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Antemortem Diagnosis of Rabies From Skin: Comparison of nested RT-PCR with TaqMan real time PCR

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Abstract

Molecular techniques were adopted to diagnose rabies viral RNA from skin biopsy samples collected from 20 animals. Nested RT-PCR and TaqMan real time PCR techniques have successfully diagnosed rabies viral RNA in 9 and 11 skin samples, respectively. The sensitivity of both techniques was calculated in comparison to FAT applied on brain samples. Sensitivity of 76.47% was obtained with nested RT-PCR on skin biopsy samples while Taqman real time PCR revealed sensitivity of 92.85%. It was concluded that TaqMan real time PCR is a useful, specific, sensitive and better molecular approach for antemortem rabies diagnosis from skin samples of rabies suspected animals.

Key Words: Lyssavirus, Rabies virus, Skin biopsies, Diagnosis, PCR techniques.

Introduction

Rabies is an acute lyssavirus disease of warm blooded animals which may also be transmitted to humans (zoo-anthroponosis). It mainly affects CNS causing fatal encephalomyelitis in humans and animals. The disease is caused by highly neurotropic, single stranded, unsegmented, negative polarized RNA virus belonging to the family Rhabdoviridae of the order Mononegavirales (15). 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 (14). The clinical diagnosis of rabies is sometimes suggested by epidemiological (history of exposure) and clinical findings (6). However, making a reliable diagnosis of rabies based on clinical presentation can be difficult to distinguish from encephalitic conditions 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 antemortem.

Rabies is transmitted from animal to animal or animal to humans through bites or scratches on skin or licks on the mucosal surface. The rabies virus is also present in nerve cells surrounding the base of hair follicles (10). The antemortem diagnosis of rabies can be established from skin samples collected from live animals (2). The detection of rabies by PCR is a sensitive approach since PCR can amplify a single fragment of the viral genome. TaqMan PCR is relatively more sensitive, rapid and specific for the detection of rabies virus in tissue samples and importantly correlates with the concentration of infectious virus (7). The aim of this study is to evaluate nested RT-PCR and TaqMan real time PCR techniques for the detection of rabies virus from skin samples in comparison with FAT (Fluorescent Antibody Test) applied on brain.

Material and methods

Source of skin specimen

Skin biopsy samples were collected from 20 rabies suspected animals (7 buffaloes, 5 cattle and 8 dogs) 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 skin biopsy samples with a minimum of 10 hair follicles were collected with the help of sterilized skin biopsy punch. Skin 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 the skin 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, 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 (14). Nested set of primers used in the present study was that used earlier by (8,11,12) (Table 1).

Table 1: Names, nucleotide sequence, positions and sense of 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	-
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	-

Table 2: Names, nucleotide sequence, positions, concentration and Tmax of primers used for TaqMan real time PCR

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

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 (30 pmol/µl) (Table 1), 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 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 assay

The procedure used for the nested RT-PCR was that used earlier (8,11,12) with minor modifications. Briefly, 12 µl of cDNA was subjected to a first round amplification using RabN1 and RabN5 primers extension step at 72˚C for 5 min. For the second round, 8 µl of first round PCR product was used 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.

TaqMan real time PCR assay

The TaqMan real time assay was carried out in 25 µl PCR mixture volume consisting of 12.5 µl of TaqMan master mix (Applied Biosystem, USA) with 2.5 µl of primers Rab-1F and Rab1-R (400nm/ µl) and 1 µl probe 1Pr. (250nm/ µl) (Table 1) 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.

Comparison of nested and TaqMan real time PCR

As, FAT on neural tissue is world wide recommended standard technique by WHO for diagnosis of rabies (12). So, sensitivity of nested RT-PCR and TaqMan real time PCR employed on skin samples was calculated in comparison to FAT.

Results and discussion

Clinical details of animal

20 animals comprising of 7 buffaloes, 5 cattle and 8 dogs were incorporated in the present study. Mean average age of buffaloes was 5.57 years, while mean average age of cattle was 5.08 years and mean average age of dogs was 4.10 years.

Table 3: Results obtained with Nested RT-PCR and Taqman real time PCR for antemortem diagnosis of rabies from skin samples

Sample No.	Species	Age	Sex	Nested RT-PCR	Ct values (cycle threshold)	TaqMan real time PCR
1.	Buffalo	2 years	F	+	28.732	+
2.	Dog	5 months	F	-	ND	-
3.	Cattle	3 years	M	+	29.567	+
4.	Dog	4 years	M	-	39.561	-
5.	Buffalo	6 years	F	-	38.771	-
6.	Buffalo	4 years	F	-	39.677	-
7.	Dog	3½ months	M	-	ND	-
8.	Buffalo	6 years	F	+	33.151	+
9.	Dog	5 years	F	-	ND	-
10.	Cattle Calf	6 months	F	+	24.132	+
11.	Dog	2½ years	F	+	28.611	+
12.	Cattle calf	1 months	F	-	24.339	+
13.	Cattle	4½ years	F	-	38.563	-
14.	Buffalo	8 years	F	-	38.789	-
15.	Dog	12 years	M	-	22.377	+
16.	Dog	1 years	M	+	20.163	+
17.	Dog	7½ years	M	+	23.238	+
18.	Buffalo	7 years	F	-	38.281	-
19.	Buffalo	6 years	F	+	20.029	+
20.	Cattle	1 years	F	+	27.201	+
		% Positivity		**45%**		**55%**

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

Analysis of nucleic acid

The 260/280 ratio of RNA was in the range of 1.70-1.89 while the concentration varied from 100.17-744.40 ng/µl and the 260/280 ratio of cDNA was in the range of 1.87-2.12 and concentration varied from 2021.80-4708.40 ng/µl.

Nested RT-PCR

Amplification with primers Rab N1 and Rab N5 yielded 1477 bp first round product. Nested pair of primers (Rab Nfor and Rab Nrev) used for amplification in second round yielded 762 bp product as reported by (8,11,12). By nested RT-PCR, viral RNA could be diagnosed in 9/20 skin biopsy samples (Table 3) as compared to 10/15 human nuchal skin biopsy samples (13) and 7/10 (9) by use of nested RT-PCR on skin biopsy samples.

TaqMan real time PCR

For amplification with TaqMan real time PCR newly synthesized primers (Table 2) were used and 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. By TaqMan real time PCR, viral RNA could be diagnosed in 11/20 skin biopsy samples (Table 3).

Comparison of nested and TaqMan real time PCR

Nested RT-PCR and TaqMan real time PCR applied on 20 skin biopsy samples were able to diagnose rabies in 9 and 11 skin biopsy samples respectively. Postmortem brain tissue examination by FAT revealed 13 out of 20 (65%) rabies positive cases. Nested RT-PCR and TaqMan real time PCR applied on skin biopsy samples yields a sensitivity of 76.5% and 92.9%. Overall, in the present study TaqMan real time PCR was more sensitive than nested RT-PCR for detecting rabies virus RNA in skin samples. Sensitivity of nested RT-PCR on skin samples was more than the sensitivity of 66.6% observed by (13) on human nuchal skin biopsy samples and (12) revealed sensitivity of 57.1% on animal skin biopsy samples. Another study revealed almost similar (77.7%) sensitivity with the use of RT-PCR on neck skin samples in human patients (2).

So far, conventional RT-PCR has been reported to be a reliable test for antemortem diagnosis (1) 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 antemortem rabies detection from skin 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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