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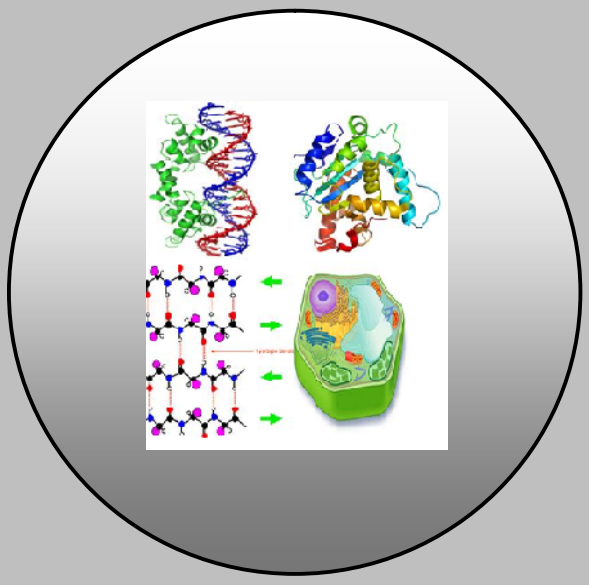

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RESEARCH PAPER

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Antemortem Diagnosis of Rabies from Saliva

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ABSTRACT

In the present study molecular techniques viz. nested RT-PCR technique was applied on 11 saliva samples collected from rabies suspected animals. Sensitivity of nested RT-PCR was compared in accordance with WHO recommended gold standard test viz. Fluorescent Antibody Technique (FAT) applied on brain samples. Nested RT-PCR had successfully diagnosed rabies viral RNA in 4 saliva samples out. Sensitivity of 66.67% was obtained when compared with fluorescent antibody test. The present study concluded that Nested RT-PCR is a useful, specific, sensitive and better molecular approach for earlier diagnosis of rabies from saliva of rabid suspected cases.
Keywords: Antemortem, Fluorescent Antibody Test, Nested RT-PCR, Rabies, Saliva

INTRODUCTION

Rabies is an acute *Lyssavirus* disease causes invariably fatal encephalomyelitis. In India rabies is enzootic and is a serious public health and economic problem (Nagarajan *et al.* 2006). A national multicentric rabies survey conducted by APCRI in India in collaboration with WHO revealed an incidence of 20,565 human deaths per year due to rabies in India (Sudarshan *et al.* 2007). The clinical diagnosis of rabies is sometimes suggested by epidemiological (history of exposure) and clinical findings (Hemachudha, 1994). However, making a reliable diagnosis of rabies based on clinical presentation can be difficult to distinguish from encephalitic condition causes by other viral infections (Emmons, 1979). Therefore, diagnosis is often confirmed late in the course of the disease or postmortem (Fishbein *et al.* 1991). With the advent of molecular approaches, it is now possible to detect rabies antemortem. Thus, early detection of this dreadful disease is of utmost importance to eliminate number of contacts that require post exposure prophylaxis and expenses of unnecessary diagnostic tests and inappropriate therapy.

Since rabies virus appears in the saliva of dogs before and during the appearance of clinical signs (Schneider, 1975), thus molecular approaches can be employed for reliable antemortem diagnosis on saliva samples. Ante-mortem diagnosis of rabies by molecular techniques based on detecting virus or viral RNA has been attempted in body fluids of live animals such as saliva (Crepin *et al.* 1998). Thus the present study was undertaken to evaluate nested RT-PCR technique for the detection of rabies virus from saliva samples.

MATERIAL AND METHODS

Saliva samples were collected for diagnosis of rabies from 11 rabies suspected animals presented to the Veterinary Clinics, GADVASU, Ludhiana, Punjab and Civil Veterinary Hospital from different districts of Punjab. The animals were presented at the clinics 3-4 days after onset of clinical symptoms. Saliva samples were collected either in a sterilized vial directly or with the help of sterile syringe from oral cavity of animal. Saliva samples obtained from two healthy animals served as negative controls. Inactivated VP12 rabies virus strain (Rabigen vaccine) was used as positive control.

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 (30 pmol/µl) and subjected to 65ºC for 10 min and was later snap cooled on ice and briefly spun down. cDNA synthesis was done using high-capacity cDNA reverse transcription kit (Applied Biosystems, USA). 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). 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.

The procedure used for the nested RT-PCR was that used earlier (Nadin-Davis 1998, Nagaraj *et al*. 2006 and Kaw *et al*. 2011) 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 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 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.

The sensitivity of Nested RT-PCR was calculated using formulae:

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

RESULTS

The 260/280 ratio of RNA was in the range of 1.71-1.89 while the concentration varied from 91.23-321.56 ng/µl and the 260/280 ratio of cDNA was in the range of 1.89-2.07 and concentration varied from 1956.60-4153.20 ng/µl.

Table 1. Nested RT-PCR for ante-mortem detection of rabies.

S. No.	Species	Age	Sex	Saliva	FAT (Brain)
1.	Cattle	3 years	M	-	+
2.	Dog	4 years	M	-	-
3.	Dog	3.5 months	M	-	-
4.	Buffalo	6 years	F	+	+
5.	Cattle Calf	6 months	F	-	+
6.	Dog	2.5 years	F	-	+
7.	Buffalo	8 years	F	-	-
8.	Dog	12 years	M	-	+
9.	Dog	7.5 years	M	+	+
10.	Buffalo	6 years	F	+	+
11.	Cattle	1 years	F	+	+
Total				**4/11**	**8/11**

+ Positive, - Negative

Table 2:.Sensitivity comparison of Nested RT-PCR with FAT.

Test	FAT on brain smears	FAT on brain smears	Total
Nested RT-PCR (Positive)	4	0	4
Nested RT-PCR (Negative)	4	3	7
Total	**8**	**3**	**11**

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

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 (Fig.1) as reported by (Nadin-Davis 1998, Nagaraj *et al*. 2006 and Kaw *et al*. 2011). By Nested RT-PCR, viral RNA could be diagnosed in higher number 4/11 (36.36%) of saliva samples (Table 1) (Fig. 1 & 2).

Sensitivity of 66.67% was obtained when compared with fluorescent antibody test (Table 2).

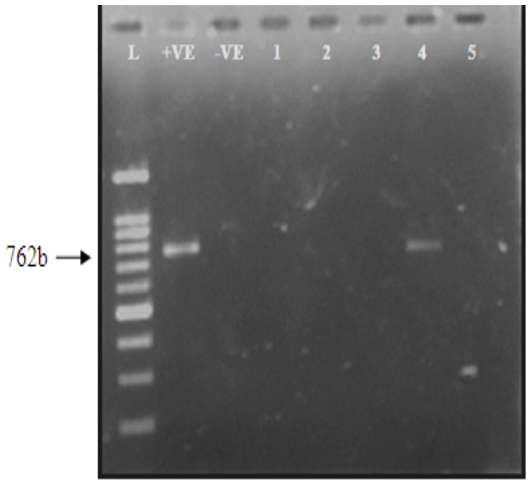

Fig. 1: Nested RT-PCR amplified product of rabies virus from saliva samples (No. 1-5) and control samples

Lane 1: Ladder, Lane2: +ve control, Lane 3: -ve control , Lane 4: Sample 1, Lane 5: Sample 2, Lane 6: Sample 3, Lane 7: Sample 4, Lane 8: Sample 5

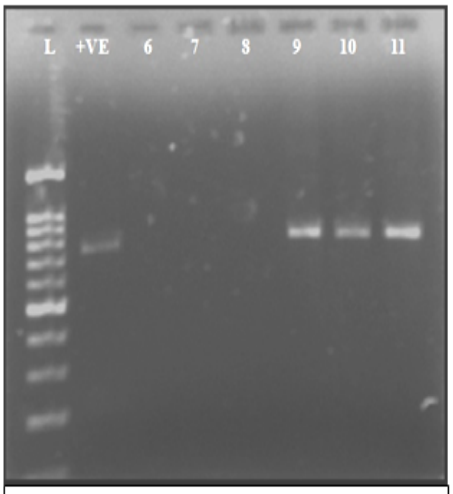

Fig. 2: Nested RT-PCR amplified product of rabies virus from saliva samples (No. 6-11) and control samples.

Lane 1: Ladder, Lane2: +ve control, Lane 3: Sample 6, Lane 4: Sample 7, Lane 5: Sample 8, Lane 6: Sample 9, Lane 7: Sample 10, Lane 8: Sample 11

DISCUSSION

Nested RT-PCR applied on saliva samples of rabies suspected animals for diagnosis of rabies confirmed positive cases more than (Crepin *et al.* 1998 and Nagaraj *et al.* 2006), who reported detection of rabies virus in 11/37 (29.72%) and 6/21 (28.57%) respectively in saliva samples of human patients by conventional RT-PCR. However, (Noah *et al*. 1998) found rabies virus in 9/15 (60.0%) saliva samples by use of RT-PCR. Similarly, (Wacharapluesadee and Hemachudha, 2010) diagnosed rabies virus in 47/62 (75.80%) saliva samples by nucleic acid amplification based technique. (Kaw *et al.* 2011) confirmed rabies virus in 3/12 (25.0%) saliva samples of animals by nested RT-PCR which was less as compared with the present study. It was concluded that Nested RT-PCR applied on saliva of rabies suspected animal helps in antemortem diagnosis of rabies.

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