doi:10.5455/vetworld.2013.189-192

ORIGINAL RESEARCH

Sensitivity comparison of nested RT-PCR and TaqMan real time PCR for intravitam diagnosis of rabies in animals from urine samples

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Received: 18-07-2012, Accepted: 26-08-2012, Published online: 14-01-2013

How to cite this article: Dandale M, Singh CK, Ramneek V, Deka D, Bansal K and Sood NK (2013) Sensitivity comparison of nested RT-PCR and TaqMan real time PCR for intravitam diagnosis of rabies in animals from urine samples, *Vet. World* 6(4):189-192, doi:10.5455/vetworld.2013.189-192

Abstract

Aim: Early diagnosis of dreadful rabies is of utmost importance to restrict number of contacts and timely administration of post exposure prophylaxis. The present study was conducted to evaluate the sensitivity comparison of Nested RT-PCR with TaqMan real time PCR technique for intravitam diagnosis of rabies in animals from urine samples.

Materials and Methods: Advance molecular approaches Nested RT-PCR and TaqMan real time PCR was employed on 21 urine samples for intravitam diagnosis of rabies. Comparison of both the techniques was done with standard immunofluorescence test (FAT) applied on brain for postmortem confirmation of rabies.

Result: Rabies viral RNA was detected in 6/21 (28.57%) and 11/21 (52.38%) urine samples by application of Nested RT-PCR and TaqMan real time PCR respectively. Sensitivity obtained from both the techniques was 62.50% and 78.94% respectively when compared with gold standard immunofluorescence test (FAT).

Conclusion: TaqMan real time PCR can serve as more sensitive and viable approach for the intravitam diagnosis of rabies as compared to Nested RT-PCR for detection of rabies from urine of suspected animals.

Clinical importance: This study may serve as background for future intravitam rabies diagnostics**.**

Key words: ante mortem, immunofluorescence, rabies, real time PCR, urine

Introduction

Since time immemorial rabies continues to be a major health threat to mankind as well as all warm blooded animals. According to results of global surveillance by the World Health Organization, about 50,000 cases of human rabies occur each year [1], the majority of them in developing countries [2]. Rabies causes fatal encephalomyelitis. In India rabies is enzootic and is a serious public health and economic problem [3]. The appearance of specific rabies disease symptoms is preceded by prodromal period in which there are a number of non-specific symptoms of malaise [4]. Differentiation from other neurological diseases may require extensive investigations. Therefore, diagnosis is often confirmed late in the course of disease or post-mortem [5]. With the advent of molecular approaches, it is now possible to detect rabies ante-mortem from range of biological samples e.g. nuchal skin biopsy [6], saliva [7], Cerebrospinal fluid (CSF) [8] and urine samples [9].

Knowing the feasibility of detection of rabies from soiled urine sample especially in case of aggressive rabid animals, the present study was envisaged to evaluate the importance of TaqMan real time PCR technique for ante mortem diagnosis of rabies from urine samples.

Materials and Methods

Sample collection, RNA extraction and cDNA synthesis:

Urine samples were collected from 21 rabies suspected animals (14 buffaloes, 4 cattle's 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 urine samples were collected from the animals suspected to be rabid. Urine samples were collected directly in sterile containers while urinating or with urethral catheterization. Urine samples obtained from two healthy animals served as negative controls. Rabies positive brain homogenate was used as positive control.

Total RNA from urine 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.

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	-	

Table-2. Details of primers and probe for TaqMan real time assay

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

Nested RT-PCR assay: The procedure used for the nested RT-PCR based on N (Nucleoprotein) gene was that used earlier [10-12] 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 Rab Nfor and Rab Nrev 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).

TaqMan real time PCR assay: Considering the N gene that is most conserved in *Lyssavirus* and sequence data concerned with gene are most exhaustive [7]. All TaqMan primers and probes (Table-2) were newly designed at School of Animal Biotechnology, GADVASU by the Primer Express 3.0 computer program (Applied Biosystems, Foster City, California) 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 (Applied Biosystem, USA) with 1 µl of primers Rab-8F and Rab-8R (400nm/ µl) and 1 µl probe Rab-8Pr. (250nm/µl), 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 50ºC for 2 min, 95ºC for 10 min, followed by 40 cycles in two steps: 95ºC for 15 s, 44ºC 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 [13]. So, results obtained in TaqMan real time PCR on urine samples were compared with FAT for detecting the sensitivity of this molecular technique.

Table-3. Comparison of TaqMan real time PCR and Nested RT-PCR with FAT

Sr. no	Species	Age	Sex	Urine		FAT
				TaqMan real time PCR	Nested RT-PCR	Brain
1.	Buffalo	6 years	F	-	-	+
2.	Cow calf	6 months	F	+	+	+
3.	Buffalo	5½ years	F	+	+	+
4.	Dog	2 years	M	-	-	-
5.	Buffalo	6 years	F	-	-	-
6.	Buffalo	5 years	F	-	-	-
7.	Cow calf	10 months	F	+	-	+
8.	Cow Calf	1 ½ years	F	+	+	+
9.	Buffalo	3 ½ years	F	+	+	+
10.	Buffalo	5 years	F	+	+	+
11.	Cow.	2 ½ years	F	-	-	-
12.	Dog	4 years	F	+	-	+
13.	Dog	10 months	M	+	-	+
14.	Buffalo	6 years	F	-	-	+
15.	Buffalo	7 years	F	-		+
16.	Buffalo	5 ½ years	F	-	-	-
17.	Buffalo	7 years	F	+	-	+
18.	Buffalo	6 years	F	+	-	+
19.	Buffalo	4 ½ years	F	+	+	+
20.	Buffalo	5 years	F	-	-	-
21.	Buffalo	6 years	F	-	-	+
22.		+ve Control	F	NA	NA	+
23.		-ve Control	F	-	-	-
		-ve Control	F	-	-	-
	Total			11/21	6/21	15/21

NA- Not Available, + Positive, - Negative

Results

Amplification with primers Rab N1 and Rab N5 yielded 1477 bp first round product. Nested pair of primers (Rab Nfor and Rab Nrev) used for amplification in second round yielded 762 bp product as reported [10-12]. By nested RT-PCR, viral RNA could be diagnosed in 6/21 (28.57%) in urine samples (Table-3) with a sensitivity of 62.50%.

In TaqMan real time PCR 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 [14]. With this technique viral RNA could be diagnosed in 11/21 (52.38%) urine samples sensitivity of 78.94% was obtained when compared with gold standard immunofluorescence test (FAT) on brain (Table-3). Percent of positivity (52.38 %) was higher in current study as compared with that of 44.4% (16/36) obtained with the NASBA [9] assay though sensitivity was less.

Discussion

Outward spread of rabies virus from the CNS leads to infection of almost all organs, including infection of the heart, lung, gastrointestinal tract, bone marrow, cornea, neck tissues, kidneys, and salivary glands. This has made the ante moretm diagnosis of rabies from various biological samples [7, 15-17]. The specimens for ante-mortem testing such as saliva, urine and cerebrospinal fluid (CSF) cannot be processed by Direct Fluorescent Antibody Technique so nucleic acid amplification tests are the method of choice [6, 15]. The percent positivity obtained with NASBA on urine was 44.4% (16 of 36 specimens) and also showed superior sensitivity to nested RT-PCR, which targets the N gene, in ante-mortem specimens while percent positivity [15]. In another study with NASBA assay on urine samples positivity percentage was 39% (16/41) [9]. Rabies virus RNA could be recovered from urine samples obtained from 4 dogs and could also be recovered from tissues like bladder, bladder trigone, urethral sphincter, nerve, ureter, renal pelvis, renal medulla, and renal cortex tissues suggesting that neural spread of the virus to the bladder is the primary event in the progression of infection, with a subsequent propagation of the virus to renal structures [18].

Results obtained by molecular methods on urine samples in the present study correlates well with earlier studies. While focusing on the comparative efficiency of Real time PCR assay and other conventional RT-PCR assay earlier was more sensitive (sensitivity 75% versus 37%,) [11]. The more sensitivity of the TaqMan real-time assay was reported when compared with conventional RT-PCR or nested PCR [11, 19-22]. The TaqMan-based assay was reported 1000-fold more sensitive than traditional RT-nPCR in raccoon rabies viral standard stock [22].

Molecular methods, although useful and extremely sensitive, may not always give positive results for patients with rabies. This may be due to the intermittence of virus shedding, the timing of sample collection, and the type of specimens collected. Moreover, the extent the clinical type of rabies (particularly paralytic rabies and cases with atypical features) influences the outcome of laboratory results [23]. Thus, it was concluded that TaqMan real time PCR could be a feasible approach as compared to Nested RT-PCR for ante mortem diagnosis of rabies. This study highlights its utility in establishing ante mortem diagnosis of rabies using urine samples within a few hours.

Conclusion

TaqMan real time PCR can serve as more sensitive and viable approach for the intravitam diagnosis of rabies as compared to Nested RT-PCR for detection of rabies from urine of suspected animals.

Authors' contribution

CKS-Substantial contribution to conception and design, MD and KB-Acquisition of data and drafted the manuscript, CKS, DD, BSS, R and NKS-analysed and interpreted the results, revised manuscript critically for important intellectual content. All authors read and approved the final manuscript.

Acknowledgements

Authors are grateful to Dr. S. N. S. Randhawa, Director of Research, Guru Angad Dev Veterinary and Animal Science University for providing the necessary facilities and Dr H S Sandha, Director, Animal Husbandry for sponsoring research scheme entitled "Development of Research-cum-Diagnostic laboratory for Rabies". The study relates to the naturally prevalent cases and, thus, does not include any experiments.

Competing interests

Authors declare that they have no competing interest.

References

1. Menezes R. (2008) Rabies in India. *Can. Med. Assoc.*, 178 (5): 564–566.
2. Madhusudana SN., Sukumaran SM. (2008) Antemortem diagnosis and prevention of human rabies. *Ann. Ind. Acad. Neurol.*, 11(1): 3–12.
3. Nagarajan T., Mohanasubramanian B., Seshagiri EV., Nagendrakumar SB., Saseendranath MR., Satyanarayana ML., Thiagarajan D., Rangarajan PN., Srinivasan VA. (2006) Molecular Epidemiology of Rabies Virus Isolates in India. *J. Clin. Microbiol*, 44 (9): 3218–3224.
4. Bishop DHL. (1979) *Rhabdoviruses*. CRC Press, Boca Raton, Florida. Vol I: 23-41.
5. Fishbein DB., Smith JS., Rupprecht C., Clark K. (1991) Unexplained rabies in three immigrants in the United States: A virological investigation. *New. Engl. J. Med*, 324: 205-11.
6. Dacheux L., Reynes JM., Buchy P., Sivuth O., Diop BM., Rousset D., Rathat C., Jolly N., Dufourcq JB., Nareth C., Diop S., Rajerison ICR., Sadorge C., Bourhy H. (2008) A Reliable Diagnosis of Human Rabies Based on Analysis of Skin Biopsy Specimens. *Clin. Infec. Dis*, 47: 1410–1417.
7. Kaw A., Singh CK., Ramneek., Sood NK., Sandhu BS., Deka D., Awahan S. (2012) Intravitam diagnosis of rabies from saliva by Nested RT-PCR and real time PCR. *Braz. J. Vet. Pathol*, 5(2): 70-73.
8. Saengseesom W., Mitmoonpitak C., Kasempimolporn S.,

Sitprija V. (2007) Real-time PCR analysis of dog cerebrospinal fluid and saliva samples for ante-mortem diagnosis of rabies. *Southeast Asian J. Trop. Med. Public Health,* 38(1): 53-57.
9. Wacharapluesadee S., Hemachudha T. (2010) Ante-mortem and post-mortem diagnosis of rabies using nucleic acid-amplification tests. *Expert Rev Mol. Diagn,* 10(2): 1-12.
10. Nadin-Davis SA. (1998). Polymerase chain reaction protocols for rabies virus discrimination. *J. Virol. Methods,* 75: 1-8.
11. Nagaraj T., Joel PV., Desai A., Kamat A., Madhusudana SN., Ravi V. (2006) Ante mortem diagnosis of human rabies using saliva samples: Comparison of real time and conventional RT-PCR techniques. *J. Clin. Virol,* 36: 17–23.
12. Kaw A., Singh CK., Sandhu BS., Sood NK., Ramneek., Deka D., Awahan S. (2011) Diagnosis of rabies in animals by Nested RT-PCR. *Indian. J. Anim. Sci*, 81(4): 367-369.
13. Hanlon AC., Smith SJ., Anderson RG. (1999) Article II: Laboratory diagnosis of rabies. *J. Am. Vet. Med. Assoc,* 215: 1444-1447.
14. Hughes GJ., Smith JS., Hanlon CA., Rupprecht CE. (2004). Evaluation of a Taq-Man PCR assay to detect rabies virus RNA: influence of sequence variation and application to quantification of viral loads. *J. Clin. Microbiol,* 42: 299–306.
15. Fooks AR., Johnson N., Conrad MF., Wakeley PR., Banyard AC., McElhinney LM., Marston DA., Dastjerdi A., Wright E., Weiss RA., Muller T. (2009). Emerging Technologies for the Detection of Rabies Virus: Challenges and Hopes in the 21st Century. *PLoS Negl. Trop. Dis,* 3(9): e530. doi: 10.1371/journal.pntd.0000530.
16. Wacharapluesadee S., Hemachudha T. (2002). Urine samples for rabies RNA detection in the diagnosis of rabies in humans. *Clin. Infect. Dis*, 34:874-875.
17. Hemachudha T., Laothamatas J., Rupprecht CE. (2002). Human rabies: a disease of complex neuropathogenetic mechanisms and diagnostic challenges. *Lancet Neurol*, 1: 101-109.
18. Sitprija V., Sriaroon C., Lumlertdaecha B., Wacharapluesadee S., Phumesin P., Khawplod P., Wilde H., Hemachudha T. (2003). Does Contact with Urine and Blood from a Rabid Dog Represent a Rabies Risk? *IDSA,* 37 (10): 1399-1400.
19. Smith J., McElhinney LM., Heaton PR., Black EM., Lowings JP. (2000). Assessment of template quality by the incorporation of an internal control into a RT-PCR for the detection of rabies and rabies-related viruses. *J. Virol. Methods,* 84(2):107–115.
20. Coertse J., Weyer J., Nel LH, Markotter W. (2010). Improved PCR Methods for Detection of African Rabies and Rabies-Related Lyssaviruses. *J. Clin. Microbiol*. 48(11):3949–3955.
21. Franka R., Johnson N., Muller T., Vos A., Neubert L., Freuling C., Rupprecht CE.,Fooks AR. (2008). Susceptibility of North American big brown bats (*Eptesicus fuscus*) to infection with European bat lyssavirus type 1. *J. Gen. Virol,* 89: 1998–2010.
22. Nadin-Davis SA., Sheen M., Wandeler AI. (2009). Development of real-time reverse transcriptase polymerase chain reaction methods for human rabies diagnosis. *J. Med. Virol,* 81(8):1484–1497.
23. Hemachudha T., Wacharapluesadee S. (2004). Ante mortem Diagnosis of Human Rabies. *Clin. Infect. Dis*, 39 (7):1085-1086.

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