Title: INTRAVITAM DIAGNOSIS OF RABIES FROM URINE BY NESTED RT-PCR

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Keywords Immunofluroescence Intravitam Diagnosis, Nested RT-PCR, rabies urine

Abstract To standardize molecular technique eg. Nested RT-PCR for diagnosis of rabies viral RNA from urine samples from animal suspected rabies. To analyse the sensitivity of Nested RT-PCRr for intravitam diagnosis in comparison with gold standard Immunofluroescence applied on neural tissue.

Original Article

Intravitam diagnosis of rabies from urine by Nested RT-PCR

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Abstract

Objectives: 1. To standardize molecular technique e.g. Nested RT-PCR for diagnosis of rabies viral RNA from urine samples from animal suspected for rabies.

2. To analyze the sensitivity of Nested RT-PCR for intravitam diagnosis in comparison with gold standard Immunofluorescence applied on neural tissue.

Period of Study: Dec 2010 – May 2012

Material and Methods: Nested RT-PCR technique was applied on 21 urine samples collected from rabies suspected animals. First round amplification with nested set of primers (RabN1 and RabN5) yielded 1477 bp product while amplification with second round primers (RabNfor and RabNrev) yielded 762 bp product. Sensitivity of the technique was compared in accordance with WHO recommended gold standard test viz. Immunofluorescence (FAT) applied on brain samples.

Results: By employing nested RT-PCR, viral RNA could be detected in 6/21 (28.57%) urine samples. Confirmatory diagnosis by immunofluorescence of brain samples revealed 15 true positive cases. Sensitivity of 62.5% was recorded by application of Nested RT-PCR on urine samples in comparison to immunofluorescence performed on brain samples.

Interpretation & Conclusion: It was concluded that Nested RT-PCR applied on urine samples of animals suspected for rabies can be helpful for early diagnosis of rabies.

Keywords: Immunofluorescence Intravitam, Diagnosis, Nested RT-PCR, Rabies, Urine

Introduction

Rabies is a disease caused by RNA virus, belonging to the genus *Lyssavirus* of the family Rhabdoviridae, which can affect almost all mammals, including humans. The clinical diagnosis of rabies is sometimes suggested by epidemiological (history of exposure) and clinical (e.g., paralysis, hydrophobia) findings[1]. However, the disease is often mistaken for other disorders[2]. Differentiation from other neurologic diseases may require extensive investigations[3]. Therefore, diagnosis is often confirmed late in the course of the disease or postmortem. Most PEP is rendered without knowledge as to whether the animal was indeed rabid, this lays unnecessary financial burden to render post exposure prophylaxis.

The standard diagnostic test consists of direct fluorescent antibody test (FAT) on impression smears from fresh brain samples[4]. This test remains the corner stone of postmortem rabies diagnosis. However, the FAT requires expensive reagents and instruments, well-trained technicians and necropsy material. The sensitivity of the test is substantially reduced once brain specimens start to decompose. This can pose a problem in tropical countries like India, where transportation of specimens to a regional diagnostic laboratory often entails delay.

Outward spread of rabies virus from CNS leads to infection of almost all organs[5]. This has allowed intravitam diagnosis of rabies on the basis of biopsies of the nape of the neck[6], saliva[7], CSF[8] and urine[9]. It was reported that Rabies virus was present within tubular cells of the kidneys and mice inoculated with urine from a rabid fox died of rabies[10]. The polymerase chain reaction (PCR) technique is a rapid, sensitive and specific method that can be used as an alternative tool for early diagnosis of rabies, as this approach is efficient even with highly, degraded samples[11].

Objectives

1. To standardize molecular technique viz. Nested RT-PCR for diagnosis of rabies viral RNA from urine samples collected from animal suspected for rabies.

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

Primer Name	Sequence	Gene	Positions	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 CAAACATTCTTCTTA 3'	N	876-896	-

2. To compare the sensitivity of Nested RT-PCR applied for Intravitam diagnosis with conventional technique (Immunofluorescence) applied on neural tissue.

Material & Methods

Source of urine samples: Urine samples were collected from 21 animals (14 buffaloes, 4 cows and 3 dogs) suspected for rabies presented to the Veterinary Clinics, GADVASU, Ludhiana, Punjab and Civil Veterinary Hospital from different districts of Punjab. Soon after the clinical diagnosis was made, urine samples were collected either in a sterilized vial directly while animal was urinating or by urethral catheterization. Urine samples were also obtained from two healthy animals, as negative controls for the molecular assays. Rabies positive brain homogenate was used as positive control.

RNA extraction and cDNA synthesis: Total RNA in urine 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 65°C for 10 min, chilled on ice and briefly spun down. Reverse transcriptase (Applied Biosystems, USA) mix was prepared and subjected to conditions 25°C for 10 min, 37°C for 2 h, 85°C for 5 min and chilling on ice for 5 min in a thermal cycler (Eppendorf).

Analysis of nucleic acid: RNA and cDNA concentration was measured using Nano Drop Spectrophotometer (Nanodrop Technologies, CA) in ng/μl and quality was checked as a ratio of OD 260/280. This cDNA was used for amplification in the nested RT-PCR assay.

Primers : Primers usedwere based on N gene because it is the most conserved in the *Lyssaviruses* and the sequence data concerning this gene are the most exhaustive that were shown to allow amplification of a wide range of genetically diverse Lyssaviruses. Nested set of primers used in the present study was that used earlier[12, 13, 14] (Table I). RabN1 and RabN5 primers are used for first round amplification while RabNfor and RabNrev primers are used for second round amplification.

Nested RT-PCR : The procedure used for the nested RT-PCR was that used earlier[12, 13, 14] with minor modifications. Briefly, 12 μl of cDNA was subjected to a first round amplification using RabN1 and RabN5 primers (30 pmol/μl), dNTP's and Taq DNA 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 amplified with RabNfor and RabNrev (Table I) and subjected to initial denaturation at 95°C 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 amplified PCR products were loaded on agarose gels along with positive control, negative control and DNA ladder (100 base pair plus, Fermentas). The agarose gels were visualized under Geldoc (Bio-Rad) and photographed with the same software.

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[12], so, Nested RT-PCR PCR technique employed on urine samples was compared with FAT for detecting the sensitivity of the molecular technique.

Statistical analysis: The sensitivity of Nested RT-PCR applied on urine sample was calculated using formula as under:

Table II
Nested RT-PCR for ante mortem diagnosis of rabies from urine samples of animals

S. No.	Species	Age	Sex	Nested RT-PCR (Urine)
1.	Buffalo	6 years	F	-
2.	Cow calf	6 months	F	+
3.	Buffalo	5½ years	F	+
4.	Dog	2 years	M	-
5.	Buffalo	6 years	F	-
6.	Buffalo	5 years	F	-
7.	Cow calf	10 months	F	-
8.	Cow Calf	1½ years	F	+
9.	Buffalo	3½ years	F	+
10.	Buffalo	5 years	F	+
11.	Cow	2½ years	F	-
12.	Dog	4 years	F	-
13.	Dog	10 months	M	-
14.	Buffalo	6 years	F	-
15.	Buffalo	7 years	F	-
16.	Buffalo	5 ½ years	F	-
17.	Buffalo	7 years	F	-
18.	Buffalo	6 years	F	-
19.	Buffalo	4 ½ years	F	+
20.	Buffalo	5 years	F	-
21.	Buffalo	6 years	F	-
	Total			**6/21**

*+ ve – positive, -ve negative, ND- not detected

Results

Analysis of nucleic acid: The concentration and purity of RNA was accessed before cDNA synthesis. Similarly, quality of cDNA was accessed before application of Nested RT-PCR Technique.

Nested RT-PCR: Amplification with primers Rab N1 and Rab N5 yielded 1477bp first round product. Nested pair of primers (Rab Nfor and Rab Nrev) used for amplification in second round yielded 762 bp product. By nested RT-PCR, viral RNA could be diagnosed in 6/21 saliva samples (Table II).

Sensitivity comparison of molecular techniques with FAT: The nested RT-PCR applied on 21 urine samples was able to diagnose rabies in 6 urine samples. Postmortem brain tissue examination by FAT revealed rabies in 15 out of 21 (71.42%) cases. Application of Nested RT-PCR on urine samples for viral nucleic acid yielded a sensitivity of 62.5%.

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

Test	FAT on brain smears (Positive)	FAT on brain smears (Negative)	Total
Nested RT-PCR on urine (Positive)	6	0	6
Nested RT-PCR on urine (Negative)	9	6	15
Total	**15**	**6**	**21**

Sensitivity = True Positive / True Positive + False Negative × 100 = 8/ 8 + 4 = 66.67%

Discussion

Diagnosis is an inevitable part of rabies prevention and control. Rapid and reliable diagnosis renders a valuable help in post exposure immunization decision making.

Nested RT PCR

The biological fluids viz. saliva, urine and cerebrospinal fluid (CSF) render limited significance for diagnosis of rabies by fluorescent antibody test, thus, nucleic acid amplification tests for such biological fluids becomes the method of choice[12, 13]. So far, conventional RT-PCR has been reported to be a useful test for ante mortem diagnosis. RT-PCR also offer additional benefit of epidemiological classification of the virus using the amplified products[15].

As speed of obtaining results in diagnosis of rabies is of utmost importance it was pointed out[16] that RT-PCR requires more time (18 hours) as compared to that of FAT on brain samples. However, in case of urine samples the time of detection of rabies was lesser with molecular approach as compared to that of immunofluorescence. Results of intra-vitam detection of rabies from urine correlate well with those of the post-mortem fluorescent antibody test performed on brain specimens of same animals.

Conclusion

The current study was an innovative approach where ante mortem diagnosis in animals has been successfully attempted from urine samples of animals suspected to be rabid. It can be concluded that Nested RT-PCR is a useful, specific, and sensitive molecular approach for ante-mortem detection from urine samples of animals clinically suspected to be rabid.

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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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