



Journal of Experimental Biology and Agricultural Sciences

<http://www.jebas.org>

ISSN No. 2320 – 8694

DIAGNOSIS OF ANIMAL RABIES: COMPARISON OF DIRECT FLUORESCENT ANTIBODY TEST (dFAT), REVERSE TRANSCRIPTASE -PCR AND REAL-TIME PCR

Manjunathareddy GB^{1,*}, Sumana K¹, Yogisharadhya R¹, Susan Cherian², Prajapati A¹, Patil SS¹, Balamurugan V¹, Singh KP², Singh R² and Rahman H¹

¹ICAR-National Institute of Veterinary Epidemiology and Disease Informatics (NIVEDI), Yelahanka, Bengaluru, Karnataka 560064, India

²Division of Veterinary Pathology, ICAR-Indian Veterinary Research Institute (IVRI), Izatnagar, Bareilly, U.P 243122, India

Received – August 01, 2016; Revision – October 08, 2016; Accepted – October 25, 2016

Available Online – October 30, 2016

DOI: [http://dx.doi.org/10.18006/2016.4\(Spl-3-ADPCIAD\).S69.S74](http://dx.doi.org/10.18006/2016.4(Spl-3-ADPCIAD).S69.S74)

KEYWORDS

Animal

Diagnosis

Rabies

RT-PCR

RT-qPCR

ABSTRACT

Rabies is endemic in most parts of India, with the exception of Andaman and Nicobar, Lakshadweep islands and to some extent Nagaland. For prevention and control it is essential to rapidly and precisely diagnose rabies. In this study, we used three diagnostic methods, direct fluorescent antibody test (dFAT), reverse transcriptase polymerase chain reaction (RT-PCR) and real time reverse transcriptase polymerase chain reaction (RT-qPCR) to detect the rabies virus in suspected animal brains. Out of the 80 animal brain samples tested, 64 (80%) were positive for rabies according to the RT-qPCR. Compared to the RT-qPCR, the sensitivities of dFAT and RT-PCR were 95.31% and 96.88%, respectively. The specificities of dFAT and RT-PCR were on par with qRT-PCR. Even though the dFAT findings did not completely coincide with results obtained from RT-PCR and RT-qPCR, dFAT appears to be a fast and reliable assay that can be used to analyze fresh brain samples. But in countries like India where temperature reaches 50°C during summer and lack of diagnostic facilities and trained personnel to carry out the dFAT at field level, the suspected samples will be usually sent to National/Regional Disease Diagnostic Laboratory /State veterinary or agricultural universities for rabies diagnosis. In summary the molecular methods RT-PCR and RT-qPCR can serve as quick and rapid diagnostic methods for animal rabies in India.

* Corresponding author

E-mail: gbmpatho@gmail.com (Dr. G. B. Manjunathareddy)

Peer review under responsibility of Journal of Experimental Biology and Agricultural Sciences.

Production and Hosting by Horizon Publisher India [HPI]
(<http://www.horizonpublisherindia.in/>).
All rights reserved.

All the article published by Journal of Experimental Biology and Agricultural Sciences is licensed under a Creative Commons Attribution-NonCommercial 4.0 International License Based on a work at www.jebas.org.



1 Introduction

Rabies is fatal non-suppurative encephalitis caused by RNA virus of genus *Lyssavirus* 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 detection of negribodies by sellar's staining is an old method for diagnosis. The development of direct fluorescent antibody technique (dFAT) which according to WHO, is a gold standard for rabies diagnosis because of short duration, low cost and higher sensitivity. As an adjunct to dFAT mouse inoculation is also carried out especially in developing countries, which is also 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 central laboratories for diagnosis with high probability of break in cold chain leading to decomposition, In such condition the reverse transcriptase polymerase chain reaction (RT-PCR) and real time reverse transcriptase polymerase chain reaction (RT-qPCR) which have high sensitivity may serve as better diagnostic assays. Many studies carried out to develop and validate RT-PCR and RT-qPCR for diagnosis of rabies in human (Hughes et al., 2004; Nagaraj et al., 2006; Wacharapluesadee et al., 2008; Nadin-Davis et al., 2009). Whereas, 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 dFAT, RT-PCR with syber green Real time PCR for the diagnosis of rabies in domestic and wild animals in India.

2 Materials and Methods

2.1 Study samples

A total of eighty rabies suspected samples collected from different species and different geographical regions (Andhra Pradesh:3, Gujarat:6, J&K:1, Karnataka:11, Kerala:10, Maharashtra: 8, Orisa:2, Punjab:8, Rajasthan:12, Tamil Nadu: 4, Uttarakhand:3 and Uttar Pradesh:12) of country were used in the study.

2.2 Direct fluorescent antibody technique (d FAT)

The FAT was performed according to the procedure described by the Office International des Épizooties (OIE) and World Health Organization (WHO). Briefly, the impression smears were prepared from different portions of brain and were fixed in chilled acetone for 2 hrs. The slides were encircled around the smear and were immersed in PBS (pH 7.2) for 5 min. They were incubated with FITC conjugate anti-rabies antibody (BioRad, USA) for 30 min in humidified dark chamber at

37°C. The slides were washed with PBS thrice in slide holding glass trough by creating current with magnetic stirrer. After washing, slides were mounted examined under fluorescent microscope at 400 nm (Nikon, Japan). The presence of dusty apple green fluoresce was taken as positive signal. Brain samples from mice infected with challenge virus standard (CVS) and normal mouse brain were as positive and negative controls, respectively.

2.3 Isolation of RNA

Total RNA from brain tissue was extracted by TRIZOL method. 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 (Sigma, USA) was added and mixed vigorously for 15 seconds and incubated for 10 min on ice. The aqueous phase collected after centrifugation @ 12000 rpm for 10 min at 4 °C was mixed with 900 µl of isopropanol, followed by centrifugation at 12000 rpm for 10 min at 4 °C. The RNA pellet was washed with 1 ml of 70% ethanol, dried and dissolved in 50 µl of nuclease free water and concentration of RNA was estimated by spectrophotometer (NanoDrop ND-1000, USA).

2.4 cDNA preparation

The cDNA synthesis was carried out using Reverse Transcription System (Promega, USA) as per the recommended protocol. Briefly, 2 µl of total RNA (~ 1.0 µg) was incubated for 10 min at 70°C and quickly chilled on ice and centrifuged briefly. Then final reaction volume of 20 µl was prepared by adding 4.0 µl of MgCl₂ (25 mM), 2.0 µl of RT 10X buffer, 2.0 µl of dNTP mixture, (10 mM), 0.5 µl of RNasin, 0.6 µl of AMV Reverse Transcriptase (20 IU/µl), 1.0 µl of Random Primers (0.5mg/ml), and 7.9 µl of Nuclease free water. The reaction mixture was incubated at room temperature for 10 min followed by 42 °C for 60 min. Reverse Transcriptase enzyme was inactivated at 95°C for 5 min with final incubation at 4°C for 5 min.

2.5 Reverse transcriptase Polymerase chain reaction (RT-PCR)

RT-PCR for detection of rabies virus targeting most conserved nucleoprotein gene specific two sets of primers (Table 1) designed based on sequences available from our previous study was carried out (Reddy et al., 2011). Briefly, 25 µl reaction mixture (2.5x master mix- 10 µl, MgCl₂,10 mM- 1.0 µl, Primer Forward- 0.5 µl, Primer Reverse-0.5 µl, Template cDNA-1 µl and Nuclease free water- 12 µl) using 2.5x master mix (5 PRIME, USA) was prepared. The amplification was carried out in thermocycler (Eppendorf, Germany) with an initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C/1 min, 53°C/1 min and 72°C/1 min, and a final extension step of 72°C for 10 min. The amplified products were electrophoresed on 1% agarose and were photographed.

Table 1 Oligonucleotide primers used for RT-PCR and RT-qPCR to diagnose rabies.

Primer	Nucleotide sequences (5'-3')	Nucleotide position*	Sense	Rabies gene	Size of amplicon (bp)
RABNF1	GATTGAGCATCTATATTCAGC	648-688	+	N	200
RABNR2	GAGGAACGGCGGTCTCCTG	888-869	-		
RABNF2	ACTGATGTAGAAGGGAATTG	340-359	+	N	533
RABNR2	GAACGGAAGTGGATGAAATA	872-853	-		

2.6 Real time PCR (RT-qPCR)

Syber green RT-qPCR was performed in 20µl reaction volume comprised of 8.0 µl of nuclease-free water, 0.5 µl each of the primers (table.1), 10 µl of 2x syber green master mix (Finnzymes, Finland) and 1 µl of cDNA. The reactions were carried out in thin wall clear PCR strip tubes with clear strip caps (Axygen, USA) in an MX3000P quantitative PCR system (Stratagene, USA). The reaction was performed for 40 cycles with 55°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).

2.7 Calculation of sensitivity and specificity

Sensitivity was calculated with the formula $[TP/(TP+FN)] \times 100$ where TP was the number of samples with true-positive results as determined by the reference assay (qRT-PCR) and FN was the number of samples with false-negative results. Specificity was defined as $[TN/(TN+FP)] \times 100$ where TN was the number of samples with true-negative results and FP was the number of samples with false-positive results.

3 Results

In the present study comparison of three diagnostic methods (dFAT, RT-PCR and RT-qPCR) was carried out with a total of 80 suspected rabies brain samples from wide host range (beef; 2, buffalo;8, cattle;15, dogs; 28, horse; 5, human; 6, hyena; 5, goat; 8 and jackal; 3) and from different geographical regions of the country were used in the study: Uttar Pradesh-12, Uttarakhand-3, J&K-1, Punjab-8, Rajasthan-12, Gujarat- 6, Maharashtra- 8, Karnataka-11, Kerala-10, Tamil Nadu-4 and Orisa-2 Andhra Pradesh 3. When these 80 animal and human brain samples were tested; 61 were positive according to the

FAT (Figure. 1A & Figure.1 B), 62 were positive by RT-PCR and 64 were positive by RT-qPCR. All the three assays gave negative for remaining 16 brain samples. Sensitivities of dFAT and RT-PCR were 95.31 and 96.88% respectively and specificities of dFAT and RT-PCR were on par with RT-qPCR (Table. 2).

Conventional RT-PCR using a primer set that amplified the N gene of rabies virus was able to detect viral RNA in 62 samples with both sets of primers covering short (RABNF1 and RABNR1) as well as high length of genome (RABNF2 and RABNR2). The band intensity of the amplified gene varied among the samples (Figure. 2). However, RT-qPCR could detect the N gene in 64 samples without any non-specific reactions with specific melting temperature (82.24-83.11°C).

4 Discussions

Rabies is endemic in most parts of India, with the exception of Andaman and Nicobar, Lakshadweep islands and to some extent Nagaland. National survey by the Association of the Prevention and Control of Rabies in India estimated a total of 20,000 human deaths due to dog bite each year (Sudarshan et al., 2007). Although the loss of livestock due to rabies is significant, there are only few publications on estimates of the incidence of rabies in livestock (Knobel et al., 2005). 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). Monitoring and surveillance of any disease requires rapid diagnostic tests. In the present study we first screened the all suspected rabies brain by dFAT as it is considered as the gold standard for rabies diagnosis by WHO, but in our study with dFAT we were able to detect only 61 out of 64 cases which found positive by RT-qPCR (Table. 2).

Table 2 Sensitivity and specificity of dFAT, RT-PCR, and qRT-PCR for diagnosis of rabies virus in the field brain samples.

		qRT-PCR		Sensitivity	Specificity
		P	N		
dFAT	P	61	0	95.31	100
	N	3	16		
RT-PCR	P	62	0	96.88	100
	N	2	16		

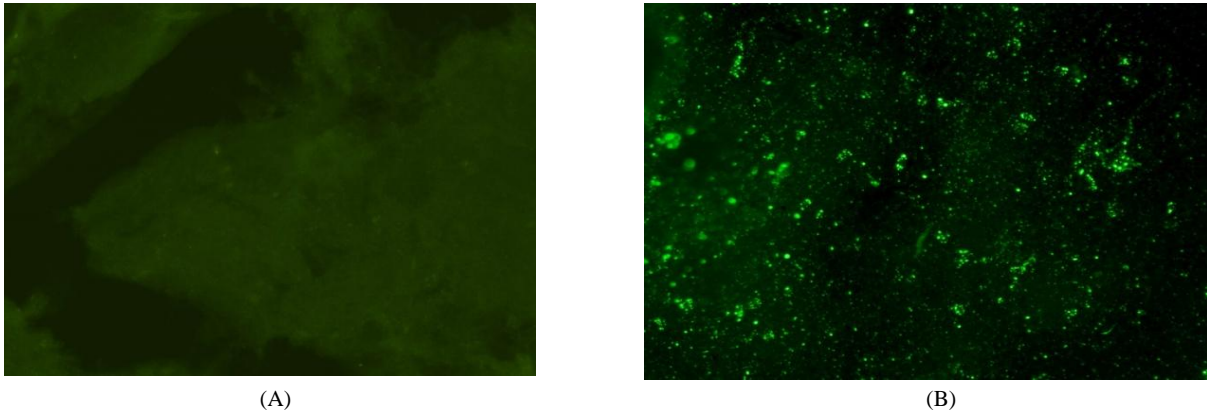


Figure 1 Brain impression smears showing no specific (a) and specific (b) rabies fluorescence signals

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 & Smith, 2002; Wacharapluesadee & 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 RT-PCR and RT-qPCR 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 (Silva et al., 2013).

Out of 64 cases, 62 cases were found positive with rabies virus 'N' gene specific primers with specific amplification with 96.88% sensitivity by RT-PCR. The higher sensitivity and specificity as compared to dFAT has also been reported earlier and might be due to decomposition of tissue during which the RNases degrade the genome into smaller segments (Smith et al., 2000; David et al., 2002). Rojas et al. (2006) reported

successful detection of rabies virus genome up to 23 days in the brain samples stored at ambient temperature. The low sensitivity in the present study might also be attributed to lower number of nucleic acid copies leading which in turn might have led to negative by RT-PCR and positive by RT-qPCR. The RT-PCR besides many other advantages like earlier detection of rabies viral infection compared to DIF and Seller staining can also be useful in strain identification with sequencing for molecular epidemiology (Biswal et al., 2012).

Nowadays RT-qPCR based diagnostic assays are more favored than conventional reverse-transcription PCR methods by several laboratories, because RT-PCRs will generate the risk of post amplification processing, cross-contamination, does not allow an exact quantification of genome copies and does not include tests for specificity (Belak & Thoren, 2001; Wacharapluesadee & 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 64 samples as positive. In the present study the more number of samples were found rabies positive by RT-qPCR compared to RT-PCR even though the length of genome covered is same (200bp) this might be due to amount of viral genome in the sample also determines the sensitivity of these two techniques.

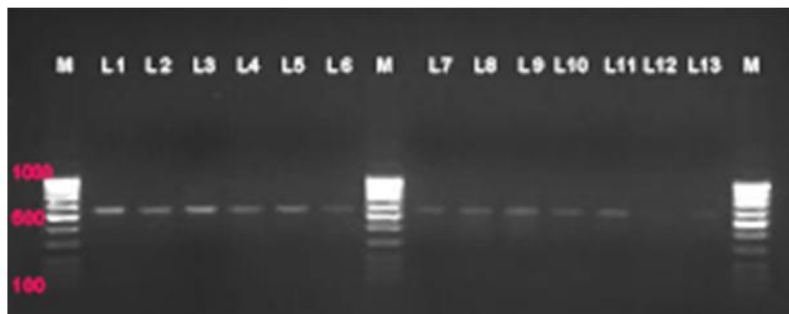


Figure 2 Gel photo showing amplified N (533bp) gene of rabies virus from different field rabies isolates. M - 100bp marker, L1- Positive control (CVS) and L2-L13 are field samples.

Hughes et al. (2004) reported single mutations for the North American RABV strains in the region of the primers or the probe can alter the sensitivity of the PCR. The above observations were eliminated in the present study by designing primers after sequencing more than 40 isolates. But more studies are warranted targeting different portions of rabies genome as the genetic diversity among rabies viruses may hamper the use of a single assay and rabies surveillance may benefit more from the use of a pan-lyssavirus primer SYBR green assay rather than a strain or specific based assay (Fooks et al., 2009).

Conclusion

The results of the present study demonstrated the high potential of RT-qPCR over RT-PCR and dFAT for the diagnosis of rabies in domestic and wild animals in India.

Conflict of interest

All Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

References

Belak S, Thoren P (2001) Molecular diagnosis of animal diseases: some experiences over the past decade. *Expert Review in Molecular Diagnostics* 1:434-443. DOI: 10.1586/14737159.1.4.434.

Biswal M, Ratho RK, Mishra B (2012) Role of reverse transcriptase polymerase chain reaction for the diagnosis of human rabies. *Indian Journal of Medical Research* 135: 837–842.

Chhabra M, Bhardwaj M, Ichhpujani RL, Lal S (2005) Comparative evaluation of commonly used laboratory tests for post-mortem diagnosis of rabies. *Indian Journal of Pathology and Microbiology* 48: 190-193.

David D, Yakobson B, Rotenberg D, Dveres N, Davidson I, Stram Y (2002) Rabies virus detection by RT-PCR in decomposed naturally infected brains. *Veterinary Microbiology* 87: 111-118. DOI:10.1016/S0378-1135(02)00041-X.

Fooks AR, Johnson N, Freuling CM, Philip R, Wakeley (2009) Emerging technologies for the detection of rabies virus: challenges and hopes in the 21st Century. *PLoS Neglected Tropical Diseases* 3: 1-12. DOI: 10.1371/journal.pntd.0000530.

Gupta PK, Singh RK, Sharma RN, Rao YU, Butchaiah G (2001) Preliminary report on a single-tube, non-interrupted reverse transcription-polymerase chain reaction for the detection of rabies virus in brain tissue. *Veterinary Research Communication* 25: 239-47. doi:10.1023/A:1006437810594

Hughes GJ, Smith JS, Hanlon CA, Rupprecht CE (2004) Evaluation of a TaqMan PCR assay to detect rabies virus RNA: influence of sequence variation and application to quantification of viral loads. *Journal of Clinical Microbiology* 42: 299-306. DOI: 10.1128/JCM.42.1.299-306.2004.

Jayakumar R, Tirumurugaan KG, Ganga G, Sheela KP, Vijayarani K, Nainar AM (2003) In situ reverse transcription-polymerase chain reaction: a novel technique for detection of rabies virus RNA in murine neuroblastoma cells. *Acta Virologica* 47:57-59. DOI: 10.1007/s11259-007-3492-8.

Kaw A, Singh CK, Ramneek, Sandhu BS, Sood NK, Ramneek, Deka D, Awahan S (2011) Diagnosis of rabies in animals by nested RT-PCR. *Indian Journal of Animal Sciences* 81: 367–69.

Knobel DL, Cleaveland S, Coleman PG, Fèvre EM, Meltzer MI, Miranda ME, Shaw A, Zinsstag J, Meslin FX (2005) Re-evaluating the burden of rabies in Africa and Asia. *Bull World Health Organ* 83: 360-368. DOI: /S0042-96862005000500012.

Loza-Rubio E, Rojas-Anaya E, Banda-Rui'z VM, Nadin-Davis SA, Cortez-Garci'a B (2005) Detection of multiple strains of rabies virus RNA using primers designed to target Mexican vampire bat variants. *Epidemiology and Infection* 133: 927–934. DOI: 10.1017/S095026880500405X.

Nadin-Davis SA, Sheen M, Wandeler AI (2009) Development of real-time reverse transcriptase polymerase chain reaction methods for human rabies diagnosis. *Journal of Medical Virology* 81: 1484-1497. DOI: 10.1002/jmv.21547.

Nagaraj T, Vasanth JP, 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. *Journal of Clinical Virology* 36: 17–23. DOI: 10.1016/j.jcv.2006.01.009.

Praveena PE, Jayakumar R, Balachandran C, Thirumurugan G, Raj GD, Murali Manohar B (2007) Detection of rabies virus genes by in-situ polymerase chain reaction. *Veterinary Research Communication* 31: 775-81. DOI: 10.1007/s11259-007-3492-8.

Reddy GB, Singh R, Singh RP, Singh KP, Gupta PK, Mahadevan A, Shankar SK, Ramakrishnan MA, Verma R (2011) Molecular characterization of Indian rabies virus isolates by partial sequencing of nucleoprotein (N) and phosphoprotein (P) genes. *Virus Genes* 43:13-17. DOI: 10.1007/s11262-011-0601-0.

Rojas AE, Loza-Rubio E, Banda RVM, Hernández BE (2006) Use of Reverse Transcription-Polymerase Chain Reaction to Determine the Stability of Rabies Virus Genome in Brains Kept at Room Temperature. *Journal of Veterinary Diagnostic Investigation* 18: 98–10. DOI: 10.1177/104063870601800115

Silva SR, Katz IS, Mori E, Carnieli PJ, Vieira LF, Batista HB, Chaves LB, Scheffer KC (2013) Biotechnology advances: a perspective on the diagnosis and research of Rabies Virus. *Biologicals* 41: 217-223. DOI: 10.1016/j.biologicals.2013.04.002.

Smith J, McElhinney LM, Heaton PR, Black E M, Lowings JP (2000) Assessment of template quality by incorporation of an internal control into a RT-PCR for the detection of rabies and rabies-related viruses. *Journal of Virological Methods* 84: 107–115. DOI: 10.1016/S0166-0934(99)00124-X.

Sudarshan MK, Madhusudana SN, Mahendra BJ, Rao NS, Ashwath NDH, Abdul Rahman S, Meslin F, Lobo D, Ravikumar K, Gangaboraiah (2007) Assessing the burden of human rabies in India: results of a national multi-center

epidemiological survey. *International Journal of Infectious Diseases* 11: 29–35. DOI: 10.1016/j.ijid.2005.10.007.

Trimarchi C, Smith J (2002) Diagnostic evaluation. In: Rabies, edited by Jackson, AC and Wunner WH (Academic Press, London, UK), 307–349. ISBN-13: 978-0123693662.

Wacharapluesadee S, Hemachudha T (2010) Ante and post-mortem diagnosis of rabies using nucleic acid-amplification tests. *Expert Review of Molecular Diagnostics* 10: 207–218. DOI: 10.1586/erm.09.85.

Wacharapluesadee S, Sutipanya J, Damrongwatanapokin S, Phumesin P, Chamnanpood P, Leowijuk C, Hemachudha T (2008) Development of a TaqMan real-time RT-PCR assay for the detection of rabies virus. *Journal of Virological Methods* 151: 317-320. DOI: 10.1016/j.jviromet.2008.05.004.