Indian J.L.Sci.1(2) : 93-96, 2012

INTRAVITAM DIAGNOSIS OF RABIES FROM SALIVA BY TAQMAN REAL TIME PCR

M. DANDALE[a], C. K. SINGH[b1], KARAN BANSAL[c], RAMNEEK[d], D. DEKA[e], B. S. SANDHU[f] AND N. K. SOOD[g]

[a]Department of Veterinary Pathology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, India
[a]E-mail: drmangesh9922@gmail.com
[b]E-mail: rabiesck@gmail.com
[c]E-mail: karanbansal35@gmail.com
School of Animal Biotechnology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, India
[d]E-mail : ramneekv@rediffmail.com
[e]E-mail: deepakdeka@gmail.com
Department of Veterinary Pathology,Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, India
[f]E-mail: bhupisandhu@rediffmail.com
[g]E-mail: nareshsood47@yahoo.com

ABSTRACT

Reliable and early diagnosis of rabies is of utmost importance to eliminate exposure and timely administration of post exposure prophylaxis.Present study reports efficiency of TaqMan real time PCR technique for intravitam diagnosis of rabies from saliva. Samples of saliva were collected from 24 (14 buffaloes, 7 cattle's and 3 dogs) animals suspected for rabies. Comparison of TaqMan real time was done with immunofluorescence test of brain for ascertaining the sensitivity of this molecular technique. By TaqMan real time PCR, viral RNA could be diagnosed in 12/24 saliva samples with a sensitivity of 77.27%. The present study concluded that use of TaqMan real time PCR on saliva samples is a viable approach for the intravitam diagnosis of rabies. This study may facilitate early diagnosis of rabies and thus enable the control efforts to decrease contact exposures by rabid animal and consequent cost of post exposure prophylaxis.

KEYWORDS: Intravitam, immunofluorescence, Real Time PCR, rabies, saliva

Rabies is progressive devastating zoonotic disease of mammals that causes fatal encephalomyelitis. In India, rabies is enzootic and is a serious public health and economic problem (Nagarajan et al., 2006). The appearance of specific rabies disease symptoms is preceded by prodromal period in which there are a number of non-specific symptoms of malaise (Bishop ,1979). Since a systematic approach to the clinical diagnosis of rabies in living animals is needed so that to allow or exclude a presumptive diagnosis of rabies and its differentiation from other neurological diseases. With the advent of molecular approaches, it is now possible to detect rabies intravitam. The early detection of this dreaded disease is of utmost importance to eliminate possible exposure and further post exposure prophylaxis.

Ante-mortem diagnosis of rabies by molecular techniques based on detecting virus or viral RNA has been attempted in body fluids of live animals such as saliva and CSF (Crepin et al. ,1998). Thus the present study was envisaged to evaluate the importance of TaqMan real time PCR technique for intavitam diagnosis of rabies from saliva samples.

MATERIALS AND METHODS

Saliva samples were collected from 24 rabies suspected animals (14 buffaloes, 4 cows, 3 cow calves and 3 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 saliva samples were collected either in a sterilized vial directly or with the help of sterile syringe from oral cavity of animal. Saliva samples obtained from two healthy animals served as negative controls. Rabies positive brain homogenate was used as positive control.

Total RNA from saliva 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 25˚C for 10 min, 37˚C for 2 h, 85˚C for 5 min and chilling on ice for 5

[1]**Corresponding author**

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.

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 a., 1998). 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 1). TaqMan probe was labeled at the 5' end with a fluorescent reporter dye (FAM) and at the 3' end with a quencher dye (TAMRA). Primer and probe concentrations were optimized according to the manufacturer's recommendations.

The TaqMan real time assay was carried out in 20 µl PCR mixture volume consisting of 12.5 µl of TaqMan master mix (Applied Biosystem, USA) with 1 µl of primers Rab-8F and Rab-8R (400nm/ µl) and 1 µl probe-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 15s, 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 (Ct) 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 standard technique 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 saliva samples were compared with FAT for detecting the sensitivity of this molecular technique.

RESULTS

Newly designed primers (Table 1) were used for amplification with TaqMan real time PCR. 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 (Hughes et al.,2004). By TaqMan real time PCR, viral RNA could be diagnosed in 12/24 saliva samples (Table 2).

DISCUSSION

TaqMan assay revealed that a positive Ct value was still obtained when only 1 pg of total RNA was added to the assay whereas 100 times more RNA is required to generate a visible band on an ethidium bromide-stained agarose gel (Wakekey et al., 2005).

The study confirms results of the others, who have

Table1: Details of primers and probe

Primer Name	Sequence	Gene	Length (nt)	Positions	Tmax (°c)	Remarks
Primer 8F	5'-TTG ACG GGA GGA ATG GAA CT -3'	N	20	434-453	62	Newly designed
Primer 8R	5'-GAC CGA CTA AAG ACG CAT GCT-3'	N	21	477-497	64	Newly designed
Probe 8Pr	5'-FAM- AGG GAC CCC ACT GTT TAMRA -3'	N	15	458-472	48	Newly designed

Table 2: TaqMan real time PCR on saliva samples

S.N.	Species	Age	Sex	Ct values (cycle threshold)	TaqMan real time PCR
1.	Buffalo	6 years	F	38.879	-
2.	Dog	3 months	M	32.432	+
3.	Cow calf	6 months	F	32.551	+
4.	Buffalo	5.5 years	F	32.629	+
5.	Dog	2 years	M	37.925	-
6.	Buffalo	6 years	F	35.687	-
7.	Buffalo	5 years	F	37.925	-
8.	Cow Calf	10 months	F	31.009	+
9.	Cow Calf	1.5 years	F	30.551	+
10.	Buffalo	3.5 years	F	31.823	+
11.	Buffalo	5 years	F	26.872	+
12.	Cow	.5 years	F	38.004	-
13.	Dog	10 months	M	33.195	+
14.	Buffalo	6 years	F	36.462	-
15.	Cow	7 years	F	33.151	+
16.	Buffalo	7 years	F	38.004	-
17.	Buffalo	5.5 years	F	35.626	-
18.	Buffalo	7 years	F	33.532	+
19.	Buffalo	6 years	F	39.319	-
20.	Cow	4 years	F	36.038	-
21.	Buffalo	4.5 years	F	27.457	+
22.	Cow	6.5 years	F	28.611	+
23.	Buffalo	5 years	F	39.546	-
24.	Buffalo	6 years	F	36.038	-
Total					**12/24**

applied the real-time PCR for the detection of rabies virus genotypes in saliva (Hughes et al., 2004, Nagaraj et al., 2006, Wakeley et al., 2006). The advantage with TaqMan real time assay is that it does not require multiple transfers of materials as it is done in a single closed tube that makes the detection of virus fast and minimizes the risk of cross-contamination.

Further, incorp1oration of TaqMan technology into a real-time assay not only enables the Lyssavirus template to be detected but also allows it to be genotyped (Orlowska et al., 2008).

ACKNOWLEDGEMENT

Authors are grateful to Dr. S N S Randhawa, Director of Research, GADVASU for providing the necessary research facilities and Dr H S Sandha, Director Animal Husbandry, Punjab for sponsoring research scheme entitled "Development of Research-cum-Diagnostic laboratory for Rabies".

REFERENCES

Bishop D.H.L., 1979. Rhabdoviruses. CRC Press, Boca Raton, Florida, Vol I:23-41.

Crepin P., Audry L and Rotivel Y., 1998. Intravitam diagnosis of human rabies using saliva and cerebrospinal fluid. J. Clin. Microbiol., **36**(4): 1117-1121.

Hughes G.J., Smith J.S., Hanlon C.A.and Rupprecht C.E., 2004 Evaluation of a TaqMan PCR Assay to Detect Rabies Virus RNA: Influence of Sequence Variation and Application to Quantification of Viral Loads. J. of Clin. Microbiol., **42**(1): 299-306.

Hanlon A.C., Smith S.J.and Anderson R.G., 1999. Article II: Laboratory Diagnosis of Rabies. J. Am. Vet. Med. Assoc., **215**: 1444-1447.

Heaton P.R., Johnstone P., McElhinney L.M., Cowley R., O'Sullivan E. and Whitby J.E., 1997. Heminested PCR assay for the detection of six genotypes of rabies and rabies-related viruses. J. Clin. Microbiol., **35** : 2762-2766.

Nagarajan T., Mohanasubramanian B., Seshagiri E.V., Nagendrakumar S.B.,Saseendranath M.R., Satyanarayana M.L., Thiagarajan D., Rangarajan P.N. and Srinivasan V.A. ,2006. Molecular Epidemiology of Rabies Virus Isolates in India. J. Clin. Microbiol., **44** (9): 3218-3224.

Nagaraj T., Vasanth J.P., Desai A., Kamat A., Madhusudan S.N.and Ravi V.,2006. Ante mortem diagnosis of human rabies using saliva samples: comparison of real time and conventional RT-PCR techniques. J. Clin. Microbiol., **36**:17-23.

Orłowska A., Smreczak M., Trębas P. and Żmudziński J.f., 2008. Comparison of real-time pcr and heminested rt-pcr methods in the detection of rabies virus infection in bats and terrestrial animals. Bull. Vet. Inst. Pulawy., **52**: 313-318.

Wakeley P.R., Johnson N.and McElhinney L.M. ,2005. Development of a Real-Time TaqMan Reverse Transcription-PCR Assay for Detection and Differentiation of Lyssavirus Genotypes 1, 5, and 6 J. Clin. Microbiol., **43**(6): 2786-2792.

Wakeley P.R., Johnson N., McElhinney L.M., Marston D., Sawyer J.and Fooks A.R. ,2006. Development of a real-time, differential, RT-PCR TaqMan assay for Lyssavirus genotypes 1, 5, and 6. Dev. Biol. (Basel), **126**: 227-236.