Indian J Med Res 135, June 2012, pp 809-811

# Commentary

## Role of the RT-PCR method in ante-mortem & post-mortem rabies diagnosis

Rabies is endemic in Asia and Africa, where the primary reservoir and vector of the rabies virus is the domestic dog. Worldwide human mortality from enzootic canine rabies is estimated to be in excess of 55,000 deaths per year, of which approximately 56 per cent occur in Asia (20,000 in India alone), and 44 per cent in Africa, 99 per cent of which are transmitted by dogs[1]. Although all age groups are susceptible, rabies is most common in children aged <15 yr. Rabies is one of the more neglected viral zoonotic diseases worldwide especially in developing countries, with the greatest burden in poor rural communities. There are several reasons for this situation including lack of accurate data systems, under-reporting of cases by local communities and central authorities, unreliable diagnosis of cases (which is generally based on clinical symptoms) and inadequate legislation for compulsory notification of cases[2].

The gold standard diagnostic technique which is recommended by the WHO[1] is the demonstration of virus antigen in brain tissue by the direct fluorescence assay (DFA). The DFA provides near 100 per cent sensitivity in post-mortem rabies diagnosis of humans and animals and can be rapidly completed in less than 2 h. Confirmatory tests for DFA are the tissue culture infection test (RTCIT) and mouse inoculation test (MIT). Both assays require several days or weeks for final diagnosis. In addition to these classical methods for rabies virus detection, molecular amplification methods like reverse transcriptase polymerase chain reaction (RT-PCR) have been developed. *In vitro* DNA amplification by PCR was initiated in 1983 by Kary Mullis, and since then PCR assays have been developed for veterinary virology. The first publication related to PCR diagnosis of virus infection of veterinary importance was published in the late 1980s and the first descriptions of diagnostic PCR for rabies were published in 1991 by Sacramento *et al*[3] and in 1993 by Kamolvarin *et al*[4]. However, the use of the RT-PCR and other amplification techniques is not currently recommended for routine post-mortem diagnosis of rabies in animals and in humans, but is recommended for epidemiological survey in laboratories with strict quality control procedures and experience and expertise with this technique[1].

Molecular epidemiology based on RT-PCR is an important tool for the classification of animal virus diseases, including rabies virus, and provides a better understanding of epidemiological relationships. Using the RT-PCR method followed by direct sequencing and phylogenetic analysis novel canine rabies virus clades were identified in the Middle East and North Africa[5]. Two reports on transmission of rabies by solid organ transplantations occurred in the USA[6] and Germany[7]. There are no 100 per cent sensitive *in vivo* tests that exclude rabies in donors, however, RT-PCR might be a feasible approach to test donors with a history of acute progressive encephalitis.

The article by Biswal and coworkers[8] in this issue shows the role of RT-PCR as a better tool for the diagnosis of human rabies. Their study demonstrated that for post-mortem rabies diagnosis RT-PCR had 100 per cent sensitivity and specificity as compared to the classical method which showed only 83.3 per cent sensitivity. In addition, the RT-PCR and MIT had 100 per cent sensitivity and specificity while one false negative result was attributed to DFA. False negative by the DFA test depends upon the expertise of the examiner, the quality of the anti-rabies conjugate and the fluorescence microscope. In order to increase the sensitivity of the test, impression of tissue samples from three brain regions *viz*. the brainstem, cerebelum and Ammon' horns should be taken. The sensitivity of DFA can be reduced especially when the brain tissue submitted for testing is decomposed. In such

a case the RT-PCR is preferable and has a higher sensitivity compared to the classical methods. Our previous study on naturally decomposed brain tissue from infected animals showed false negative results in DFA and in RTCIT while the samples were found positive in RT-PCR[9]. These findings demonstrate that diagnostic ability of this test takes into account time and environmental problems that limit the use of DFA and RTCIT. However, whenever a negative result is obtained, there is no certainty regarding the true or false presence of rabies viral sequences. There is also a possibility that the sample had deteriorated, and was no longer suitable for RNA extraction, so in terms of public health no definite recommendation can be made when a field case is negative by RT-PCR. Recently it was shown that RT-PCR was found 1.6 times more sensitive than RTCIT suggesting that RT-PCR can complement RTCIT as a confirmatory assay[10].

Ante-mortem rapid diagnosis in humans is essential if human rabies therapy is anticipated. In addition, rapid diagnosis decreased the contacts that require post-exposure prophylaxis. For ante-mortem human rabies diagnosis different biological samples fluids such as saliva, cerebrospinal fluid, tears, urine as well nuchal skin biopsy containing hair follicles are needed for viral detection. Since DFA cannot be applied to liquid samples, RT-PCR and other molecular techniques are strongly recommended in conjunction with other conventional techniques[1]. Biswal and coworkers[8] applied the RT-PCR on human saliva samples ante-mortem and about 10 per cent of samples were found positive. Since there is intermittent shedding in fluids, it is extremely important to repeatedly collect fluids samples for virus detection. The sample collection process can have significant impact on the outcome of the PCR assay of tissue and fluids so refrigerated or freezing of samples and rapid processing should be done in order to preserve the RNA for amplification. The time of saliva sample collection might influence the positive results of saliva by using molecular approaches. The virus detection is highest during the first two days after the onset of symptoms and remains stable from day 2 through day 7 or even later[11]. A recent study showed a 100 per cent sensitivity in the RT- hemi nested PCR targeting the L polymerase gene in ante-mortem human rabies diagnosis[12]. The study involved 43 patients, showed higher sensitivity of detection from skin biopsies (98.3%) than from saliva (70.2%), but when three daily serial samples of saliva were tested, the sensitivity reached 100 per cent.

The use of RT-PCR and other molecular methods in ante-mortem human rabies diagnosis overcomes the low sensitivity of viral antigen detection methods. Rabies virus antibody is detected in only 20 per cent of unvaccinated rabies patient tested within 1-26 days after the disease onset[13]. Antibody positive serum samples can be obtained within 9 days after the disease onset. Antibodies appeared in CSF later or are not detected at all. Rabies virus antigen in neural innervations of hair follicle can be demonstrated by the fluorescence antibody technique on a frozen section of a skin biopsy from the nape of the neck. Test sensitivity was 82 per cent when it was performed within four days compared with 60 per cent between days 5 and 8. Both corneal and salivary impressions for detection of rabies virus antigen may be unreliable because of variation in interpretation[13]. The obstacle of RT-PCR and other molecular methods is the case by which sample cross-contamination can occur especially in nested PCR. Extensive precautions should be taken to avoid carryover contamination in these methods. The positive PCR product should be sequenced to confirm the origin of the virus and to rule out possible contamination. In conclusion, the presently available evidence indicates the advantages of using molecular diagnostic assays greater than disadvantages of these methods compared to classical virological assays for diagnosis.

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## References

1. WHO expert consultation on rabies. *World Health Organ Tech Rep Ser* 2005; *931* : 1-88.
2. Bourhy H, Dautry-Varsat A, Hotez PJ, Salomon J. Rabies, still neglected after 125 years of vaccination. *PLoS Negl Trop Dis* 2010; *4* : 1-3.
3. Sacramento D, Bourhy H, Tordo N. PCR technique as an alternative method for diagnosis and molecular epidemiology of rabies virus. *Mol Cell Probe* 1991; *5* : 229-40.
4. Kamolvarin N, Tirawatnpong T, Rattanasiwamoke R, Tirawatnpong S, Panpanich T, Hemachudha T. Diagnosis of rabies by polymerase chain reaction with nested primers. *J Infect Dis* 1993, *167* : 207-10.
5. David D, Hughes GJ, Yakobson BA, Davidson I, Hun H, Aylan O, *et al*. Identification of novel canine rabies virus clades in

the Middle East and North Africa. *J Gen Virol* 2007; *88* : 967-80.

6. Srinivasan A, Burton EC, Kuehnert MJ, Rupprecht C, Sutker WL, Ksiazek TG, *et al*. Transmission of rabies virus from an organ donor to four transplant recipients. *N Engl J Med* 2005; *352* : 1103-11.
7. Hellenbrand W, Meyer C, Rasch G, Steffens I, Ammon A. Cases of rabies in Germany following organ transplantation. *Euro Surveill* 2005; *10* : 8.
8. Biswal M, Ratho RK, Mishra B. Role of reverse transcriptase polymerase chain reaction for the diagnosis of human rabies. *Indian J Med Res* 2012; *135* : 837-42.
9. David D, Yakobson BA, Rotenberg D, Dveres N, Davidson I, Stram Y. Rabies virus detection by RT-PCR in decomposed naturally infected brains. *Vet Microbiol* 2002; *87* : 111-8.
10. Panning M, Baumgarte S, Pfefferle S, Maier T, Martens A, Drosten C. Comparative analysis of rabies virus RT-PCR and virus isolation using samples from a patient infected rabies virus. *J Clin Microbiol* 2010; *48* : 2960-2.
11. Wacharapluesadee S, Hemachudha T. Ante mortem post mortem diagnosis of rabies using nucleic acid amplification tests. *Expert Rev Mol Diagn* 2010; *10* : 207-18.
12. Dacheux L, Reynes JM, Buchy P, Sivuth O, Diop BM, Rousset D, *et al*. A reliable diagnosis of human rabies based on analysis of skin biopsy specimens. *Clin Infect Dis* 2008; *47* : 1410-7.
13. Hemachudha T, Laothamatas J, Rupprecht CE. Human rabies: a disease of complex neuropathogenetic mechanisms and diagnostic challenges. *Lancet Neurol* 2002; *1* : 101-9.