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Molecular Detection of Rabies by SYBR Green Real Time PCR

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Abstract : *The present study was envisaged to compare the sensitivity of SYBR Green real time PCR with immunofluorescence PCR for diagnosis of rabies. SYBR Green real time PCR technique was applied on brain samples collected from 39 rabies suspected animals. Sensitivity of SYBR Green technique was compared in accordance with WHO recommended gold standard test viz. Fluorescent Antibody Technique (FAT) applied on brain samples. SYBR Green real time PCR applied on brain samples had successfully confirmed rabies in 16/39 with a Sensitivity of 80% . It was concluded that SYBR Green real time PCR is a useful, specific, sensitive and better molecular approach for diagnosis of rabies from brain of rabid suspected cases and can be used as confirmatory test for rabies diagnosis.*

Keywords: *Brain, Molecular, Rabies, Real time PCR, SYBR Green.*

I. INTRODUCTION

Rabies is enzootic and is a serious public health and economic problem in India. A national survey by the Association of the Prevention and Control of Rabies APCRI, 2003 estimated that in India a total of 18,500 human deaths occur as a result of rabies each year. Although the loss of livestock due to rabies is significant, there are few publications on estimates of the incidence of rabies in livestock [1]. Molecular approaches like RT- PCR and real-time PCR seem to be more reliable and more sensitive than traditional methods used for rabies diagnosis [2, 3, 4]. They allow for the detection of genetic material of viruses in a relatively short time. The present study was conducted to study the efficacy of SYBR Green real time PCR for detection of rabies in brain tissue samples.

II. MATERIALS AND METHODS

2.1 Source of brain specimens

39 brain samples from different animal species, all suspected of rabies, previously diagnosed by FAT and stored as 10% homogenate in phosphate buffered saline at -20˚C were obtained. Lyophilized anti rabies vaccine was used as positive control and brain of normal animal that had natural death was used as negative control.

2.2 RNA extraction and cDNA synthesis

Total RNA in the brain specimens, positive and negative controls was extracted using Qiazol (Qiagen, USA) according to the manufacturer's instructions. The RNA was stored at -80˚C until further use. The RNA was subjected to cDNA synthesis using a primer RabN1 (TABLE 1) and subjected to 65ºC for 10 min, followed by 37º C 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 mins and chilling on ice for 5 mins in a thermal cycler (Eppendorf). This cDNA was used for amplification in real time PCR assay in this study. Considering that the N gene is the most conserved in the Lyssaviruses (except some domains of the L protein gene) and that the sequence data concerning this gene are the most exhaustive, we used primers in the N gene that were shown to allow amplification of a wide range of genetically diverse lyssaviruses [5].

2.3 SYBR GREEN Real time PCR assay

The procedure used for the real time PCR was essentially those described earlier by [5] Nagaraj et al., (2006) with minor modifications. The 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 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. This software coupled to the ABI system determines the cycle threshold (*C*t) that represents the number of cycles in which the fluorescence intensity is significantly above the background fluorescence.

III. RESULTS AND DISCUSSION

Clinical details of animals

The salient clinical features of the 39 suspected animals are presented in TABLE 2. Amongst the 39 animals that were included in this study 7 were buffaloes, 10 cows, 20 dogs and 1 each horse and cat.

Real time PCR assay

For amplification in real time PCR oligonucleotides O1 and R6 (Table 1) were used. A typical amplification plot and melting curve analysis for the determination of the specificity is presented in Fig. 1 and 2 respectively. As can be observed from the (FIG. 1), the cycle threshold (*C*t) of the positive control was at the 26th cycle whilst most of the clinical samples had *C*t values ranging from 26 to 29 cycles. It can be observed from (FIG. 2) that a 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 SYBR Green real time analysis shows that, amongst the 39 samples, 16 samples (41.02%) were positive. The sensitivity of this test was 80% and specificity was 1. The sensitivity of nested RT-PCR on the same samples was 70% and specificity was 1. Thus confirming that real time PCR is more sensitive than conventional PCR.

PCR has become a valuable tool in various laboratories being faster than both the RTCIT and MIT and more sensitive and specific than the FAT. When combined with sequence analysis the sample genotype can be rapidly confirmed and epidemiological analysis undertaken [6]. PCR detection of RV (rabies virus) has been described by a number of investigators [7, 8, 9, 10]. We preferred SYBR Green real time assay as the application of this assay was for more sensitive diagnosis of the infection. [11] HUGHES designed a TaqMan PCR based method for detection of rabies virus RNA in tissue samples. The assay was found to be sensitive and specific and correlated well with the concentration of infectious virus but the number of mismatches reduced the efficiency of the reaction such that four differences could result in a weakly positive or negative result. When using SYBR Green it is however necessary to rely on melting curves to ensure that the obtained signal is specific. An advantage of real time PCR is that it is a closed tube system that significantly reduces risk of cross contamination of PCR products and thus results in increased confidence in the results acquired [5].

IV. TABLES

Table 1: Primers used for Nested RT-PCR

Primer Name	Nucleotide Sequence 5'-3'	Positions	Sense
O1	CTACAATGGATGCCGAC	66–82	+
R6	CCTAGAGTTATACAGGGCT	201-183	-

Table 2: Clinical features of 39 rabies suspected animals

Clinical feature	Proportion of animals (out of 39)
Fever	13
Hyper-salivation	18
Difficult intake	13
Behavioral change	22
Off feed	33
Micturition	9
Paralysis	17
Recognized owner	17
Vaccination status- nil	38
-proper	01

V. FIGURES

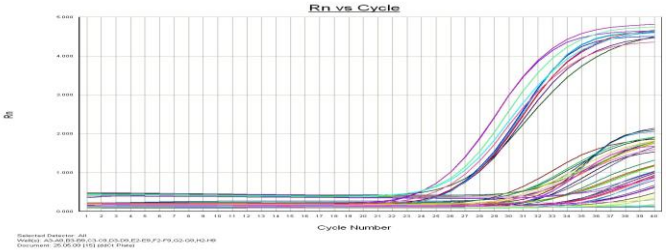

Fig. 1: Graph showing cycle threshold at 26th cycle for positive control and cycle threshold of positive samples ranges between 26- 29th cycles.

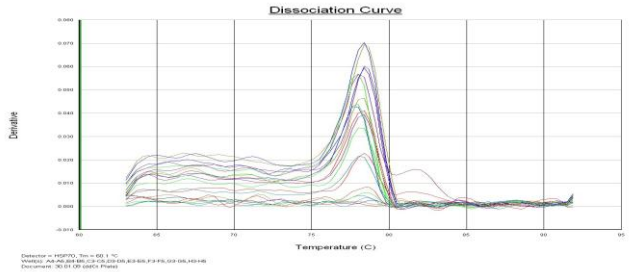

Fig. 2: Graph showing dissociation curve peak at 78 ºC. The shallow peaks before 70 ºC represent primer dimer.

VI. CONCLUSION

Newer, more advanced molecular approaches like SYBR Green real time PCR was applied for the detection of rabies virus in brain tissue specimens of animals. Highest sensitivity was observed with real time PCR than conventional RT-PCR. The results were in concordance with the FAT applied on the impression smears of the same samples. So this technique can be successfully used for the epidemiological study and if combined with sequencing the genotypes prevalent in that area can be identified.

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

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