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Comparative evaluation of Nested RT-PCR and TaqMan real time PCR for ante mortem diagnosis of rabies from body secretion/excretion of animals

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ABSTRACT

Nested RT-PCR and TaqMan real time PCR techniques were compared for rabies diagnosis in 62 biological fluid samples collected ante mortem from animals clinically suspected to be rabid. The average sensitivity of Nested RT-PCR technique obtained from fluid samples was detected as 61.68% while for TaqMan real time PCR it was 72.07%. Usage of molecular approaches for ante mortem rabies diagnosis from fluid samples lead to conclusion that body secretion (saliva, milk) and excretion (urine) can be a viable, alternative tool for the ante mortem diagnosis of rabies where standard sample collection is not feasible in aggressive rabid animals.

Key words: Ante mortem, Diagnosis, Saliva, Milk, Urine

Rabies is largely a fatal disease which can be prevented by timely and appropriate anti rabies prophylaxis. Primary care of wounds, proper categorization of bite and use of Rabies Biologicals as RIG and vaccine can prevent this dreaded disease. However, diagnosis is highly significant component of rabies prevention and control. Rapid and reliable diagnosis can help in decreasing potential exposure of the affected individuals thus preventing further spread of rabies to humans and animals. Further, it also helps in controlling the cost of post exposure immunization. Therefore, present study was conducted to explore the role of biological fluids for intravitam diagnosis of rabies.

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 was identified from the salivary glands, lungs, kidneys, heart and liver (Vieira *et al*. 2011). 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 salivary glands, the adrenal medulla, nasal mucosa, cornea and epidermis are frequently infected via spread in peripheral nerves (Balachandran and Charlton 1994). The possibility of transfer of virus from axons to glandular epithelial cells of mammary gland cannot be ruled out however perusal of literature revealed dearth of published work on detection of rabies viral RNA in milk samples of lactating rabid animals.

It was reported that rabies virus was present within tubular cells of the kidneys and mice inoculated with urine from a rabid fox died of rabies (Debbi and Trimachi 1970). With the help of advance molecular approaches rabies virus RNA can be detected in a range of biological fluids and samples (e.g. saliva, CSF, tears, skin biopsy sample and urine) (Fooks *et al*. 2009).

Thus, the present study was envisaged to study the comparative sensitivity of nested RT-PCR and TaqMan real time PCR for intravitam diagnosis of rabies from biological fluid samples.

MATERIALS AND METHODS

Sample collection, RNA extraction and cDNA synthesis: In the present study, samples were collected for diagnosis of rabies from 25 rabies suspected animals presented to the Veterinary Clinics, GADVASU, Ludhiana, Punjab and Civil Veterinary Hospital from different districts of Punjab over a period of 17 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 (Table 1). Thus the total of 62 biological fluid samples served as a basis for current investigation.

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Soon after the clinical diagnosis was made, saliva (collected directly or by syringe aspiration), milk (strip milking), urine samples (direct while urinating or soiled samples post-urination) were collected in sterilized vials from animals clinically suspected to be rabid. The vials were stored at –80°C 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 2 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 according to the manufacturer's instructions. 400 µl quantity of fluid sample was used for RNA extraction.

The 10 µl of RNA was subjected to cDNA synthesis using a primer RabN1 (30 pmol/µl) and subjected to 65°C 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.

Reverse transcriptase 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. RNA and cDNA concentration was measured using nano drop spectrophotometer 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 (Nadin-Davis 1998, Nagaraj *et al*. 2006, 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) (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 extension step at 72°C for 5 min.

For the second round, 5 µl of first round PCR product was amplified using Rab Nfor and Rab Nrev and subjected to thermo cycling conditions as first round except annealing at 55°C 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). The agarose gels were visualized under Geldoc.

TaqMan real time PCR assay: Considering the N gene that is most conserved in *Lyssavirus* and sequence data concerned with gene are most exhaustive (Crepin *et al*. 1998). All TaqMan primers and probes (Table 1) were newly designed at School of Animal Biotechnology, GADVASU by the Primer Express 3.0 computer program. Primer and probe concentrations were optimized according to the manufacturer's recommendations.

The TaqMan real time assay was standardized with 20 µl PCR mixture volume consisting of 12.5 µl of TaqMan master mix with 1 µl of primers Rab-8F and Rab-8R (400nm/ µl) and 1 µl probe Rab-8Pr. (250nm/ µl) (Table 1), 2.5 µl of the cDNA prepared using RabN1 primer and 2 µl of RNAse free water was added to make a final volume. 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, 44°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 (*C*t) that represents the number of cycles in which the fluorescence intensity is significantly arose above the background fluorescence.

Since, FAT is recommended worldwide as a gold standard for diagnosis of rabies on neural tissue, after death of animal by World Health Organization (Hanlon *et al*. 1999). So, results obtained in TaqMan real time PCR on fluid samples were compared with FAT for detecting the sensitivity of this molecular technique.

RESULTS AND DISCUSSION

An attempt was made to detect rabies viral RNA from secretions (saliva, milk) and excretion (urine samples) by nested and TaqMan PCR techniques. The details of the results obtained with both the techniques are given in Table 2.

Sensitivity recorded from saliva samples using TaqMan and nested PCR was 77.27% and 68% respectively. Like Nagaraj *et al.* (2006) we also found real time PCR assay on saliva samples to be more sensitive than conventional RT-PCR assay. Test sensitivity of 78.94 and 62.5% with TaqMan PCR and nested PCR respectively on urine samples was obtained in this study. Dacheux *et al*. (2008) revealed sensitivity of 14.6% in group 1 and 9.5% in group 2 by the use of hnRT-PCR on human urine samples. The test sensitivity of 39% was obtained in a study on human urine samples with the use of nucleic acid-amplification tests

Table 1. Primers used for nested RT-PCR and TaqMan PCR

Primer name	Sequence	Gene	Positions
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 TTCTTC TTA 3'	N	876–896
Rab-8F	5'-TTG ACG GGAGGA ATG GAA CT-3'	N	434–453
Rab-8R	5'-GAC CGA CTA AAG ACG CAT GCT-3'	N	477–497
Probe Rab-8Pr	5'-FAM- AGG GAC CCC ACT GTT-TAMRA-3'	N	458–472

Table 2. Details of the results obtained with nested RT-PCR and TaqMan real time PCR

Species	Age	Sex	TaqMan results			FAT	Nested RT-PCR		
			Saliva	Milk	Urine	Brain	Saliva	Milk	Urine
Buffalo	6 years	F	38.879	39.657	39.137	+	-	-	-
Dog	3 months	M	32.432	NA	NA	+	+	NA	NA
Cow calf	6 months	F	32.551	NA	28.393	+	-	NA	+
Buffalo	5½ years	F	32.629	26.554	28.740	+	+	+	+
Dog	2 years	M	37.925	NA	39.642	-	-	NA	-
Buffalo	6 years	F	35.687	39.035	38.319	-	-		-
Buffalo	5 years	F	39.487	38.319	39.657	-	-		-
Cow calf	10 months	F	31.009	NA	27.611	+	+	NA	-
Cow calf	1 ½ years	F	30.551	NA	29.393	+	+	NA	+
Buffalo	3 ½ years	F	31.823	25.425	26.740	+	+	+	+
Buffalo	5 years	F	26.872	31.023	27.751	+	+	-	+
Cow	2 ½ years	F	39.642	NA	39.035	-	-	NA	-
Dog	4 years	F	NA	NA	33	+	NA	NA	-
Dog	10 months	M	33.195	NA	31.354	+	+	NA	-
Buffalo	6 years	F	36.462	35.93	39.487	+	-	-	-
Cow	7 years	F	33.151	39.035	NA	+	-	-	NA
Buffalo	7 years	F	38.004	36.362	38.121	+	-	-	-
Buffalo	5 ½ years	F	35.626	36.053	35.93	-	-	-	-
Buffalo	7 years	F	33.532	39.867	34.823	+	+	-	-
Buffalo	6 years	F	39.319	39.546	34.009	+	-	-	-
Cow	4 years	F	36.038	37.194	NA	-	-	-	NA
Buffalo	4 ½ years	F	27.457	32.023	29.346	+	+	-	+
Cow	6 ½ years	F	28.611	36.053	NA	+	-	-	NA
Buffalo	5 years	F	39.546	38.765	39.546	-	-	-	-
Buffalo	6 years	F	38.121	39.567	38.319	+	-	-	-
Total			12/24	4/17	11/21	18/25	9/24	2/17	6/21
77.27%	60%	78.94%	100%	68%	54.54%	62.5%			

+ve, Positive for rabies; -ve, negative for rabies; NA, not available.

(Wacharapluesadee and Hemachudha 2010).

More sensitive molecular techniques such as conventional and real-time PCR assays have produced satisfactory results for detection of rabies virus RNA from brain tissue and saliva (Black *et al*. 2002, Nagaraj *et al*. 2006).

Thus, it was concluded that TaqMan real time PCR could be employed for intravitam diagnosis of rabies using biological fluid samples e.g. saliva, milk, urine.

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