Title: ANTE MORTEM DIAGNOSIS OF RABIES FROM SALIVA: COMPARISON OF NESTED RT-PCR WITH TAQMAN REAL TIME PCR

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Keywords Ante mortem, Nested RT-PCR, Rabies, TaqMan real time PCR

Abstract To evaluate efficacy of molecular techniques nested RT-PCR and TaqMan real time PCR for the detection of rabies virus from saliva. To compare the sensitivity of molecular techniques applied on saliva for ante mortem diagnosis of rabies with immuno flourescence applied on brain.

Original Article

Ante mortem diagnosis of rabies from saliva: comparison of Nested RT-PCR with TaqMan real time PCR

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Abstract

Objectives: 1. To evaluate efficacy of molecular techniques viz. nested RT-PCR and TaqMan real time PCR for the detection of rabies virus from saliva.

2. To compare the sensitivity of molecular techniques applied on saliva for ante mortem diagnosis of rabies with immunofluorescence applied on brain

Period of Study: July 2010 – Dec. 2012

Material and Methods: Molecular techniques viz. Nested RT-PCR and TaqMan real time PCR techniques were applied on 11 saliva samples collected from rabies suspected animals. Sensitivity of both the techniques was compared in accordance with WHO recommended gold standard test viz. Fluorescent Antibody Technique (FAT) applied on brain samples.

Results: Nested and TaqMan real time PCR had successfully diagnosed rabies viral RNA in 4 and 6 saliva samples, respectively out of 8 FAT confirmed cases. Sensitivity of 66.67% was obtained with nested RT-PCR on saliva samples while TaqMan real time PCR revealed sensitivity of 80.0%.

Interpretation & Conclusion: It was concluded that TaqMan real time PCR is a useful, specific, sensitive and better molecular approach for earlier diagnosis of rabies from saliva of rabid suspected cases.

Keywords: Ante mortem, Nested RT-PCR, Rabies, Saliva, TaqMan real time PCR.

Introduction

Rabies is a zoonotic disease of concern caused by *Lyssavirus*. It mainly affects CNS causing invariably fatal encephalomyelitis in humans and animals. The disease is caused by single stranded, unsegmented, negative sense RNA virus belonging to the family *Rhabdoviridae* of the order *Mononegavirales*. 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[1].

The clinical diagnosis of rabies is at times suggested by epidemiological history of exposure and clinical findings[2]. However, making a reliable diagnosis of rabies based on clinical presentation can be difficult to distinguish from encephalitic condition caused by other viral infections[3]. Therefore, diagnosis is often confirmed late in the course of the disease or postmortem[4]. With the advent of molecular approaches, it is now possible to detect rabies ante-mortem. 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.

Rabies is transmitted from animal to animal or animal to humans through bites scratches on skin or licks on the mucosal surface. Since rabies virus appears in the saliva before and during the clinical signs[5], 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[6]. TaqMan PCR is very sensitive, rapid and specific for the detection of rabies virus in tissue samples and importantly correlates with the concentration of infectious virus[7].

Objectives

1. To evaluate efficacy of molecular techniques viz. nested RT-PCR and TaqMan real time PCR for the detection of rabies virus from saliva.
2. To compare the sensitivity of molecular techniques applied on saliva for ante mortem

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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 CAA ACA TTC TTC TTA 3'	N	876-896	-

diagnosis of rabies with immunofluorescence applied on brain.

Material & Methods

Source of saliva samples: Saliva samples were collected from 11 animals (3 buffaloes, 3 cows and 5 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, saliva samples were collected either in a sterilized vial directly or with the help of sterile syringe from oral cavity of animal. Saliva samples were also obtained from 2 healthy animals, as negative controls for the molecular assays. Inactivated VP12 rabies virus strain (Rabigen vaccine) was used as positive control.

RNA extraction and cDNA synthesis: Total RNA in 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 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 both the nested and the TaqMan real time PCR assays.

Primers: Primers used were 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 by[8, 9, 10] (Table 1). All TaqMan primers and probes were newly designed by the Primer Express 3.0 computer program (Applied Biosystems, Foster City, Calif.). 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 labeled 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.

Nested RT-PCR: 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 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.

TaqMan real time PCR assay: The TaqMan real time assay was standardized and carried out in 25

Table-2
Details of primer's used for TaqMan real time PCR

Primer Name	Sequence	Gene	Length (nucleotides)	Positions	Conc. (nm/ μl)	Tmax (°c)
Rab-1F	5'-TTG ACG GGA GGA ATG GAA CT-3'	N	20	434-453	400	62
Rab-1R	5'-GAC CGA CTA AAG ACG CAT GCT-3'	N	21	477-497	400	64
Probe-1Pr.	5'-FAM- AGG GAC CCC ACT GTT-TAMRA-3'	N	15	458-472	250	48

μl PCR mixture volume consisting of 12.5 μl of TaqMan master mix (Applied Biosystems, USA) with 2.5 μl of primers Rab-1F and Rab1-R (400nm/ μl) and 1 μl probe 1Pr. (250nm/ μl) and 5 μl of the cDNA prepared using RabN1 primer. 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, 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 arose above the background fluorescence.

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. So, Nested RT-PCR and TaqMan real time PCR techniques employed on saliva samples was compared with FAT for detecting the sensitivity of these molecular techniques.

Statistical analysis: The sensitivity of various tests applied was calculated using formulae:

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

Results

Analysis of nucleic acid: 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.

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 4/11 saliva samples (Table 3).

TaqMan real time PCR: Newly designed primers (Table 2) were used for amplification with TaqMan real time PCR. The samples in which

Table-3
Nested RT-PCR and Taqman real time PCR for ante mortem diagnosis of rabies from saliva samples

Sample No.	Species	Age	Sex	Nested RT-PCR	TaqMan Real time PCR	Ct values (cycle threshold)
1.	Cattle	3 years	M	-	-	ND
2.	Dog	4 years	M	-	-	39.543
3.	Dog	3.5 months	M	-	-	ND
4.	Buffalo	6 years	F	+	+	18.147
5.	Cattle Calf	6 months	F	-	+	25.246
6.	Dog	2.5 years	F	-	+	25.921
7.	Buffalo	8 years	F	-	-	39.999
8.	Dog	12 years	M	-	-	ND
9.	Dog	7.5 years	M	+	+	26.787
10.	Buffalo	6 years	F	+	+	26.241
11.	Cattle	1 years	F	+	+	26.458
		% Positivity		**36.36%**	**54.54%**	

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

Table 4
Sensitivity comparison of Nested RT-PCR with FAT.

Test	FAT on brain smears (Positive)	FAT on brain smears (Negative)	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%

Table 5
Sensitivity comparison of TaqMan real time PCR with FAT.

Test	FAT on brain smears (Positive)	FAT on brain smears (Negative)	Total
TaqMan real time PCR (Positive)	6	0	6
TaqMan real time PCR (Negative)	2	3	5
Total	**8**	**3**	**11**

Sensitivity = True Positive / True Positive + False Negative × 100 = 8/ 8 + 2 = 80.0%

threshold cycle number (Ct) values were found to be in the range of 20-35 were considered positive and above 35 were considered negative[7]. By TaqMan real time PCR, viral RNA could be diagnosed in 6/11 saliva samples (Table 3).

Sensitivity comparison of molecular techniques with FAT: The nested RT-PCR and TaqMan real time PCR applied on 11 saliva samples were able to diagnose rabies in 4 and 6 saliva samples, respectively. Postmortem brain tissue examination by FAT revealed rabies in 8 out of 11 (72.72%) cases. Application of Nested RT-PCR on saliva samples for viral nucleic acid yielded a sensitivity of 66.67% while application of TaqMan real time PCR yielded a sensitivity of 80.0%.

Discussion

Nested RT PCR: By nested RT-PCR, viral RNA could be diagnosed in 4 out of 11 saliva samples (Table 3) which was more as compared with other studies[6,8,10], that reported detection of rabies virus in 11 out of 37 (29.72%), 6/21 (28.57%) and 3/12 (25.0%) respectively in saliva samples by conventional RT-PCR. However, use of RT-PCR and nucleic acid amplification by[11,12] found rabies virus in 9/15 (60.0%) and 47/62 (75.80%) saliva samples respectively.

TaqMan real time PCR: By TaqMan real time PCR, viral RNA could be diagnosed in 6/11 saliva samples (Table 3) which was less as compared to 87% as detected by real time PCR on saliva samples of human patients[13]. Another study[8] reported 18/21 saliva samples positive by real time PCR.

Sensitivity comparison of molecular techniques with FAT: Application of Nested RT-PCR on saliva samples for viral nucleic acid yielded a sensitivity of 66.67% which is more than the sensitivity of 30% reported by[6] and 37% reported by[8] who also used nested RT-PCR on human saliva samples. The sensitivity detected in present study was more as compared to 37.5% reported by[10] by use of nested RT-PCR on saliva samples of animals. Another study[11] observed a higher sensitivity of >98% by the use of RT-PCR on saliva samples for ante mortem diagnosis of rabies in human saliva samples.

TaqMan real time PCR on saliva samples for viral nucleic acid yielded a sensitivity of 80% which was more as compared with findings of[12,13] that reported sensitivity of 75.8% and 75%, respectively on saliva samples by real time PCR.

Conclusion

So far, conventional RT-PCR has been reported to be a reliable test for ante mortem diagnosis[14] 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. However, in the present study we found that the sensitivity of TaqMan real time PCR is even better than RT-PCR. It can be concluded that TaqMan 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.

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

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Announcement

The APCRI Journal is published twice a year. Once in January and again in July. The APCRI Journal invites Contributions from the Scientific Community, on All aspects of Rabies and Related Matter, in the form of Original Articles and Review Articles, Brief Reports, Case Reports, Personal Viewpoint, Letters to the Editor, Notes and News, Your Questions and Book Review.

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