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RABIES VIRUS DETECTION IN MILK SAMPLES: EFFICIENCY COMPARISON OF NESTED RT-PCR AND TAQMAN REAL-TIME PCR FOR INTRAVITAM DIAGNOSIS

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Abstract
Milk sample was obtained from 17 lactating animals suspected to be rabid. An attempt was made by employing advanced molecular approach Nested RT-PCR and TaqMan real time PCR on milk samples with an aim to detect the presence of rabies viral RNA. Confirmatory diagnosis of rabies was made by applying conventional techniques; Immunofluorescence on brain tissue. This study depicts the presence of rabies viral RNA in 2/17 milk sample with a sensitivity of 54.54% by Nested RT-PCR and 4/17 milk samples with a sensitivity of (60%) by TaqMan real time PCR when compared with FAT applied on brain tissue. This study confirms the presence of rabies viral RNA in milk.

Keywords: Immunofluorescence, Intravitam, Nested RT-PCR, Rabies, Saliva.

INTRODUCTION

Rabies caused by Lyssavirus is an important fatal viral disease afflicting animals and humans alike. It causes 10th highest mortality of all infectious diseases worldwide. It is the most feared of all zoonotic diseases and remains a major public health challenge in India [1]. More than 55,000 people die of rabies worldwide and roughly 36% of the world's rabies deaths occur in India. Forty percent of people who are bitten by suspect rabid animals are children under 15 years of age.Outward spread of rabies virus from the CNS leads to infection of almost all organs [2] and have been reported in various secretions and excretions viz. saliva [1], urine [3] and CSF [4]. Although transmission of rabies virus from consuming raw milk from an infected animal is theoretically possible, yet there is dearth of reports of transmission of rabies by consumption of milk of rabid animal. The present study was, therefore, envisaged to access the sensitivity of Nested RT-PCR and TaqMan Real time PCR for diagnosis of rabies viral RNA in milk samples.

I. MATERIALS AND METHODS

2.1 Collection of milk samples

Milk samples were collected from 17 rabies suspected animals (14 buffaloes, 3 cows) presented to the Veterinary Clinics, GADVASU, Ludhiana, Punjab and Civil Veterinary Hospital from different districts of Punjab. Soon after the clinical diagnosis was made, the milk samples were collected from the lactating animals suspected for rabies in sterilized vials by strip milking of teats. The vials were stored at -80ºC until further processing. Milk

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samples obtained from two healthy animals served as negative controls. Rabies positive brain homogenate was used as positive control.

2.2 RNA extraction and cDNA synthesis

Total RNA from milk 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) (Table 1.) 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.

2.3 Nested RT-PCR

The procedure used for the nested RT-PCR was that used earlier [1, 5, 6] 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 using nested set of primers RabNfor and RabNrev (Table 1) and subjected to initial denaturation at 95ºC for 2 min 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.

2.4 TaqMan Real time PCR

Considering the N gene that is most conserved in Lyssavirus and sequence data concerned with gene are most exhaustive. All TaqMan primers and probes were newly designed at School of Animal Biotechnology, GADVASU by the Primer Express 3.0 computer program (Applied Biosystems, Foster City, California). Sequences were obtained by using the default settings of the program. From this alignment, areas of relative conservation were selected as target regions for placement of the TaqMan primers and probes. These regions were used as input for Primer Express to generate the optimal primer and probe sequences according to the default settings. TaqMan primer and probe details are shown in (Table 2). TaqMan probe was labelled at the 5' end with a fluorescent reporter dye (FAM) and at the 3' end with a quencher dye (TAMRA). Primer and probe concentrations were optimized according to the manufacturer's recommendations.

The TaqMan real time assay was standardized with 20 µl PCR mixture volume consisting of 12.5 µl of TaqMan master mix (Applied Biosystem, USA) with 1 µl of primers Rab-8F and Rab-8R (400nm/ µl) and 1 µl probe Rab-8Pr. (250nm/ µl), 2.5 µl of the cDNA prepared using RabN1 primer and 2 µl of RNAse free water was added to make a final volume. Amplification was carried out at 50˚C for 2 min, 95˚C for 10 min, followed by 40 cycles in two steps: 95˚C for 15 s, 44˚C for 1 min. Amplification, data acquisition and analysis were carried out by using

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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 arose above the background fluorescence.

2.5 Statistical Analysis

Since, FAT is a gold standard for diagnosis of rabies on neural tissue, after death of animal by World Health Organization [7]. So, nested RT-PCRand TaqMan real time PCR employed on milk samples were compared with conventional approach FAT for detecting the efficacy of these molecular techniques.

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

III. RESULTS AND DISCUSSION

Amplification with primers RabN1 and Rab N5 yielded 1477bp first round product. Nested pair of primers (RabNfor and RabNrev) used for amplification in second round yielded 762 bp product as reported by [1, 5, 6]. By nested RT-PCR, viral RNA could be diagnosed in 2/17milk samples (Table 3). Perusal of literature reveals that there is no reported work of detection of rabies viral RNA in milk.

Sensitivity of nested RT-PCR on milk samples was found to be 54.54 %.

The samples in which threshold cycle number (Ct) values were found to be in the range of 20-35 were considered positive and above 35 were considered negative [8]. By TaqMan real time PCR, viral RNA could be diagnosed in 4/17 (23.53%) milk samples (Table 3).

Sensitivity of 78.94% was obtained with application of TaqMan real time PCR assay on milk samples when compared with gold standard immunofluorescence test (FAT) on brain.

IV. TABLES

Table 1: Primers 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	
RabNfor	5' TTG T(AG)G A(TC)CA ATA TGA GTA CAA 3'	N	135-156	+
RabNrev	5' CTG GCT CAA ACA TTC TTC TTA 3'	N	876-896	

Table 2: Details of TaqMan Primers and probe

Primer Name	Sequence	Gene	Length (nt)	Positions	Tmax (°c)	Remarks
Primer Rab-8F	5'-TTGACGGGAGGAATG	N	20	434-453	62	Newly designed

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		GAA CT-3'					
Primer	Rab-8R	5'-GAC CGA CTA AAG ACG CAT GCT-3'	N	21	477-497	64	Newly designed
Probe	Rab-8Pr	5'-FAM- AGG GAC CCC ACT GTT-TAMRA-3'	N	15	458-472	48	Newly designed

Table 3: Details of Nested RT-PCR and TaqMan real time PCR for intravitam diagnosis of Rabies

Sample	**Species**	**Age**	**Nested**	**TaqMan real**	**FAT (Brain)**
No.1.	Buffalo	6 years	**RT--PCR**	**time PCR**	+
2.	Buffalo	5.5	+	+	+
3.	Buffalo	6years years			
4.	Buffalo	5 years			
5.	Buffalo	3.5	+	+	+
6.	Buffalo	5years years		+	+
7.	Buffalo	6 years			+
8.	Cow	7 years			+
9.	Buffalo	7 years			+
10.	Buffalo	5.5	-	-	
11.	Buffalo	7years years	-	-	+
12.	Buffalo	6 years			+
13.	Cow	4 years			
14. 15.	Buffalo Cow	4.5	-	+	+
16.	Buffalo	years6.5	-	-	+
		5years years	-	-	
17.	Buffalo	6 years			+
Total			2/17	4/17	12/17

+ Positive, - Negative

V. CONCLUSION

The present study confirms the presence of rabies virus in milk. TaqMan real time PCR can serve as more sensitive and viable approach for the intravitam diagnosis of rabies as compared to Nested RT-PCR for detection of rabies from milk of suspected animals. This study also suggests that milk of rabid animal should not be consumed raw.

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