VirusDis. (January–March 2014) 25(1):120–124
DOI 10.1007/s13337-013-0184-6

SHORT COMMUNICATION

Diagnostic evaluation of RT-PCR–ELISA for the detection of rabies virus

R. P. AravindhBabu · S. Manoharan · P. Ramadass

Received: 30 September 2013 / Accepted: 11 November 2013 / Published online: 30 November 2013

Abstract Rabies is primarily a disease of terrestrial and airborne mammals. In most cases, rabies is diagnosed primarily on the basis of clinical symptoms and signs, and a corroborative history of or evidence of an animal bite, death of an animal and incomplete or no vaccination following exposure. The facility for laboratory diagnosis and confirmation of rabies is available in only a few institutions in India. Diagnostic tests using conventional assays like fluorescent antibody test (FAT) are unreliable at times, despite the clinical diagnosis. Currently, there are a number of molecular tests that can be used to complement conventional tests in rabies diagnosis. We have developed and evaluated an RT-PCR–ELISA using a panel of brain tissue samples from rabies suspected animals of various species. This assay was able to detect rabies virus genome in all the 43 samples that were previously tested positive for rabies. Moreover this assay was shown to be 100 % sensitive and specific in detecting the rabies virus genome in post-mortem brain tissue samples from different species of animals. Our pilot study shows the potential of this assay as an alternative diagnostic test when the samples are unsuitable for use in FAT and also a supplementary test to FAT. In addition, the region of nucleoprotein gene amplified using this assay can be used for the molecular investigation of geographical origin of the field strains.

R. P. AravindhBabu · S. Manoharan (✉) · P. Ramadass
Rabies Laboratory, Department of Animal Biotechnology, Madras Veterinary College, Chennai 600007, India
e-mail: ulagaimano@yahoo.com

Present Address:
R. P. AravindhBabu (✉)
National Institute for Animal Biotechnology, 1-121/1, 4th Floor, Axis Clinicals Building, Miyapur, Hyderabad 500049, India
e-mail: rparavindhbabu@yahoo.com

Keywords Rabies · India · Diagnosis · FAT · RT-PCR · RT-PCR-ELISA

Rabies is one of the oldest recognized diseases and most important of the zoonotic diseases in India. The causative agent, *Rabies virus* (family: Rhabdoviridae; genus: Lyssavirus) is maintained in animal reservoirs. Although, dogs are the major reservoirs, other domesticated mammals and wildlife also plays a significant role in transmission [1, 3]. Rapid diagnosis of rabies in animals is therefore significant as in-contact humans are at a risk of contracting the disease and should start the post exposure treatment as early as possible. The World Organisation for Animal Health (OIE) and World Health Organisation (WHO) approved fluorescent antibody test (FAT) for rabies gives reliable results on fresh brain tissue specimens. However autolysed tissue samples can reduce the sensitivity of this test and often are unsuitable for confirming the presence of rabies antigen [2]. Molecular tools based on the detection of the viral genome are becoming widely accepted and accessible for the diagnosis of rabies. Moreover for numerous diagnostic laboratories in rabies-endemic regions of the developing world, cost and simplicity are critical factors in disease diagnosis which cannot be neglected, even when the principal consideration is for rapid diagnosis [6]. Recently we have evaluated a reverse transcription-polymerase chain reaction assay for routine and cost effective diagnosis of *Rabies virus* in post-mortem brain samples from different species of animals [2]. The sensitivity and specificity of this assay was comparable to that of FAT and the assay was able to detect up to 0.01 FFD_{50} (50 % fluorescent focus forming doses) of the rabies virus.

In order to improve the analytical sensitivity of this assay for extending its use in diagnosis of rabies virus from ante-mortem samples such as saliva and cerebrospinal

Table 1 Comparison between FAT, RT-PCR and RT-PCR–ELISA assays for the detection of rabies virus in post-mortem brain samples

SI no.	Host	Place of origin	Results			
			FAT	RT-PCR	RT-PCR–ELISA	
					OD values	Positivity
1	Dog	Chennai, Tamilnadu	+	+	0.99	+
2	Cow	Theni, Tamilnadu	+	+	1.39	+
3	Dog	Chennai, Tamilnadu	−	−	0.18	−
4	Dog	Chennai, Tamilnadu	−	−	0.15	−
5	Dog	Chennai, Tamilnadu	+	+	0.87	+
6	Cow	Theni, Tamilnadu	+	+	1.09	+
7	Dog	Chennai, Tamilnadu	+	+	0.78	+
8	Cow	Chennai, Tamilnadu	+	+	0.98	+
9	Dog	Mannuthy, Kerala	+	+	0.76	+
10	Dog	Mannuthy, Kerala	+	+	1	+
11	Dog	Mannuthy, Kerala	+	+	0.93	+
12	Dog	Chennai, Tamilnadu	−	−	0.21	−
13	Dog	Mannuthy, Kerala	+	+	0.93	+
14	Dog	Mannuthy, Kerala	−	−	0.77	+
15	Dog	Bangalore, Karnataka	−	+	0.84	+
16	Dog	Chennai, Tamilnadu	+	+	1.13	+
17	Dog	Chennai, Tamilnadu	−	−	0.14	−
18	Goat	Chennai, Tamilnadu	+	+	1.36	+
19	Dog	Mannuthy, Kerala	−	+	0.61	+
20	Dog	Mannuthy, Kerala	+	+	1.13	+
21	Dog	Mannuthy, Kerala	+	+	1.38	+
22	Dog	Bangalore, Karnataka	+	+	0.89	+
23	Dog	Bangalore, Karnataka	+	+	0.94	+
24	Dog	Bangalore, Karnataka	+	+	1.37	+
25	Dog	Bangalore, Karnataka	+	+	1.39	+
26	Mouse	Bangalore, Karnataka	+	+	1.71	+
27	Mouse	Bangalore, Karnataka	+	+	1.64	+
28	Mouse	Bangalore, Karnataka	+	+	1.56	+
29	Goat	Chennai, Tamilnadu	+	+	1.21	+
30	Cat	Chennai, Tamilnadu	-	+	0.85	+
31	Mouse	Chennai, Tamilnadu	+	+	1.02	+
32	Mouse	Chennai, Tamilnadu	+	+	1.03	+
33	Dog	Chennai, Tamilnadu	+	+	1.43	+
34	Dog	Chennai, Tamilnadu	+	+	1.6	+
35	Dog	Mannuthy, Kerala	+	+	1.29	+
36	Dog	Mannuthy, Kerala	+	+	1.6	+
37	Dog	Mannuthy, Kerala	+	+	1.47	+
38	Dog	Mannuthy, Kerala	+	+	1.23	+
39	Cow	Chennai, Tamilnadu	−	−	0.17	−
40	Dog	Chennai, Tamilnadu	+	+	1.19	+
41	Dog	Bangalore, Karnataka	#	+	0.78	+
42	Dog	Bangalore, Karnataka	+	+	1.13	+
43	Dog	Bangalore, Karnataka	#	+	0.83	+
44	Dog	Bangalore, Karnataka	#	+	1.01	+
45	Dog	Bangalore, Karnataka	+	+	1.75	+
46	Dog	Bangalore, Karnataka	#	+	0.93	+

Table 1 continued

SI no.	Host	Place of origin	Results			
			FAT	RT-PCR	RT-PCR–ELISA	
					OD values	Positivity
47	Human	Chennai, Tamilnadu	+	+	2.22	+
48	Dog	Chennai, Tamilnadu	+	+	1.49	+
49	Goat	Chennai, Tamilnadu	+	+	2.08	+
50	Dog	Chennai, Tamilnadu	+	+	1.38	+
51	Goat	Chennai, Tamilnadu	+	+	2.14	+
52	Mouse	CVS 11 strain (laboratory passaged)	+	+	1.41	+

+ Presence of rabies virus antigen/genome; − no rabies virus antigen/genome detected; # samples unsuitable for use in FAT

fluid, we have modified the classical gel based detection to an ELISA based detection of the amplified products. Although RT-PCR–ELISA using hybridization probes for the detection of rabies and other lyssa viruses are previously available [4, 8], our assay uses a direct detection format without the use of hybridization probes.

The brain tissue samples used in this study (Table 1) were collected from animals suspected to have rabies and submitted to Rabies Laboratory, Department of Animal Biotechnology, Madras Veterinary College, Chennai between the years 2005 and 2007. In addition, brain samples from mouse which was intra-cerebrally inoculated with Challenge Virus Standard-11 (CVS-11) strain of rabies virus was used as positive control. Brain samples from rabies free cattle, sheep, goat, mouse and dog were used as negative controls. The same panel of brain tissue samples was previously used to validate an in house RT-PCR assay for rabies diagnosis [1].

Monolayers of baby hamster kidney (BHK)-21 epithelial cells were maintained in Eagle's minimum essential medium (Gibco BRL, Life Technologies, Paisley, United Kingdom) supplemented with 10 % heat inactivated foetal calf serum (Gibco BRL, Life Technologies, Paisley, United Kingdom), 2 mM L-glutamine (Sigma, Poole, United Kingdom) and antibiotics at 37 °C. The CVS-11 strain of rabies virus was propagated and titrated on BHK-21 cells as described previously (http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.01.13_RABIES.pdf). Impression smears were made from the brain samples on a clean glass slide and fixed in acetone for 30 min at −20 °C and tested for the presence of viral antigen by direct FAT [5]. The staining was done using anti-rabies nucleocapsid rabbit immunoglobulin conjugated with fluorescein isothiocyanate (BioRad, India).

Total RNA was extracted directly from the infected brain tissues or rabies virus infected BHK-21 cell monolayer using the TRIzol reagent according to the manufacturer's instructions (Gibco BRL, Life Technologies, Paisley, United Kingdom). Reverse transcription was performed with 2 µg of the total brain RNA using the RevertAid TM cDNA synthesis kit (MBI Fermentas, USA) as per manufacturer's instructions. Briefly, RNA was denatured at 70 °C for 5 min, cooled on ice which was then added to a final volume of 20 µl containing Moloney Murine Leukemia Virus (M-MLV) reverse transcription buffer, 10 mM dNTPs, 20 U of RNase inhibitor, 10 pmol of primer JW12 (5′-ATGTAACACCTCTACAATG-3′) and 200 U of MMLV reverse transcriptase. This mixture was incubated at 42 °C for 60 min in a water bath and heated at 70 °C for 5 min to terminate the reaction.

Amplification of 5 µl of the cDNA template was done using *Taq* polymerase (5U; Roche), PCR Dig labeling mix (200 µM; Roche) and 10 pmol each of primers JW12 and JW6 (5′Biotin-CAATTAGCACACATTTTGTG-3′) in a 50 µl reaction volume. The amplifications were performed on a PTC100 Thermal Cycler (MJ Research, USA). After denaturation at 95 °C for 10 min, the reactions were cycled five times at 95 °C for 60 s, 50 °C for 60 s and 72 °C for 60 s and then 30 times at 95 °C for 30 s, 45 °C for 30 s, 72 °C for 60 s. This was followed by a final elongation step at 72 °C for 10 min. On completion 5 µl of amplified digoxigenin and biotin labeled PCR product was detected using an ELISA format using the RT-PCR–ELISA Dig detection kit (Roche). Briefly, 5 µl of the RT-PCR reaction is mixed with 95 µl of hybridization buffer and was added to each well and incubated at 37 °C for 1 h in a shaker incubator. The wells were washed 5 times with washing buffer. Two hundred micro liters of anti-Dig peroxidase conjugate was added to each well and incubated at 37 °C for 1 h. The wells were washed five times with washing buffer. Two hundred micro liters of ABTS (3,3-azino diethyl benzothiazoline sulphonic acid) substrate was then added to each well and incubated for 10 min. The optical density at 405 nm was read using an ELISA plate reader (Biotek, USA). The amplified products were also analysed on 1 % agarose gel electrophoresis.

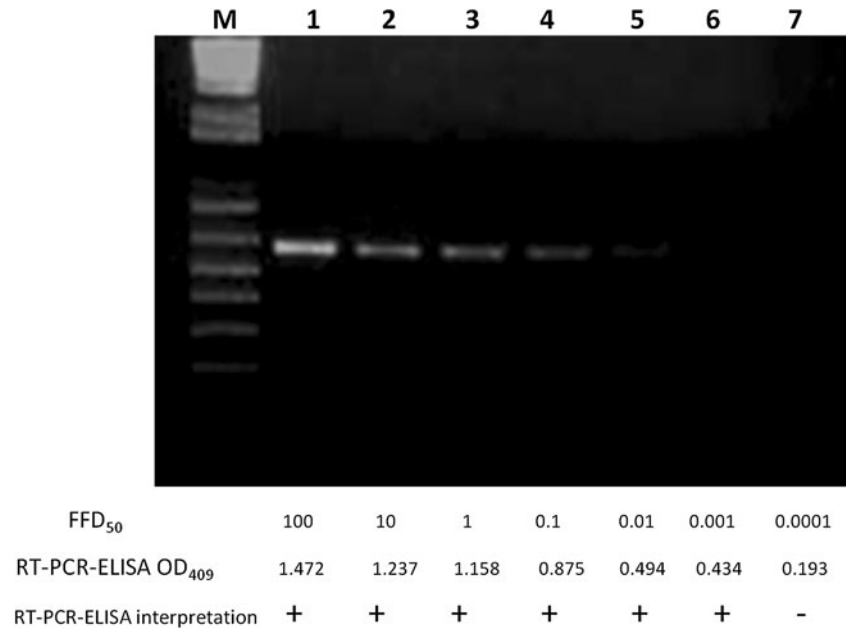

Fig. 1 RT-PCR–ELISA of various dilutions of CVS-11 strain *Rabies virus*. Five micro liter of the amplified product was run on 1 % agarose gel and 5 µl was used in the ELISA. Titre (FFD_{50}) of the virus used for RNA extraction, RT-PCR–ELISA OD_{405nm} values and the interpretation are shown below the corresponding *lanes*. Molecular weight marker: 1 kb plus DNA ladder (Invitrogen)

The analytical sensitivity of the RT-PCR–ELISA was evaluated by extracting the total cellular RNA from one ml of the serial dilutions of BHK cells passaged CVS-11 virus suspension containing 10^2 50 % fluorescent focus forming doses (FFD50) per ml. Agreement between the FAT and RT-PCR–ELISA assay was measured by calculating the Cohen's kappa coefficient (κ). The cut off value for RT-PCR–ELISA was determined using the mean absorbance of 20 rabies negative samples + three times the standard deviation. The OD_{405} of the negative samples ranges from 0.10 to 0.23. A sample with OD_{405} above 0.26 was considered positive for the presence of rabies genome and below the cut off value was considered negative.

Among the panel of 52 brain tissue samples, 43 were previously known to be positive using FAT and RT-PCR. RT-PCR–ELISA was able to detect the presence of rabies genome in all of these 43 samples (Table 1). As the RT-PCR–ELISA was able to detect rabies genome in samples that were unsuitable to use in FAT the sensitivity and specificity of the assay was determined by comparing it with a polished gold standard which included the FAT result along with the RT-PCR result [7]. This RT-PCR assay was previously validated and did not gave false positive results or non specific amplifications when used on brain tissue samples from different species of animals [2]. The sensitivity and specificity of the RT-PCR–ELISA assay were found to be 100 % in comparison with the polished gold standard. The kappa coefficient for the agreement between the RT-PCR–ELISA and FAT assays was 0.97. Analytical sensitivity of RT-PCR–ELISA was found to be 0.001 FFD_{50} of rabies virus (Fig. 1) which is tenfold higher than our routine RT-PCR assay. The RT-PCR–ELISA OD_{405} values had a linear correlation with the FFD_{50} of rabies virus used for RNA extraction and the R^2 value was found to be 0.966.

Although there is a tenfold increase in the analytical sensitivity of RT-PCR–ELISA when compared with RT-PCR followed by gel electrophoresis, both assays detected the presence of rabies genome in 100 % of the brain samples that were positive in FAT and also from the four autolysed samples that were unsuitable for use in FAT. However the increased sensitivity may be useful when ante-mortem samples with lesser viral load are presented for diagnosis. In addition to the increased sensitivity, RT-PCR–ELISA format can handle more number of diagnostic samples in a single run when a 96 well format is used both for PCR amplification and subsequent ELISA detection. Although false positive results are relatively a common occurrence with the highly sensitive molecular assays, our assay did not detect any false positive in the brain samples from dog, cattle, buffalo, sheep, goat and mouse. However this assay needs to be validated using a large number of samples before using it for routine diagnostic purpose.

Although recent techniques such as the real time RT-PCR has proven much more sensitive in detecting rabies virus than a conventional RT-PCR based assays, the expensive instrumentation and the higher cost incurred per diagnostic sample are the major hurdles for its use as

routine diagnostic test [2]. Therefore, there is a clear need to simplify molecular diagnostic techniques so these tests can be applied universally in developing and developed countries. The semi-automated or automated instruments and robotics-based techniques are useful only when large numbers of the same test are undertaken such as surveillance and companion animal testing [6]. Validated diagnostic tests that confirm the presence of rabies virus or a lyssavirus variant have been the foundation of rabies control strategies in many countries. This preliminary evaluation shows the potential of this RT-PCR–ELISA to be a sensitive and cost effective assay for rabies diagnosis. Although the WHO does not currently recommend RT-PCR based assays for routine diagnosis of rabies, this assay can be used in circumstances where the samples are unsuitable for FAT. Further, it also has the potential for use in the detection of rabies in ante-mortem clinical samples.

References

1. AravindhBabu RP, Manoharan S, Ramadass P, Chandran NDJ. Rabies in South Indian cows: an evidence of Sri Lankan Rabies virus variant infection based on the analysis of partial nucleoprotein gene. Indian J Virol. 2011;22:138–41.
2. AravindhBabu RP, Manoharan S, Ramadass P, Chandran NDJ. Evaluation of RT-PCR assay for routine laboratory diagnosis of Rabies in post mortem brain samples from different species of animals. Indian J Virol. 2012;23:392–6.
3. Bhatia R, Ichhpujani RL, Madhusudana SN, Hemachudha T. Rabies in South and Southeast Asia. Proceedings of the WHO expert consultation on Rabies. Geneva; 2004.
4. Black EM, McElhinney LM, Lowings JP, Smith J, Johnstone P, et al. Molecular methods to distinguish between classical rabies and the rabies-related European bat lyssaviruses. J Virol Methods. 2000;87:123–31.
5. Dean DJ, Abelseth MK, Athanasiu P. The fluorescent antibody test. In: Meslin FX, Kaplan MM, Kowprowski H, editors. Laboratory techniques in rabies: World Health Organization. Geneva: Switzerland; 1996. p. 88–93.
6. Fooks AR, Johnson N, Freuling CM, Wakeley PR, Banyard AC, et al. Emerging technologies for the detection of rabies virus: challenges and hopes in the 21st century. PLoS Negl Trop Dis. 2009;3:e530.
7. McAdam AJ. Discrepant analysis: how can we test a test? J Clin Microbiol. 2000;38(6):2027–9.
8. Whitby JE, Heaton PR, Whitby HE, Sullivan EO, Johnstone P. Rapid detection of rabies and rabies related viruses by RT-PCR and enzyme linked immunosorbent assay. J Virol Methods. 1997;69:63–72.