

Original Research Article

<https://doi.org/10.20546/ijcmas.2017.606.142>

Comparative Assessment of Seller's Staining Test (SST), Direct Fluorescent Antibody Test and Real Time PCR for Diagnosis of Rabies in Dogs

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A B S T R A C T

Keywords

Rabies, Seller's staining test, Direct fluorescent antibody test, Real time reverse transcriptase polymerase chain reaction.

Article Info

Accepted:
19 May 2017
Available Online:
10 June 2017

Rabies is a highly fatal viral infection of central nervous system affecting all warm blooded animals including humans. The virus belongs to the *Rhabdoviridae* family, genus *Lyssa* virus. Several techniques have been standardized internationally for the diagnosis of rabies. This study was designed to assess the efficacy of Seller's staining test (SST) and Direct Fluorescent Antibody Test (dFAT) for diagnosis of rabies antigen, Mouse Inoculation Test (MIT) and real time reverse transcriptase polymerase chain reaction (qRT-PCR) were used as the Gold Standards. Antibodies against the N Nucleoprotein were used for the dFAT, whereas the nucleoprotein gene was targeted in the qRT-PCR assay. A total of 10 rabies suspected dog brain samples received at the Department of Microbiology at Bomabay Veterinary College, Mumbai, were tested by Seller's staining test (SST), Direct Fluorescent Antibody Test (dFAT) and Mouse Inoculation Test (MIT) as per the protocol described by WHO (2007). Out of 10 brain specimens Negri bodies were observed in 05 (50%) brain samples by SST. dFAT tested positive for 06 (60%) samples, rabies virus was isolated in 07 samples by intra-cerebral inoculation in Swiss-albino mice and 07 (70%) samples positive for rabies using qRT-PCR. Compared to the qRT-PCR, the sensitivities of SST and dFAT were 71.42 % and 85.71%, respectively. It was concluded that qRT-PCR is a more sensitive and better molecular approach for diagnosis of rabies from brain of rabid suspected cases as compare to SST and dFAT.

Introduction

Rabies is a highly fatal viral zoonotic disease of mammals caused by a neurotropic virus belonging to the *Lyssavirus* genus, in the family *Rhabdoviridae*. In India the disease is enzootic in nature with dogs as the main vector of transmission accounting for 20,000 human deaths per year (Sudarshan *et al.*, 2007). The Seller's staining is an old method used for diagnosis rabies by detection of negribodies. According to WHO, the direct fluorescent antibody technique (dFAT) which

is a gold standard for rabies diagnosis because of short duration, low cost and higher sensitivity. As an accessory to dFAT mouse inoculation is also carried out especially in developing countries, which is highly sensitive method but requires several days to get the result (Chhabra *et al.*, 2005).

In tropical countries like India, where the sample shipment may take longer time to reach laboratories for diagnosis with high

probability of break in cold chain leading to decomposition, In such condition the real time reverse transcriptase polymerase chain reaction (RT-qPCR) which have high sensitivity may serve as better diagnostic assays (Manjunathareddy *et al.*, 2016).

Many studies carried out to develop and validate of RT-qPCR for diagnosis of rabies in human (Nagaraj *et al.*, 2006; Wacharapluesadee *et al.*, 2008; Nadin-Davis *et al.*, 2009). Whereas, very few independent single test based studies are carried out on animal rabies diagnosis especially in Indian sub continent (Gupta *et al.*, 2001; Jayakumar *et al.*, 2003; Praveen *et al.*, 2007; Kaw *et al.*, 2011).

The present study describes the comparative evaluation of seller's staining, dFAT, with syber green real time PCR for the diagnosis of rabies in Dogs.

Materials and Methods

A total of ten rabies suspected brain samples collected and received at the Department of Microbiology at Bombay Veterinary College, Mumbai were used in the study.

Seller's staining

A fresh touch impression smear of the hippocampus, cerebral cortex and cerebellum of each sample was made on a clean glass slide. Seller's staining was performed according to the technique described by WHO (2007). Each smear was stained with 1% solution of basic fuchsin and methylene blue in Methanol (Acetone free) for 2-5 seconds and rinsed in running water. The slides were viewed under 100X (oil immersion) objective. Negri bodies were detected on positive slides as magenta staining depending on its density with sharply defined spherical, elongated or oval bodies with dark blue medium to large granules within the matrix of the body.

Direct fluorescent antibody technique (dFAT)

Direct fluorescent antibody technique was carried out according to the technique described by WHO (2007). Impression smear preparations of the hippocampus were placed in a Coplin jar containing acetone and fixed overnight in chilled acetone at -20°C. The slides were air-dried and stained with Fluorescence Isothiocyanate (FITC) labelled rabies specific polyclonal antibodies (Millipore, Light Diagnostics). These were then incubated at 37 °C for 30 minutes in a humid chamber and further washed with Phosphate Buffered Saline (PBS) in 3 successive washes for 5-10 minutes. The slides were rinsed with distilled water; air-dried and mounting buffered glycerol applied, then visualized under an immunofluorescent microscope (Olympus) at 20x and 40x objectives. Particles emitting bright apple green color fluorescence were considered as positive for presence of rabies virus antigen and were recorded as FAT positive. Positive and negative controls were run together with the test specimens.

Mouse inoculation test

The intra-cerebral mouse inoculation test (MIT) was conducted according to by WHO (2007). The pieces of brain tissues of approximately 1cm diameter were cut out from different areas of the brain hippocampus (Ammon's horn), cerebrum, cerebellum, medulla, pons was macerated in a mortar, diluted to 10% in PBS (PH 7.2) containing penicillin 50 IU – Streptomycin 2mg/ml, centrifuged (1500 rpm for 5 minutes) and filtered through 0.2 µm syringe filters. At least 5 suckling mice (3 days old) were inoculated intracerebrally, each with 0.03ml of the supernatant and observed daily for 21 days for any signs of rabies (roughening and loss of luster of the fur, tremor, hyper-

excitability, arching of back, convulsion, paralysis of the hind legs and death). PBS, PH 7.2 was used for inoculation of the negative control.

Isolation of RNA

Total RNA from brain tissue was extracted by TRIZOL method as described by Nagaraj *et al.* (2006). In Brief, 50-100 mg of brain tissue was homogenized in 1 ml of Trizol-LS reagent (Invitrogen, USA). After incubation, 200 µl of chloroform and 2 µl of glycogen (Ambion, Life technologies Pvt. Ltd., USA) was added and mixed vigorously for 15 seconds and incubated for 10 min on ice. The aqueous phase collected after centrifugation @ 13000 rpm for 10 min at 4 °C was mixed with 500 µl of isopropanol, followed by centrifugation at 13000 rpm for 10 min at 4 °C. The RNA pellet was washed with 1 ml of 70% ethanol, dried and dissolved in 50 µl DEPC treated water (Ambion, Life technologies Pvt. Ltd., USA) and concentration of RNA was estimated by spectrophotometer (ND 1000 Thermo Scientific, USA).

cDNA preparation

The cDNA synthesis was carried out using high capacity SuperScript™ III First-Strand Synthesis Kit (Invitrogen, Life Technologies, USA) and as per the manufacturer's instructions. The RNA extracted from brains samples was subjected cDNA synthesis using Gene Specific Primers (GSPs), RabN1 forward primer for preparing a template for its further use in SYBR green real time PCR. The primary reaction mixture for cDNA synthesis prepared by adding briefly, 5 µl of total RNA, 2 µl primer RabN1 (50 pm/µl), 1 µl dNTP mix (10 mM) and 2 µl DEPC treated water. A total of 10 µl of primary reaction mixture for cDNA mixtures prepared was vortexed briefly and incubated at 65⁰C for 5 min and kept on ice for at least 1 min.

Then a volume of 10 µl cDNA synthesis mix was prepared by adding 42 µl of 10X RT buffer, 4 µl of 25 mM MgCl₂, 2 µl of 0.1 M DTT, 1 µl of RNase OUT™ (40 U/µl) and 1 µl of SuperScript™ III RT (200 U/µl). The reaction mixture was incubated at 50°C for 50 min followed by 85°C for 5 min. 1 µl of RNase H was added to each tube and incubated at 37°C for 20 min to remove RNA and stored at -20°C until further use

Real time PCR (RT-qPCR)

SYBR green real time PCR for amplification of 135 bp region N gene of rabies virus was performed as per Nagaraj *et al.*, (2006). The oligonucleotides required for RT-PCR (Table 1) were custom synthesized and supplied by M/s. Sigma Aldrich (USA). The reaction was set in a total volume of 20 µl consisting. The reaction was performed for 40 cycles with 58.2°C for 30 seconds. Positive control (cDNA from mouse brain infected with CVS) and negative control (cDNA from PBS control mouse brain) assays were run along with NTC (no-template control). The reaction mixture was vortexed briefly and set into thermal cycler, Applied Biosystems Step One plus Real Time PCR system available at Department of Virology and Immunology, Haffkine Institute, Parel, Mumbai.

Results and Discussion

In the present study comparison of three diagnostic methods (SST, dFAT, and RT-qPCR) was carried out with a total of 10 suspected rabies brain samples from dogs.

Out of 10 brain specimens Negri bodies were observed in 05 (50%) brain samples by SST. dFAT tested positive for 06 (60%) samples and rabies virus was isolated in 07 samples by intra-cerebral inoculation in Swiss-albino mice. However, RT-qPCR could detect the N gene in 07 samples without any non-specific

reactions with specific melting temperature (87.24-88.16⁰C) (Figure 1).

In India, dogs play an important role as the reservoir and transmit rabies to humans and domestic animals in urban cycle, while jackals, wolves and foxes maintain the rabies virus in sylvatic cycle (Reddy *et al.*, 2011). The need to establish a more reliable and accurate test for rabies diagnosis for proper decisions regarding the treatment of potentially exposed individuals cannot be over emphasized.

In the present study we first screened the all suspected rabies brain by SST, dFAT and MIT. Total 05 (50%) brain specimens showed oval or round magenta red coloured Negri bodies were seen in stained with seller's stain. dFAT considered as the gold standard for rabies diagnosis by WHO, but in our study with dFAT we were able to detect only 06

(60%) out of 10 brain specimens. MIT were used as a confirmative test in the present study by which 07 samples out of 10 samples were positive.

This is in agreement with similar findings by Robles and Miranda (1992), and shows that viral isolation is the most sensitive test in situations where the amount of antigen is too little to be detected by other tests. The MIT being the reference test in our study was assumed to have a specificity and sensitivity of 100%. However, it has been shown that the quality of the specimen submitted may interfere with the accuracy of the result. This study showed that the dFAT is a sensitive and reliable test (85.71%) as compare to the SST (71.42%). The slight difference in sensitivity of dFAT compared to the MIT suggests that all dFAT-negative samples should be confirmed by MIT (Table 2).

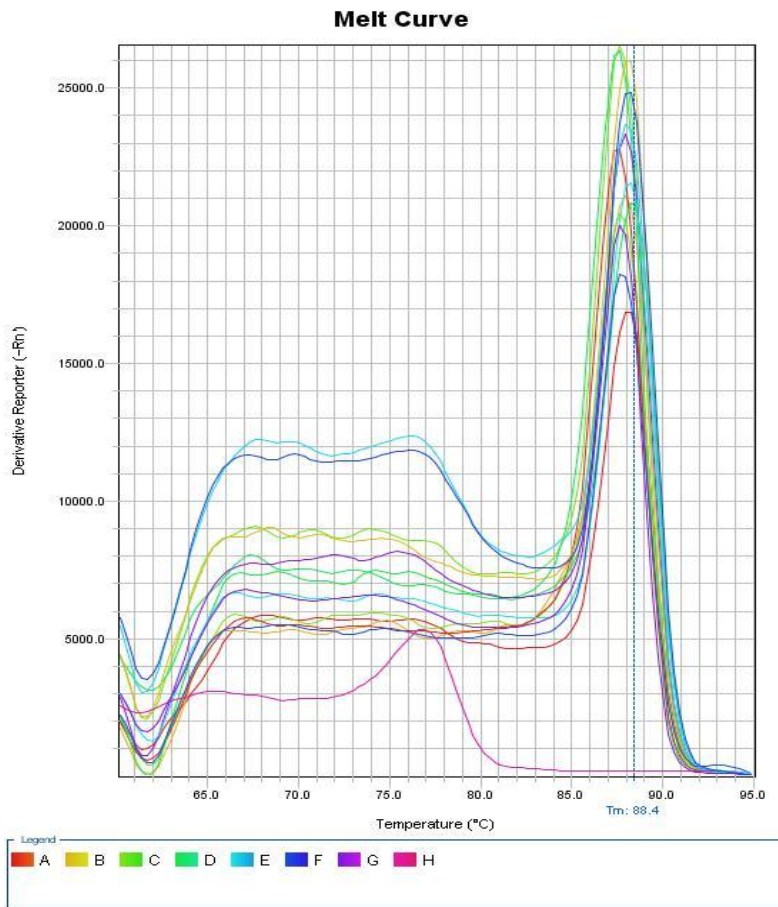
Table.1 Oligonucleotide primers for amplification of Nucleoprotein (N) gene SYBR Green Real Time PCR assay

Name	Oligonucleotide Sequence (5'-3')	Reference
O1 (+)	CTACAATGGATGCCGAC	Nagaraj <i>et al.</i> (2006)
R6 (-)	CCTAGAGTTATACAGGGCT	

Table.2 Sensitivity and specificity of SST, dFAT and qRT-PCR for diagnosis of Rabies virus in the dog brain samples

	qRTPCR				
		p	n	sensitivity	specificity
dFAT	P	06	0	85.71	100
	N	01	3		
Seller staining	P	05	0	71.42	100
	N	02	3		

Fig.1 Melt curve analysis of the RABV N gene PCR product



The low sensitivity of dFAT might be attributed to improper storage of samples, condition of sample, load of virus, stage of disease and preservative (Trimarchi and Smith, 2002; Wacharapluesadee and Hemachudha, 2010). The decomposed samples leading to loss of antigenic proteins may lead to false negative diagnosis by dFAT this can lead to problem in tropical and subtropical countries where transportation of specimens to a regional diagnostic laboratory is often delayed (Loza-Rubio *et al.*, 2005). The other disadvantage is confirmatory assays for dFAT comprise the rabies virus isolation and mouse inoculation test (MIT) but these will also require days to weeks until final diagnosis, so these disadvantages and the identification of new strains of the virus

encourage the use of new techniques like qRT-PCR that are rapid, sensitive, specific and economical for the detection and research of the Rabies Virus are being increasingly used in diagnosis and research laboratories (Manjunathareddy *et al.*, 2016).

Nowadays qRT-PCR based diagnostic assays are more favored than conventional PCR methods by several laboratories, because conventional PCRs involve tedious post amplification processing, cross-contamination, does not allow an exact quantification of genome copies and does not include tests for specificity (Belak and Thoren, 2001; Wacharapluesadee and Hemachudha, 2010). With nucleic acid detection techniques fragmented genome can

be detected depending on the size and the primers covering that length of genome as in the case of real time PCR, where we could diagnose 07 samples as positive. In the present study the more number of samples were found rabies positive by qRT-PCR though the length of genome covered is same (135bp) this might be due to amount of viral genome in the sample also determines the sensitivity of these two techniques.

Although SST has the advantages of yielding results under one hour, doesn't involved the need for expensive laboratory equipment to perform the test and the need to keep specimens cold after fixation, it is no longer be recommended for rabies routine diagnosis because it has comparatively low sensitivity and is highly subjective. It has been recommended that SST should be replaced with the dFAT (OIE, 2015), wherever it is still being employed in rabies routine diagnosis. The results of the present study demonstrated the high potential of qRT-PCR, dFAT and SST for the diagnosis of rabies in domestic and wild animals in India. Newer, more advanced molecular approaches like SYBR Green real time PCR was applied for the detection of rabies virus in brain tissue specimens of animals. Highest sensitivity was observed with real time PCR than conventional test. The results were in concordance with the dFAT applied on the impression smears of the same samples. So this technique can be successfully used for the epidemiological study and if combined with sequencing the genotypes prevalent in that area can be identified.

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How to cite this article:

Pharande, R.R., S.B. Majee, A.S. Bannalika, R.S. Gandge, R.V. Dighe, S.S. Moregoankar, A.Y. Doiphode, P. Charan and Mukherjee, S. 2017. Comparative Assessment of Seller's Staining Test (SST), Direct Fluorescent Antibody Test and Real Time PCR for Diagnosis of Rabies in Dogs. *Int.J.Curr.Microbiol.App.Sci*. 6(6): 1222-1228.
doi: <https://doi.org/10.20546/ijcmas.2017.606.142>