



Diagnosis of rabies in animals by nested RT-PCR

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Rabies is an infectious, fatal disease characterized by profound dysfunction of the central nervous system, caused by single stranded negative sense RNA virus belonging to the genus *Lyssa virus* of the family *Rhabdoviridae* (Tordo *et al.* 1986). Rabies is enzootic and is a serious public health and economic problem in India.

Although the loss of livestock due to rabies is significant, there are few estimates of the incidence of rabies in livestock (Knobel *et al.* 2005). Molecular approaches polymerase chain reaction (PCR) seem to be more reliable and more sensitive than traditional methods used for rabies diagnosis (Black *et al.* 2002, Picard Meyer *et al.* 2004, Wakeley *et al.* 2005). They allow for the detection of genetic material of viruses in a relatively short time. Perusal of literature reveals lack of information on the sensitivity of detection of rabies by molecular approaches in animals. Thus, the present study was conducted to study the efficacy of nested RT-PCR for detection of rabies in tissue samples like brain and skin of suspected animals.

Brain samples (39) from different animal species, all suspected of rabies were obtained. All cases were previously diagnosed by FAT and stored as 10% homogenate in phosphate buffered saline at –20°C. Lyophilized anti-rabies vaccine was used as positive control and normal mouse brain was used as negative control. Skin samples were also obtained from 32 cases.

Total RNA in the brain specimens, skin samples, positive and negative controls was extracted using qiazol 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 65°C for 10 min, followed by 37°C for 15 min, chilled on ice and briefly spun down. Reverse transcriptase mix was prepared and subjected to conditions 37°C for 2 h, 95°C for 5 min and chilling on ice for 5 min in a thermal cycler. The cDNA was used for amplification in nested RT-PCR assay. Considering that the N gene is the most conserved in the *Lyssaviruses* 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 *Lyssa viruses* as reported by Nagaraj *et al.* (2006).

The procedure of Nadin-Davis (1998) and Nagaraj *et al.* (2006) with some modifications was used. Briefly, 10 µl of cDNA was subjected to a first round amplification using RabN1 and RabN5 primers (30 pmol) (Table 1), DNTP's and Taq 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, along with primers Rab NF and Rab NR (30 pmol) (Table 1) and subjected to initial denaturation at 95°C for 2 min, followed by 35 cycles

Table 1. Primers used in the study

Primer name	Nucleotide sequence 5'-3'	Position	Sense
RabN1	gctctagAACACCTCTACAATGGATGCCGACAA	59–84	+
RabN5	GGATTGAC (AG) AAGATCTTGCTCAT	1514–1536	-
RabNF	TTGT (AG) GA (TC) CAATATGAGTACAA	135–156	+
RabNR	CCGGCTCAAACATTCTTCTTA	876–896	-

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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 salient clinical features of the 39 suspected animals are presented in Table 2. Amongst the 39 animals, 7 were buffaloes, 10 cows, 20 dogs and one each horse and cat.

Table 2. Clinical features of 39 animals suspected with rabies

Clinical features	Proportion of animal
Fever	13/39
Hypersalivation	18/39
Difficult intake	13/39
Behavioural change	22/39
Off feed	33/39
Micturition	9/39
Paralysis	17/39
Recognized owner	17/39
Vaccination status	38/39
Nil	
Proper	1/39

Nested RT-PCR: Amplification with primers RabN1 and RabN5 yielded a 1477 bp first round product while amplification with RabNF and RabNR yielded a 762 bp second round product (Fig. 1). Amongst the 39 brain samples, 15 samples were positive by nested RT-PCR yielding 75% sensitivity. Samples collected from normal mouse brain were negative indicating the specificity of the primers used. Out of 32 skin samples, 9 were positive by nested RT-PCR, hence yielding a sensitivity of 56.25%.

Molecular diagnosis was adopted to detect viral nucleic acid in ante-mortem or post-mortem specimens. We preferred nested RT-PCR assay since, in comparison to conventional methods of rabies diagnosis, it is a more sensitive diagnostic approach (Orlowska *et al.* 2008). PCR detection of RV (rabies virus) was described by other investigators (Wacharapluesadee *et al.* 2008, Araujo *et al.* 2008, Nadin Davis *et al.* 2009).

Perusal of literature reveals lack of information on the sensitivity of detection of rabies by molecular approaches in animals, whereas in humans the sensitivity of detection varies from 38% (Kamolvarin *et al.* 1993), 52% (Araujo *et al.* 2008) to >98% (Noah *et al.* 1998).

Romijn *et al.* (2003) detected rabies in 60 out of 72 (86%)

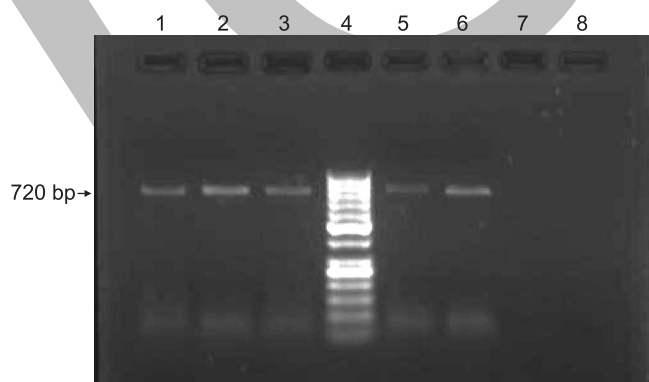


Fig 1. Nested RT-PCR amplified product of rabies virus from test and control samples. Lane 1, brain sample; lane 2, brain sample; lane 3, skin sample; lane 4, 50bp DNA ladder, molecular weight marker; lane 5, skin sample; lane 6, positive control; lane 7, negative control.

brain samples by FAT and RT-PCR. Macedo *et al.* (2006) used RT-PCR for detection of rabies virus in skin biopsy samples from 10 patients and obtained a sensitivity of 70% per sample and 77.7% per patient.

In the present study, nested RT-PCR was used for the diagnosis of rabies in animals from brain and skin samples yielding a sensitivity of 75% and 56.25%. Till now very less work has been elucidated to know the significance of molecular approaches for detection of rabies in animals. So, newer more sensitive approaches should be used for faster and accurate diagnosis of rabies.

SUMMARY

Brain tissues (39) and skin samples (32) from rabies suspected animals were diagnosed by employing nested RT-PCR. Brain samples (15: 38.46%) were positive by nested RT-PCR. The sensitivity of detection of rabies by nested RT-PCR from brain tissue in present study was 75%. Out of 32 skin samples, rabies was detected in 9 skin samples by nested RT-PCR yielding a sensitivity of 56.25%.

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