Nested RT-PCR for ante mortem diagnosis of rabies from body secretion/excretion of animals suspected for rabies

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Abstract

Aim: The present study deals with molecular technique Nested RT-PCR for detection of rabies viral RNA from biological fluid samples (Saliva, Milk and Urine) collected from animal suspected for rabies and to compare the sensitivity of Nested RT-PCR applied for ante mortem diagnosis of rabies with conventional technique (immunofluorescence) applied on neural tissue. Molecular technique

Materials and Methods: Nested RT-PCR was applied on 62 biological fluid specimens collected from rabies suspected animals. First round amplification with nested set of primers (RabN1 and RabN5) yielded 1477 bp product while amplification with second round primers (RabNfor and RabNrev) yielded 762 bp product. Sensitivity of the technique was compared in accordance with WHO recommended gold standard test viz. Immunofluorescence (FAT) applied on brain samples.

Results: By Nested RT-PCR, viral RNA could be detected in 9/24 (37.50%) saliva samples, 2/17 (11.76%) milk samples and 6/21 (28.57%) urine samples. Confirmatory diagnosis by Immunofluorescence performed on brain sample revealed 18 true positive cases. Overall, Sensitivity of Nested RT-PCR technique employed on fluid samples was 69.23% when compared with immunofluorescence performed on brain samples.

Conclusions: Early reliable ante mortem diagnosis of rabies can be obtained from biological fluid samples of animals suspected to be rabid when tested with Nested RT-PCR technique.

Key-words: milk,nested RT-PCR, rabies, saliva, urine

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Introduction

Rabies though a dreaded disease unfortunately remains as a neglected disease in majority of countries particularly in Asia [1]. Rabies virus belongs to the genus *Lyssavirus* of the family *Rhabdoviridae*. The susceptibility to rabies infection depends on the virus strain, genetic makeup of the host, concentration of the neurotransmitter receptors at the site of bite, inoculums size and very importantly proximity of the bite to the central nervous system of the host. Rabies has been a continuing problem in several countries across the globe and posed a challenge in front of researchers [2].

Rabies is a major health problem in India too and is responsible for extensive morbidity and mortality. Animals as well as human cases are reported from all over the country. It is estimated that in India, every 30 minutes a life is taken away by rabies. 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 [3]. Although the loss of livestock due to rabies is significant, there are few publications on estimates of the incidence of rabies in livestock [4].

Rabies is a neurological disease, but the rabies virus spread to several organs outside the central nervous system (CNS). The rabies virus antigen or RNA has been identified from the salivary glands, lungs, kidneys, heart and liver [5]. Infection of salivary glands (and thus viral excretion) depends on centrifugal neural spread of virus from the central nervous system (CNS) and transfer of virus from axons to glandular epithelial cells. In addition to the salivary glands, the adrenal medulla, nasal mucosa, cornea and epidermis are frequently infected via

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	-	

spread in peripheral nerves [6]. With the help of advance molecular approaches rabies virus RNA can be detected in a range of biological fluids and tissue samples e.g. saliva, CSF, tears, skin biopsy sample and urine [7].

Thus the present study was envisaged to evaluate the importance of Nested RT-PCR technique for ante mortem diagnosis of rabies from biological fluid samples and to compare the sensitivity of Nested RT-PCR assay with immunofluorescence.

Materials and Methods

In the present study, samples were collected for diagnosis of rabies from 25 animals suspected for rabies presented to the Veterinary Clinics, GADVASU, Ludhiana, Punjab and Civil Veterinary Hospital from different districts of Punjab over a period of seventeen months from December 2010 to May 2012. Out of 25 animals, saliva samples were obtained from 24 (14 buffaloes, 4 cows, 3 cow calves and 3 dogs), milk samples from 17 (14 buffaloes and 3 cows) and urine samples from 21 (14 buffaloes, 1 cow, 3 cow calves and 3 dogs) animals suspected for rabies. Thus the total of 62 biological fluid samples served as a basis for current investigation.

Soon after the clinical diagnosis was made, saliva (collected directly or by syringe aspiration), milk (strip milking), urine samples (direct while urinating or urethral catheterization) were collected in sterilized vials from the animals suspected to be rabid. The vials were closed tightly to avoid any contamination and stored at -80ºC until further processing of samples. General data about the animals, such as estimated age, sex, clinical signs, ownership status, sample details etc. were recorded. Saliva, milk and urine samples obtained from two healthy animals served as negative controls. Rabies positive brain homogenate was used as positive control.

Total RNA from all fluid 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) 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 25ºC for 10 min, 37ºC for 2 h, 85ºC 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.

Nested RT-PCR Assay: The procedure used for the nested RT-PCR based on N (Nucleoprotein) gene was that used earlier [8-10] with minor modifications. Briefly 12 µl of cDNA was subjected to a first round amplification using RabN1 and RabN5 primers (30 pmol/µl) (Table 1), dNTP's and Taq DNA polymerase for 95ºC for 2 min followed by 35 cycles of 95ºC for 1 min, 55ºC for 1 min, 72ºC for 1 min 30 s and a final extension step at 72ºC for 5 min.

For the second round, 5 µl of first round PCR product was amplified using RabNfor and RabNrev (Table 1) and subjected to thermocycling conditions as first round except annealing at 55ºC and extension for 1 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).

Results

An attempt was made to detect rabies viral RNA from secretions (saliva, milk) and excretion (urine samples) by Nested RT-PCR technique (Table 2). 9 out of total 24 saliva samples collected were detected positive for rabies by Nested RT-PCR with a sensitivity of 68%. In milk rabies viral RNA was detected in 2 out of total 17 milk samples collected with a sensitivity of 54.54% while 6 out of 21 urine samples were found positive for rabies with a sensitivity of 62.50%.

Overall, Sensitivity of Nested RT-PCR technique employed on fluid samples was 69.23% when compared with immunofluorescence performed on brain samples.

Table-2. Details of results of Nested RT-PCR on secretions and excretion

Sr. No.	Species	Sex	Nested RT-PCR			FAT
			Saliva	Milk	Urine	Brain
1.	Buffalo4RL/11	F	-	-	-	+
2.	Dog8RL/11	M	+	NA	NA	+
3.	Cow calf9RL/11	F	-	NA	+	+
4.	Buffalo	F	+	+	+	+
5.	Dog	M	-	NA	-	-
6.	Buffalo	F	-		-	-
7.	Buffalo	F	-		-	-
8.	Cow calf38RL/11	F	+	NA	-	+
9.	Cow calf39RL/11	F	+	NA	+	+
10.	Buffalo	F	+	+	+	+
11.	Buffalo	F	+	-	+	+
12.	Cow41RL/11	F	-	NA	-	-
13.	Dog	F	NA	NA	-	+
14.	Dog47RL/11	M	+	NA	-	+
15.	Buffalo	F	-	-	-	+
16.	Cow	F	-	-	NA	+
17.	Buffalo	F	-	-	-	+
18.	Buffalo	F	-	-	-	-
19.	Buffalo	F	+	-	-	+
20.	Buffalo	F	-	-	-	+
21.	Cow	F	-	-	NA	-
22.	Buffalo	F	+	-	+	+
23.	Cow	F	-	-	NA	+
24.	Buffalo	F	-	-	-	-
25.	Buffalo	F	-	-	-	+
	+ve Control CCCconbtrol	F	NA	NA	NA	+
	-ve Control Dog	F	-	-	-	-
	-ve Control	F	-	-	-	-
	Total		9/24	2/17	6/21	18/25

Discussion

More sensitive molecular technique such as conventional PCR assay has produced satisfactory results for detection of rabies virus RNA from brain tissue and saliva [9,11]. Real time PCR assay on saliva samples was more sensitive than conventional RT-PCR assay (sensitivity 75% versus 37%) [11]. Positivity of 17.6% (6/34) was obtained with the use of heminested RT-PCR on urine samples [12] whereas urine and saliva testing using nucleic acid base amplification test (NASBA)yielded highest proportion of positive results in small series (urine testing, 2/4 patients; saliva testing, 3/4 patients) as compared to testing of CSF samples [13].

However the complete perusal of literature doesn't reveal any published work on detection of rabies viral RNA in the milk samples of animals suspected for rabies. Moreover literature reveals no such work has been conducted before with all three saliva, milk and urine specimens at a time in animals.

Thus it was concluded that the molecular technique Nested RT-PCR may have a wide applicability for confirmatory ante mortem diagnosis of rabies from body secretions and excretions within a short span of time.

Author's contribution

CKS: Substantial contribution to conception and design, MD: Acquisition of data, CKS, VR, DD, BSS, NKS: Analysis and Interpretation, MD, KB: Drafting the article, CKS, VR, DD, BSS, NKS: revising it critically for important intellectual content. All authors read and approved the final manuscript.

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Competing interests

Authors declare that they have no competing interests.

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