ANTEMORTEM DIAGNOSIS OF RABIES IN BUFFALOES: MOLECULAR APPROACH

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ABSTRACT

Antemortem diagnosis of rabies is highly desirable in animals for limiting the exposure of this fatal zoonotic viral disease. Molecular approaches offer a sensitive tool for detection of rabies viral genome. However, the sensitivity of antemortem detection of rabies from skin of buffalo by molecular approaches has not been compared so far. Comparison of TaqMan real time PCR and heminested reverse transcriptase PCR (HnRT-PCR) was, therefore, undertaken to diagnose rabies from skin biopsy samples collected from eleven clinically rabies suspected buffaloes presented to veterinary clinics, GADVASU, Ludhiana, from different districts of Punjab (India). RNA was extracted from all skin samples and converted to cDNA followed by PCR amplification using both molecular methods. Out of 11 cases incorporated in the study 8 skin samples were diagnosed positive using TaqMan real time PCR and HnRT-PCR. FAT on brain tissue procured postmortem established same 8 cases as true positive for rabies. Thus, TaqMan real time PCR well as HnRT-PCR detected rabies with 100% sensitivity, specificity and accuracy. The agreement between the results obtained by these methods was 100%, thereby TaqMan real time PCR and HnRT-PCR are equally effective molecular approaches for antemortem diagnosis of rabies from skin biopsies of buffaloes.

Keywords: HnRT-PCR, TaqMan real time PCR, antemortem, buffaloes, rabies

INTRODUCTION

Rabies is an acute, progressive and fatal viral disease causing encephalomyelitis in many domestic and wild mammals including human beings. The causative agent of rabies belongs to the family Rhabdoviridae and genus *Lyssa virus* (Pringle, 1991). It has always been a cause of serious concern for veterinary and medical fraternity alike. Strict control strategies are of utmost importance to detain the existence of rabies. Antemortem diagnosis of rabies is instrumental in achieving effective rabies control by reducing the number of exposure while animal is in incubation, or for advocating post exposure therapeutics to exposed persons**.** Rapid diagnosis of rabies obviates the expenses and discomfort of unnecessary diagnostic tests and inappropriate therapy.

If the animal is not rabid, prompt diagnosis

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may save a patient from unnecessary physical and psychological trauma (Helmick, 1983). Centrifugal spread of virus to peripheral tissues starts early during the pre-clinical phase (Jogai *et al*., 2002). It localizes the virus in nerves surrounding hair follicles; therefore, skin offers a viable source for viral RNA detection for antemortem diagnosis of rabies. Molecular approaches have been reported to offer sensitive and specific tool for antemortem detection of rabies (Crepin *et al*., 1998) and have also been widely accepted as method of choice for *intravitam* rabies diagnosis in humans (Benedictis *et al*., 2011).

Out of all conventional PCR approaches, HnRT-PCR technique has been shown to offer promise in detection of rabies from brain specimens because of its higher sensitivity (Heaton *et al*., 1997). Real time PCR has been reported to be more sensitive molecular method as compared to conventional reverse transciptase PCR methods (Wacharapluesadee and Hemachudha, 2010). Orlowska *et al*. (2008) have in fact reported TaqMan real time PCR to be tenfold more sensitive than the HnRT-PCR for detection of rabies from brain samples. However, the sensitivity of detection of rabies from skin by molecular approaches from buffalo has not been compared so far. Therefore, present study was envisaged to study comparative sensitivity to diagnose rabies from skin biopsy samples by TaqMan real time PCR and HnRT-PCR in buffaloes.

MATERIALS AND METHODS

Antemortem collection of skin biopsy samples from 11 clinically rabies suspected buffaloes presented to veterinary clinics GADVASU Ludhiana, Punjab was done with the help of sterilized 3 mm skin biopsy punch from nape of the neck region. Brain tissue samples from the same animals were also collected at postmortem examination.

Skin (100 mg) was triturated with the help of sterilized sand, in pestle and mortar. One ml of PBS (pH 7.2) was used as diluents to make 10% (w/v) suspension. RNA extraction was performed using Trizol method. Total extracted RNA was converted into cDNA using High-Capacity cDNA Reverse Transcription Kit with RNAse inhibitor (Applied Biosystems, USA). Skin sample cDNA was stored at -80°C (Ultra low temperature freezer, Haier, Biomedical) for HnRT-PCR and TaqMan real time PCR amplification whereas brain tissue samples were stored at -20°C and used for confirmatory diagnosis by using gold standard fluorescent antibody test (FAT).

TaqMan real time PCR

TaqMan real time PCR amplification was performed by Applied Biosystems, Step one plus real time system in Department of Veterinary Microbiology, COVS, GADVASU, Ludhiana. The Real Time PCR reaction was carried out in 20 µl reaction volume and all the samples were run in triplicates. All samples were also run with endogenous control 18S rRNA (Applied Biosystems, USA) to test sample integrity and verification of RNA extraction. Primers targeted to highly conserved and most abundant N genes were used (Table 1.). Fresh master mix for the PCR was prepared in PCR tubes on ice (Table 2.). Thermocycling conditions were one cycle of denaturation at 95ºC for 10 minutes, 40 cycles each of denaturation at 95ºC for 15 seconds and annealing at 60°C for 1 minute.

Analysis

After completion of PCR, critical threshold cycle number (C_T) was determined corresponding to the PCR cycle number at which the fluorescence of the reaction exceeded a value determined to be statistically higher than the background by the ABI step one plus software. The C_T values were inversely proportional to the log10 of the amount of template in the PCR. A C_T value less than the mean plus two standard deviations of the negative control wells was considered positive. A C_T value above 35 was taken as no amplification. The resultant data were transferred into an Excel spread sheet, and the resulting graphs were obtained.

Table 1. Primers for TaqMan real time directed against N gene.

Primer Name	Sequence	Gene	Length (nt)	Positions	Tmax (ºc)	Reference
Primer 8F	5'-TTG ACG GGA GGA ATG GAA CT-3'	N	20	434-453	62	Bansal *et al*., 2012
Primer 8R	5'-GAC CGA CTA AAG ACG CAT GCT-3'	N	21	477-497	64	
Probe 8Pr	5'-FAM- AGG GAC CCC ACT GTT-TAMRA-3'	N	15	458-472	48	

Table 2. Master mixture constituents for TaqMan real time PCR.

Components	Volume
TaqMan Master mix	12.5 µl
Forward primer (10 pmol)	1.0 µl
Reverse primer (10 pmol)	1.0 µl
Probe (6 pmol)	1.0 µl
Nuclease Free Water	2.5 µl
cDNA	2.0 µl
Total	20 µl

Table 3. Primers targeting the nucleoprotein gene.

Primer	Sequences	Gene	Position	Sense	Reference
JW 12 Forward primer 1ST round	5'ATGTAACACCCCTACAATG3'	N	55-73	+	Orlowska *et al*. (2008)
JW 6 DPL Reverse primer 1ST round	5'CAATTGGCACACATTTTGTG3'	N	660-641	-	
JW 10 P Reverse primer 2nd round	5'GTCATCAGAGTATGGTGTTC3'	N	636-617	-	

HnRT-PCR

HnRT-PCR was performed by Applied Biosystems thermal cycler. cDNA was subjected to PCR amplification using primers targeted towards nucleoprotein (N) gene for primary PCR and HnRT-PCR (Table 3). Primary PCR was carried out in 25µl of reaction volume using Go Taq green PCR master mix (Promega) and 2 µl of cDNA, 1 µl of forward and reverse primers, subjected to PCR cycling conditions initial denaturation at 94°C for 3 minutes amplification for 35 cycles, denaturation at 94°C for 30 seconds, hybridization at 56°C for 45 seconds, elongation at 72°C for 40 seconds and final extension step of 3 minutes at 72ºC with slight modifications as reported by Dacheux *et al.* (2008). Primary PCR product served as a template for secondary PCR and the same PCR cycling conditions were employed.

FAT was performed as per the standard protocol (Meslin *et al*., 1996) and used as reference test to establish true positive cases. The sensitivity, specificity and accuracy of various tests applied were calculated using formulae:

RESULTS AND DISCUSSION

FAT is gold standard test for rabies, therefore in our study it was used as reference method of diagnosis. FAT on brain established eight cases as true positive for rabies on the basis of characteristic apple green immunofluoroscence observed in positive cases.

Eight out of eleven clinically suspected buffaloes samples were diagnosed positive for rabies by TaqMan real time PCR. C_T values equal or below 35 were considered positive and above 35 were considered negative (Table 4). Sensitivity, specificity and accuracy of TaqMan real time PCR for detecting of rabies from skin biopsies sample was 100% which is comparatively higher than the earlier finding of Bansal *et al.* (2012). They reported sensitivity of 86.67% on skin sample using TaqMan real time PCR wherein detected 11 animals positive including 3 buffaloes out of 20 suspected animals. Wacharapluesadee *et al*. (2012) reported sensitivity 81.8% on whiskers and 66.7% on hair follicles.

$$\text{Sensitivity} = \frac{\text{True positive}}{\text{True positive + False negative}} \times 100$$

$$\text{Specificity} = \frac{\text{True negative}}{\text{False positive + True negative}} \times 100$$

$$\text{Accuracy} = \frac{\text{True positive + True negative}}{\text{True positive + False positive + False negative + True negative}} \times 100$$

The present study HnRT-PCR first round JW12 and JW10 primers yielded amplified product of expected 606 bp band size and second round PCR product of expected 586 bp size, amplification with primers JW12 and JW6 yielded first round product and primers yielded 586 bp products in second round of HnRT-PCR. Similar product size has been reported in earlier study by Smith *et al*. (2003) wherein HnRT-PCR could detect rabies from skin samples confirmed the presence of classical rabies virus (genotype 1) in both the saliva and skin specimens from a 55 year old man. Out of 11 cDNA samples tested from buffalo's skin, 8 samples were found positive for rabies using HnRT-PCR. Thus, HnRT-PCR diagnosed rabies antemortem in buffaloes with 100% sensitivity, specificity and accuracy. Dacheux *et al.* (2008) employed HnRT-PCR targeting polymerase gene (L) of virus for antemortem rabies diagnosis in human beings and reported sensitivity of 98%, however in our study N gene targeted primers were used in both HnRT-PCR and TaqMan real time PCR since N gene is the most conserved region of viral genome (Crepin *et al*., 1998).

Careful perusal of literature revealed that no other work on ante-mortem detection of rabies viral RNA from skin samples in buffaloes using HnRT-PCR has been reported. Thus, this seems to be a pioneer attempt wherein this technique had been used for the ante-mortem diagnosis of rabies in skin samples from naturally infected buffaloes. Similarly no comparison between sensitivity of HnRT-PCR and TaqMan real time PCR in skin samples have been reported. However comparison of these techniques have been reported on saliva (Coertse *et al*., 2010) and on brain samples (Wakeley *et al*., 2005; Orlowska *et al*., 2008) in other species of animals and in human beings.

Present study revealed the similar sensitivities of both techniques on skin biopsies, it was similar to findings of Coertse *et al*. (2010) wherein employed these techniques for African *lyssavirus* detection in antemortem and postmortem samples of canines, felines, mongoose and human beings. In contrast, 10 fold increased sensitivity of detection of rabies in brain samples of bats and terrestrial animals by TaqMan real time PCR was reported by Orlowska *et al*. (2008). However in other study on animal brain samples including 2 of

Table 4. Comparison of detection methods for rabies positive antemortem samples.

S. No.	Laboratory identification no.	Species	Sample	TaqMan real time PCR		HnRT-PCR
				C_T value	Results	Results
1	RL 42 /12	Buffalo	Skin biopsy	21.8462	+	+
2	RL 43 /12	Buffalo	Skin biopsy	30.32783	+	+
3	RL 53 /12	Buffalo	Skin biopsy	21.52078	+	+
4	RL 54 /12	Buffalo	Skin biopsy	24.08585	+	+
5	RL 2 /13	Buffalo	Skin biopsy	26.47682	+	+
6	RL 4 /13	Buffalo	Skin biopsy	32.10932	+	+
7	RL 8 /13	Buffalo	Skin biopsy	25.77398	+	+
8	RL 11 /13	Buffalo	Skin biopsy	30.9695	+	+

bovines, Hughes *et al.* (2004) reported decreased sensitivity of detection of rabies by using TaqMan PCR as compared to HnRT-PCR. These studies represent variation in the sensitivity of detection of rabies in different samples and in different species of animals therefore studies evaluating most reliable diagnostic method in all major species of animals are highly desirable.

Present study concludes both HnRT-PCR and TaqMan real time PCR can be used as alternate diagnostic approaches depending on the feasibility of test method for antemortem rabies diagnosis in buffaloes. In developing countries despite of several advantages of TaqMan real time PCR only few laboratories employ it as routine diagnostic test method as it requires expensive equipments though technique is rapid and less prone to contamination. Therefore HnRT-PCR is feasible and equally sensitive alternate to TaqMan real time PCR for antemortem diagnosis of rabies in buffaloes using least invasive skin biopsies.

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