Title: COMPARISON of SYBR GREEN REAL TIME PCR WITHNESTED RT-PCR FOR DETECTION OF RABIES FROM HAIR FOLLICLES

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Keywords Ante-mortem, Hair Follicles, Nested RT-PCR, Rabies, SYBR Green real time PCR

Abstract The aim of the present study was to establish a rapid and sensitive molecular diagnostic method for ante-mortem diagnosis of rabies from hair follicles. To compare the sensitivity of SYBR Green real time PCR with Nested RT-PCR applies on hair follicles for ante-mortem diagnosis of rabies

Original Article

Comparison of SYBR Green Real Time PCR with Nested RT-PCR for Detection of Rabies from Hair Follicles

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ABSTRACT

Objectives:

1. The aim of the present study was to establish a rapid and sensitive molecular diagnostic method for ante mortem diagnosis of rabies from hair follicles.
2. To compare the sensitivity of SYBR Green real time PCR with Nested RT-PCR applied on hair follicles for ante mortem diagnosis of rabies.

Period of Study: Dec. 2009 – July 2010

Material and Methods: Molecular techniques viz. Nested RT-PCR and SYBR Green real time PCR techniques were applied on hair follicle samples collected from 12 rabies suspected animals. Sensitivity of both the techniques was compared in accordance with WHO recommended gold standard test viz. Fluorescent Antibody Technique (FAT) applied on brain samples.

Results: Nested and SYBR Green real time PCR applied on hair follicles had successfully confirmed rabies in 4 and 5 cases, respectively whereas FAT confirmed 8 true positive cases. Sensitivity of 50% was obtained with nested RT-PCR on hair follicle samples while SYBR Green real time PCR revealed sensitivity of 62.5%.

Interpretation & Conclusion: It was concluded that SYBR Green real time PCR is a useful, specific, sensitive and better molecular approach for earlier diagnosis of rabies from saliva of rabid suspected cases.

Keywords: Ante-mortem, Hair Follicles, Nested RT-PCR, Rabies, SYBR Green real time PCR.

INTRODUCTION

Rabies is an infectious, fatal disease characterized by profound dysfunction of the central nervous system, caused by single stranded negative sense RNA virus belonging to the genus *Lyssa virus* of the family *Rhabdoviridae*[1]. An estimated 55,000 people, mostly (88%) in Asian countries and particularly in the Indian subcontinent, die of rabies each year[2]. Although the loss of livestock due to rabies is significant, there are few publications on estimates of the incidence of rabies in livestock[3]. Rabies is transmitted from animal to animal or animal to humans through bites scratches on skin or licks on the mucosal surface[4]. It is invariably fatal, once symptoms appear. The absence of definite cure and certainty of death makes this disease more dreadful than any other known disease. Therefore, early detection of this dreaded disease is of great significance.

FAT is one of the most accurate and reliable laboratory tests available for rabies diagnosis. 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]. They are widely used as confirmatory methods in many microbiological and virological laboratories including those for rabies diagnosis as well[6,7,8,9]. SYBR Green real-time PCR has also been developed[10,11] to increase sensitivity and to obtain results even faster. The real time PCR is especially useful for that purpose as it does not require visualization of the amplification product in agarose gel and the results are observed

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during the run of the test. This makes the method more rapid than the nested RT-PCR.

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[12] and CSF[11]. The rabies virus is also present in nerve cells surrounding the base of hair follicles[13]. So in the present study, ante-mortem diagnosis of rabies was done by molecular approaches like nested RT-PCR and SYBR Green real time PCR on less invasive tissue like hair follicles.

OBJECTIVES

1. The aim of the present study was to establish a rapid and sensitive molecular diagnostic method for ante mortem diagnosis of rabies from hair follicles.
2. To compare the sensitivity of SYBR Green real time PCR with Nested RT-PCR applied on hair follicles for ante mortem diagnosis of rabies.

MATERIAL & METHODS

Clinical specimens: The hair follicles were extracted from the area around lips and muzzle from 12 rabies suspected animals and stored at -20˚C. Minimum of 20 hairs were extracted. Care was taken while extraction of hairs as virus resides in the small sensory nerves adjacent to the hair follicles. Lyophilized anti-rabies vaccine was used as positive control. Hair follicle samples from healthy animals were used as negative controls along with normal mouse brain.

RNA extraction and cDNA synthesis: Total RNA in the specimens, 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). The cDNA was used for amplification in both the nested and the real time PCR assays in this study. Considering that the N gene is the most conserved in the *Lyssa viruses* and that the sequence data concerning this gene are the most exhaustive, primers in the N gene were used, that were shown to allow amplification of a wide range of genetically diverse lyssa viruses[10].

Table 1
Details of primer's used for nested RT-PCR

Primer Name	Sequence	Gene	Position	Sense
Rab N1	5' GCTCTAG AAC ACC TCT ACA ATG GAT GCC GAC AA 3'	N	59-84	+
Rab N5	5' GGA TTG AC(AG) AAG ATC TTG CTC AT 3'	P	1514-1536	-
Rab Nfor	5' TTG T(AG)G A(TC)CA ATA TGA GTA CAA 3'	N	135-156	+
Rab Nrev	5' CTG GCT CAA ACA TTC TTC TTA 3'	N	876-896	-

Nested RT-PCR assay: The procedure used for the nested PCR was that used earlier by[10,14] 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. The nested PCR product had a size of 762 bp.

Table 2
Details of primer's used for SYBR Green real time PCR

Primer Name	Sequence	Gene	Position	Sense
01	CTACAATGGATGCCGAC	N	66-82	+
R6	CCTAGAGTTATACAGGGCT	N	201-183	-

SYBR Green real time PCR assay: A real time SYBR Green PCR 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 2) 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.

Sensitivity comparison of molecular techniques with FAT: Since, FAT is recommended worldwide as a standard technique for diagnosis of rabies on neural tissue, after death of animal by World Health Organization. So, Nested RT-PCR and SYBR Green real time PCR techniques employed on saliva samples was compared with FAT for detecting the sensitivity of these molecular techniques.

Table 3
Nested RT-PCR and SYBR Green real time PCR for ante mortem diagnosis of rabies from hair follicle samples

Sample No.	Species	Age	Sex	Nested RT-PCR	SYBR Green Real time PCR	FAT (Brain)
1	Buffalo	7 Yrs F	M	-	+	+
2	Buffalo	3 Yrs F	M	-	-	-
77443	Buffalo	2½ Mths F	M	+	+	+
4	Cow	5 Yrs F	F	+	+	+
5	Cow	7 Mths F	F	-	-	+
6	Dog	4 Mths F	F	-	-	-
7	Dog	2½ Mths F	F	-	-	-
8	Cow	1 Yr F	M	+	+	+
9	Cow	2½ Yrs F	M	-	+	+
10.	Dog	7 Mths F	F	-	-	-
11.	Dog	2½ Mths F	F	-	-	+
12.	Buffalo	2 Yrs F		+	-	+
		% Positive		33.3%	41.6%	66.6%

Table 4
Sensitivity comparison of Nested RT-PCR with FAT.

Test	FAT on brain smears (Positive)	FAT on brain smears (Positive)	Total
Nested RT-PCR (Positive)	4	0	4
Nested RT-PCR (Negative)	4	4	8
Total	**8**	**4**	**12**

Sensitivity = True Positive / True Positive + False Negative × 100 = 4/ 4 + 4 = 50%

The sensitivity of various tests applied was calculated using formulae:

Table 5
Sensitivity comparison of SYBR Green real time PCR with FAT.

Test	FAT on brain smears (Positive)	FAT on brain smears (Positive)	Total
SYBR Green real time PCR (Positive)	5	0	5
SYBR Green real time PCR (Negative)	3	4	7
Total	**8**	**4**	**12**

Sensitivity = True Positive / True Positive + False Negative × 100 = 5/5 + 3 = 62.5%

$$\text{Sensitivity} = \frac{\text{True positive}}{\text{True positive} + \text{False negative}} \times 100$$

RESULTS

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. Amongst the 12 hair follicle samples, 4 (33.3%) samples (Table 3) were positive with a sensitivity of 50% (Table 4). These results were confirmed by comparing with the results of immunofluorescence and RT-PCR on brain tissue samples obtained after death of animals.

SYBR Green real time PCR: For amplification in real time PCR oligonucleotides O1 and R6 (Table 2) were used and the PCR product was 135 bp in length. A typical amplification plot and melting curve analysis for the determination of the specificity were used. 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 30 cycles. Melting curve analysis was used to know whether the signals were genuine or spurious. Sharp peak was noted at 77-78°C for the positive control as well as all samples that were positive on or before the 30th cycle. On the contrary, samples that yielded a positive result beyond the 35th cycle showed diffuse shallow peaks obtained over 70–75°C temperature range represent primer dimmer. With this technique, 5 out of 12 hair follicle samples (41.6%) (Table 3) were positive with a sensitivity of 62.5% (Table 5).

DISCUSSION

Conventional techniques used for postmortem diagnosis of rabies are of limited value to support

the ante mortem diagnosis of the disease[15]. FAT cannot be applied with clinical samples such as 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[12]. Saliva and neck skin biopsy samples are the most widely studied specimens for attempting ante mortem diagnosis[12].

The aim of the present study was to establish a rapid and sensitive molecular diagnostic method for ante mortem diagnosis of rabies. Accordingly, two molecular techniques nested RT-PCR and SYBR Green Real time PCR, for the detection of rabies viral RNA in hair follicles in animals was used. To avoid major mismatches due to rabies virus genetic diversity, oligonucleotides that recognize specific and highly conserved sequences on the N protein were designed. 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.

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. Study by T. Hemachudha et al.[16], were able to demonstrate the presence of rabies RNA in the ends of the hair follicles. 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. Another study observed a sensitivity of 50% for hair follicles obtained from 26 patients during a study conducted on 56 patients from 1998-2009[17]. They also used 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.

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 hair follicle samples been available for testing. Conventional RT-PCR has been reported to be a reliable test for ante mortem diagnosis in two other separate case reports[18]. 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.

CONCLUSION

Rabies is an infectious, fatal disease and early detection of this dreaded disease is of paramount importance. Till now a reliable diagnosis is possible only after the death of animal. Therefore, the present study was envisaged with the objectives to use the molecular approaches like nested RT-PCR and SYBR Green real time PCR for early diagnosis of rabies in suspected animals using less invasive non neural tissues like hair follicles. 12 rabies suspected animals were presented to the Veterinary Clinics, GADVASU, Ludhiana, India. The sensitivity of nested RT-PCR for hair follicles obtained was 50%. A sensitivity of 62.5% was observed with real time PCR on hair follicles. So the molecular approaches can be used for the early and accurate diagnosis of rabies in animals. This is the first report using hair follicles for ante-mortem diagnosis of rabies in animals in India.

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

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Announcement

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