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Research Article

COMPARISON OF NESTED AND REAL TIME PCR FOR ANTE MORTEM DETECTION OF RABIES

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ABSTRACT
Biological diagnostic methods for confirmation of rabies remain limited, as sampling of brain tissue is difficult. So there is a need for a reliable method based on a simple collection of non-neural specimens as it is less hazardous. There is no ideal ante-mortem diagnostic test for rabies but skin biopsy evaluation may provide a valuable tool for this approach. However, no reports have been published demonstrating the value of intravitam laboratory diagnosis of rabies in animals. This study was aimed to determine whether rabies virus was present in skin at the onset of clinical symptoms in comparison to brain after death, and to determine the adequacy of using real-time PCR with SYBR Green I for detecting rabies virus in skin in animals. A nested RT-PCR and SYBR Green Real Time PCR were used for diagnosis of rabies from skin specimens of eleven suspected cases of rabies which were presented to the Clinics, GADVASU, Ludhiana, India, out of which 4 were positive by Nested RT-PCR and 5 by SYBR Green Real time PCR. The primers were designed to recognize conserved regions between the N nucleoprotein genes of lyssa viruses. We studied the accuracy of the diagnosis by comparing the results with post mortem results of brain obtained with use of the standard rabies diagnostic procedure. The data obtained indicate a high specificity (100%) of Nested RT-PCR and sensitivity (57%) when the nested RT-PCR was performed with skin biopsy specimens. Also, a sensitivity of 71.4% was obtained with the skin sample when we analyzed with SYBR Green Real Time PCR.

Key Words: *Rabies, PCR, Diagnosis, Skin.*

INTRODUCTION
Rabies is an acute and fatal encephalomyelitis caused by lyssaviruses transmitted to humans by rabid animals. Lyssavirus is an enveloped virus for which negative-strand RNA encodes 5 proteins, including the nucleoprotein (N) (Wunner *et al.*, 1988). Each year, at least 10 million people receive treatment after being exposed to animals suspected to be rabid; however, 55,000 people still die in Asia and Africa, based on the estimation by the World Health Organization (Knobel *et al.*, 2005). On the basis of these reports, the annual incidence of rabies in Asian and African countries ranges from 0.01 to 3 cases per 100,000 inhabitants (Cleaveland *et al.*, 2000, Kitala *et al.*, 2000 and Sudarshan *et al.*, 2007). Rabies is not a notifiable disease in many countries, and only the encephalitic form of the disease is usually recognized. The paralytic form is rarely identified and is sometimes misdiagnosed (Mallewa *et al.*, 2007). Differentiating this disease from other neurological disorders may require extensive investigation. Therefore, the diagnosis is often made late or is discovered postmortem (Anderson *et al.*, 1984 and Noah *et al.*, 1998).

To date, the use of biological methods for rabies diagnosis and notification has been very limited in developing countries, because only postmortem diagnosis of brain samples, with sensitivity near 100%, provides confirmation. The technique by which the biopsy specimens are examined consists of detecting viral nucleocapsids by immunofluorescence and is regarded as the reference protocol for retrospective confirmation of rabies (Dean *et al.*, 1996). Various intravitam diagnostic techniques for rabies in humans have been developed, but they are less sensitive than conventional postmortem diagnoses using cerebral tissue specimens (Crepin *et al.*, 1998). Currently, the highest sensitivity (range, 70%–90%) is obtained by immunofluorescence detection of viral inclusion on ultra thin sections of a skin biopsy specimen, but this technique remains difficult to implement in practice (Crepin *et al.*, loc. cit., Blenden *et al.*, 1986). More

recently, RT-PCR targeting the nucleoprotein gene was applied to skin biopsy specimens collected from 10 patients; the specificity of this test was 70% (Macedo *et al.,* 2006). In addition, most of these studies were not designed to allow an accurate comparison with the gold standard postmortem diagnostic method. The present study was undertaken to define a reliable protocol for diagnosing animal rabies with use of superficial tissue samples, which are collected in a noninvasive manner and can be used for both intravitam and postmortem diagnosis; our aim was to design a protocol to replace the classic postmortem diagnostic method that uses brain biopsy specimens. Results were obtained during an evaluation of nested reverse-transcription, PCR (RT-PCR). This test targets conserved blocks in the N gene sequence of Lyssaviruses (Bourhy *et al.,* 2005) and was performed on skin samples collected from 11 animals over 8 months time.

MATERIALS AND METHODS

The skin samples were collected from the rabies suspected animals presented to the Clinics, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, India. Most of these animals were presented at the clinics 3-4 days after onset of clinical symptoms. Soon after the clinical diagnosis was made, the skin samples were collected directly from the nape of neck with the help of biopsy punch after the animal was properly restrained. Most of the cases presented were in dumb form of rabies, so sample collection was easy in these cases. Proper precautionary measures were taken by the handlers before collection of sample. Skin samples were also obtained from 2 healthy animals, processed and stored in an identical manner. These served as negative controls for the molecular assays.

We used anti rabies vaccine as the positive control and normal mouse brain homogenate as the negative control. The entire procedure of RNA extraction, cDNA synthesis and the nested and real time PCR was standardized using these controls before actual testing of skin samples. Later, for every run of PCR skin sample from a normal healthy animal was also included as a negative control.

Skin biopsy samples were dissociated with sterile scissors and were incubated in 1 ml of Qiazol lysis buffer (Qiagen, USA) @ 1 ml for100mg tissue until further use. Total RNA in the skin specimens, positive and negative controls was extracted using Qiazol (Qiagen, USA) according to the manufacturer's instructions and stored at -80ºC until further use. The RNA (10 µl) was subjected to cDNA synthesis using a primer RabN1 (30 pmol/ µl) (Table 1) and subjected to 65ºC for 10 min, followed by 37 ºC 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 minutes and chilling on ice for 5 mins in a thermal cycler (Eppendorf). This cDNA was used for amplification in both the nested and the real time PCR assays in this study.

The procedure used for the nested PCR was essentially that described earlier by Nadin-Davis *et al.,* (1998) and Nagaraj *et al.,* (2006) with minor modifications. 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 and subjected to initial denaturation at 95ºC 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 nested PCR product had a size of 762 bp. These primers were designed to recognize conserved regions between the N genes of lyssaviruses.

A real time SYBR Green I PCR assay was carried out in 25 µl PCR mixture volume consisting of 12.5 µl of SYBR Green I 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. SYBR Green I

molecules bind to all double stranded DNA molecules emitting a fluorescent signal on binding proportional to the amplicon synthesis during the PCR reaction. This property enabled an accurate analysis of the melting temperature curve of the amplified fragments generated by real time PCR to determine the detection of specific products. Real time target amplification profile demonstrated a specific main peak with a melting temperature *T*m at 78˚C and sometimes a second weak peak at 70˚C representing non-specific products (primer dimer).

Table 1: Details of 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	−
O1	CTACAATGGATGCCGAC	66–82	+
R6	CCTAGAGTTATACAGGGCT	201–183	−

RESULTS AND DISCUSSION

The salient epidemiological and clinical features of the 11 suspected animals are presented in Table 2. Amongst the 11 animals that were included in this study 3 were buffalo, 4 cow and 4 dog cases. All cases presented were female animals. Mean average age of cattle was 2.7 years (range 7 months – 3 years) and that of dogs was 5.87 months (range 2½ months – 10 months).

Table 2: Clinical features of 11 animals with rabies at hospital admission

Clinical Feature	Proportion of Patients
Fever	03/11
Hypersalivation	08/11
Dysphagia	05/11
Behavioral Change	06/11
Off Feed	10/11
Micturition	03/11
Paralysis	05/11
Recognized Owner	04/11
Vaccination Status - Nil	10/11
- Proper	01/11

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. Amongst the 11 skin samples from patients, 4 samples were positive. Skin samples collected from 2 healthy controls as well as the normal mouse brain sample were negative indicating the specificity of the primers used.

For amplification in real time PCR oligonucleotides O1 and R6 (Table 1) were used and the PCR product was 135 bp in length. The threshold cycle (*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. There were a few samples that showed amplification plots above the threshold level beyond the 35th cycle. In order to determine whether they were signals obtained from genuine PCR products or spurious signals we used melting curve analysis.

The nested RT-PCR analysis is presented in Table 3. It can be observed that, amongst the 11 samples tested 4 samples (36.36%) were positive by this method. The sensitivity of this test was 57.1% and specificity was 100%.

Table 3: Nested RT-PCR assay analysis of rabies infected skin samples

	True positive	True negative	Total
Nested RT-PCR Positive	4	0 (False positive)	4
Nested RT-PCR Negative	3 (False negative)	4	7
Total	7	4	11

The SYBR Green real time analysis is presented in Table 4. It can be observed that, amongst the 11 samples tested 5 samples (45.45%) were positive by this method. The sensitivity of this test was 71.4% and specificity was 100%. Overall, the Real time PCR was more sensitive than nested RT-PCR for detecting rabies virus RNA in skin samples.

Table 4: SYBR Green real time PCR assay analysis of rabies infected skin samples

	True positive	True negative	Total
SYBR Green real time PCR positive	5	0 (False positive)	5
SYBR Green real time PCR negative	2 (False negative)	4	6
Total	7	4	11

Conventional techniques used for postmortem diagnosis of rabies are of limited value to support the ante mortem diagnosis of the disease (Hemachudha *et al.,* 1988 and Warrel and Warrel, 1995). The classic mouse inoculation test (Koprowski, 1996) for virus isolation can lead to a considerable delay in the estimation of an end point, requires facilities for the use of experimental animals and is labor intensive. Cell culture isolation methods (Webster and Casey, 1996) are problematic due to the inability of certain rabies virus variants to propagate easily in specific cell lines (Hughes *et al.,* 2004). Many tests have been advocated for the ante mortem diagnosis of rabies such as the corneal smear examination (Schneider, 1969) and frozen section skin biopsy (Blenden *et al.,* loc cit.). The only test considered to be reliable is the immunofluorescence (IF) test on skin biopsy samples (Dean *et al.,* loc. cit.). However in routine laboratory testing Crepin *et al.,* loc. cit. noticed that a minimum of 20 sections were needed to ensure the observation of hair follicules.

The aim of the present study was to establish a rapid and sensitive molecular diagnostic method for ante mortem diagnosis of rabies. Accordingly, we evaluated two molecular techniques nested RT-PCR and SYBR Green Real time PCR, for the detection of rabies viral RNA in animal skin samples. To avoid major mismatches due to rabies virus genetic diversity, we designed oligonucleotides (Table 1) that recognize specific and highly conserved sequences on the N protein. None of the skin samples obtained from healthy controls were positive in either the nested or the Real time PCR indicating that the primers

were indeed specific to rabies virus. Dacheux *et al.,* (1969) tested skin samples from 51 patients by heminested RT-PCR and confirmed the presence of rabies virus nucleic acid in 43 cases and observed a sensitivity >98% and specificity as 100% with skin biopsy specimens. Crepin *et al.,* loc. cit. recommended RT-PCR assay along with direct immunofluorescence test on skin biopsy specimens as a simple testing protocol for intravitam diagnosis of rabies.

We were able to detect rabies virus RNA in 5/11skin samples that we tested. In the present study most of the skin samples were collected 3-4 days after manifestation of clinical symptoms. Perhaps the diagnosis could have been confirmed by nested RT-PCR in the remaining 3 animals had a second and third sample of skin collected later in the course of the disease been available for testing. Conventional RT-PCR has been reported to be a reliable test for ante mortem diagnosis in two other separate case reports by Smith *et al.,* (2003) and Fooks *et al.,* (2003). They observed that detection of rabies specific antigen in skin biopsies from nape of the neck and hand generated a positive result with RT-PCR. Macedo *et al.,* loc. cit. concluded that probability of successful antemortem detection of rabies virus using neck skin samples is high.

In summary, real time PCR and nested RT-PCR targeting the N nucleoprotein gene can be useful, specific, and sensitive if applied to skin biopsy samples, from animals suspected to have rabies. Results correlate well with those of the fluorescent antibody test performed on brain biopsy specimens of same animals after their death. As skin sample is a superficial tissue sample which is easy to collect, we urge that skin biopsy samples be obtained from animals with risk factors for rabies and neurological symptoms that are consistent with rabies.

Therefore, we propose nested RT-PCR and SYBR Green real time PCR as an alternative to the currently recommended method of postmortem diagnosis with use of brain biopsy specimens for confirming rabies in animals and that the use of this technique should be encouraged.

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