus in dogs inoculated with two strains of rabies virus. Am. J. Vet. Res. 1984:45:724-729.