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Short Communication

Intravitam Diagnosis of Rabies From Saliva by Nested RT-PCR and Real Time PCR

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Abstract

Nested reverse transcription (RT)-PCR and SYBR Green real time PCR protocols for the intravitam detection of rabies virus genomic RNA were tested with clinical samples for the first time from rabies suspected animals (n=12). With SYBR Green real time PCR, five saliva samples were detected as positive, hence confirming that, for the sake of ante-mortem detection of rabies in saliva of animals, the sensitivity of real time PCR is more than that of RT-PCR as well as immunofluorescence that could detect rabies in three saliva samples each.

Key Words: Nested RT-PCR, rabies, real time PCR, saliva, SYBR Green.

Introduction

Rabies is a viral, zoonotic and fatal disease, which causes encephalomyelitis in humans and animals. 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 (28). The disease is caused by a RNA virus that belongs to the Lyssavirus genus of Rhabdoviridae family (19). All mammals are susceptible to the rabies virus, mainly the Carnivora and Chiroptera orders (2). The clinical diagnosis of rabies is sometimes suggested by epidemiological (history of exposure) and clinical findings (12). However, the disease is often mistaken for other disorders (23). Differentiation from other neurologic diseases may require extensive investigations. Therefore, diagnosis is often confirmed late in the course of the disease or postmortem (10). Delay in diagnosis significantly increases the number of exposures by rabid animal thus significantly augmenting cost of post-exposure prophylaxis.

The presence of virus in saliva is the most important factor for transmission of disease. Rabies virus may be excreted in the saliva of dogs before clinical signs appear and may lead to infection of an unexpecting and untreated bite victim (9). The particular time of salivary virus excretion before sickness is, therefore, crucial. Typically, rabid dogs shed virus concomitant with illness or a few days before (8). In naturally infected dogs the rate at which virus is present in the salivary glands ranges from 75-100% (11). Sensitivity up to 70.2% has also been reported (7) in skin biopsies.

The molecular techniques, mainly Polymerase Chain Reaction (PCR) are useful tools in rabies diagnosis (3, 14, 20, 21). The aim of this study is to evaluate nested RT-PCR and SYBR Green real time PCR techniques for the detection of rabies virus from saliva samples in comparison with post mortem results of brain obtained with the use of the standard rabies diagnostic procedure.

Material and methods

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

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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).

Source of saliva specimens

The saliva samples were collected from ten 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 and were stored at −20˚C until further processing. Saliva samples were also obtained from two healthy animals, as negative controls for the molecular assays. Lyophilized anti-rabies vaccine was used as positive control.

RNA extraction and cDNA synthesis

Total RNA in the saliva 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 (stored at -80˚C) was used for amplification in both the nested and the real time PCR assays.

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 (Ct) that represents the number of cycles in which the fluorescence intensity is significantly above the background fluorescence. For amplification in real time PCR oligonucleotides O1 and R6 (Table 1) were used and the PCR product was 135 bp in length.

Results and Discussion

Clinical features of 12 animals suspected with rabies are presented in Table 2.

We used primers in the N gene because it is the most conserved in the Lyssavirus (except some domains of the L protein gene) and the sequence data concerning this gene are the most exhaustive that were shown to allow amplification of a wide range of genetically diverse Lyssa viruses (18). Amongst the 12 saliva samples, 3 (25%) samples were positive.
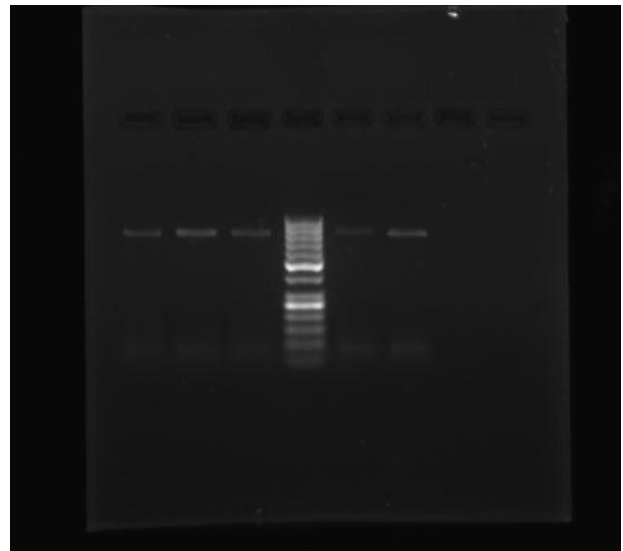

Figure 1. Nested RT-PCR amplified product of rabies virus from test and control samples Lane 1: Saliva Sample; Lane 2: Saliva Sample; Lane 3: Positive brain Sample; Lane 4: 50bp DNA Ladder, Molecular weight marker; Lane 5: Saliva Sample; Lane 6: Positive control; Lane 7: Negative control.

A typical amplification plot and melting curve analysis for the determination of the specificity is presented in Fig. 2a and 2b respectively. The cycle threshold (Ct) of the positive control was at the 26th cycle while most of the clinical samples had Ct values ranging from 26 to 30 cycles (Fig. 2a). 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 77- 78˚C for the positive control as well as all samples that were positive on or before the 30th cycle (Fig. 2b). The samples that yielded a positive result beyond the 35th cycle showed diffuse shallow peaks obtained over 70–75˚C temperature range, represent primer dimer.

The nested RT-PCR analysis showed that, amongst the 12 samples tested, 3 samples (25%) were positive yielding a sensitivity of 37.5%. Amongst 12 samples, 5 samples (41.6%) were positive by SYBR Green real time PCR yielding a sensitivity of 62.5%. Overall, in the present study real time PCR was more sensitive than nested RT-PCR for detecting rabies virus RNA in saliva samples. We also used immunofluorescence test for the detection of rabies from salivary impression smears. Out

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Table 1. Primer names, nucleotide sequences, positions and sense

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	-

Table 2. Clinical features of 12 animals suspected with rabies.

Clinical feature	Proportion of patients (out of 12)	(%)
Fever	3	25%
Hypersalivation	9	75%
Difficult intake	5	41.6%
Behavioral change	7	58.3%
Off feed	11	91.6%
Micturition	4	33.3%
Paralysis	5	41.6%
Recognized owner	5	41.6%
Vaccination status- nil	11	91.6%
Vaccination status- Proper	1	8.3%

Figure 2. A. Cycle threshold of positive control and positive samples lies between 26-30th cycles B. Dissociation curve showing two peaks of positive samples at 78 ℃ and one peak of positive sample at 77 ℃.

of 12 samples tested, 3 were positive by FAT, though upon post-mortem brain tissue examination, rabies could be detected in 8 out of 12 (75%) suspected cases by immunofluorescence.

Thus, immunofluorescence continues to be the technique of choice for post-mortem rabies diagnosis. Likewise, saliva samples were tested from 28 patients by conventional RT-PCR (6) and confirmed the presence of rabies virus nucleic acid in five cases. Higher sensitivity (62.5%) was obtained by using SYBR Green real time PCR than conventional RT-PCR (37%) for ante-mortem diagnosis of rabies using saliva samples (18).

However, most of the conventional techniques used for post-mortem analysis of the brain are of limited value to support the intravitam diagnosis of rabies (5, 13, 27). The corneal smear examination first developed with mice (22), employed for ante-mortem examination is too insensitive for accurate clinical diagnosis (6, 1, 4, 16, 26). The only test that has demonstrated reliable results is the immunofluorescence (IF) test on skin biopsy samples (27).

In the present study, most of the saliva 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 5 animals had a second and third sample of saliva been collected later in the course of the disease and tested with RT-PCR. So far, conventional RT-PCR has been reported to be a reliable test for ante-mortem diagnosis (23, 11) where it was observed that in comparison to isolation of virus from saliva by rapid tissue culture infective test (RTCIT) and mouse inoculation test (MIT) as well as detection of rabies specific antigen in skin biopsies from nape of the neck, RT-PCR yielded a better result. In a study of urine samples of 4 patients (24), 3 were positive. A 75.8% positivity was obtained (25) for saliva samples tested from 62 patients. RT-PCR on saliva for viral nucleic acid yielded a sensitivity of 50-70% and a specificity of 100% (15). However, in the present study we found that the sensitivity of SYBR Green real time PCR is even better than RT-PCR.

It can be concluded that SYBR Green real time PCR is a useful, specific, and sensitive and better molecular approach for ante-mortem rabies detection from saliva samples of rabies suspected animals. Results correlate well with those of the post-mortem fluorescent antibody test performed on brain specimens of same animals after death. Salivary secretion of the virus is
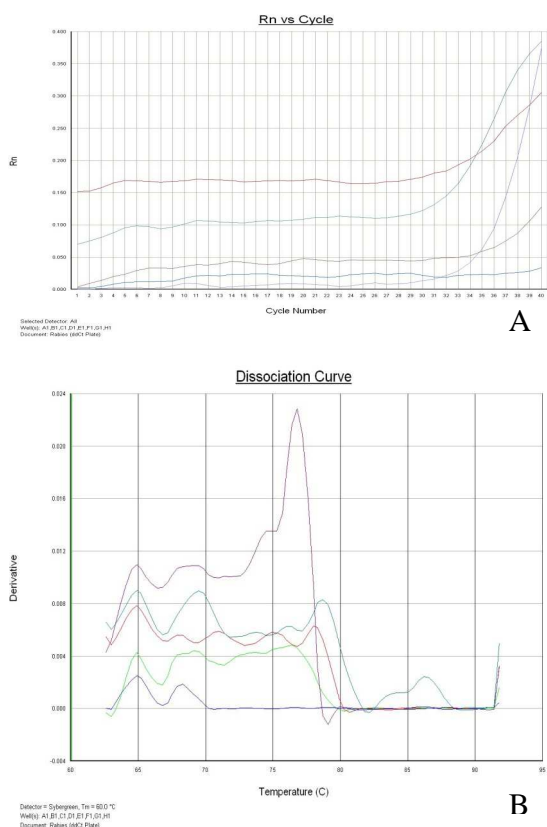
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intermittent and therefore the testing of at least three samples taken at different time intervals is necessary as also reported (9).

Acknowledgements

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