

# Detection of *Mycobacterium tuberculosis* from paraffin-embedded tissues by INNO-LiPA Rif.TB assay: retrospective analyses of Health Protection Agency National Mycobacterium Reference Laboratory data

Srinivasulu Reddy, Timothy Brown and Francis Drobniowski

## Correspondence

Srinivasulu Reddy  
srinivasulu.reddy@  
bartsandthelondon.nhs.uk

Health Protection Agency National Mycobacterium Reference Laboratory, Institute of Cell and Molecular Science, 2 Newark Street, London E1 2AT, UK

Received 24 July 2009  
Accepted 2 February 2010

Molecular diagnostic methods are of potential value in identifying tuberculosis (TB) and drug resistance where tissue specimens have been submitted for histology but not for microbiological culture. All paraffin-embedded tissue (PET) specimens ( $n=60$ ) referred to a single national centre over a 42 month study period were analysed using the INNO-LiPA Rif.TB assay; 29/60 patients had been reported to the UK Enhanced Tuberculosis Surveillance database with a diagnosis of TB based on clinical, radiological and histological evidence. *Mycobacterium tuberculosis* (MTB) DNA was detectable in 5 out of 29 reported TB cases (17.2%); 12 out of 29 of the reported TB cases had a positive MTB culture from a secondary clinical specimen and MTB DNA was detectable in 2 of the 12 (16.7%) laboratory-confirmed TB cases. Referring clinicians should be aware of the limitations of this assay on PETs, and should request molecular testing only in patients with a high clinical probability of TB and when acid-fast bacilli are seen in tissue specimens.

## INTRODUCTION

Extrapulmonary tuberculosis (TB) can occur at any body site. The Enhanced Tuberculosis Surveillance (ETS) data produced by the UK Health Protection Agency (HPA) showed that only 55% of extrapulmonary TB cases were culture confirmed (HPA, 2004) and the rest were diagnosed on the basis of histology. The problem arises when TB is not initially considered as a differential diagnosis in patients undergoing biopsy or needle aspiration, but the subsequent histology shows evidence of caseating granulomas with or without acid-fast bacilli. National Institute for Clinical Excellence guidelines recommend molecular assays for identification of *Mycobacterium tuberculosis* (MTB) complex in biopsy specimens only when a sample is inappropriately placed in formalin and if acid-fast bacilli are seen on histology (NICE, 2006).

The INNO-LiPA Rif.TB assay (LiPA PCR) (Innogenetics) is based on reverse hybridization between *rpoB* amplicons and membrane-bound capture probes (one specific for MTB complex, five overlapping wild-type probes spanning the *rpoB* target region and four of the most common mutations). The LiPA PCR can detect the presence of both MTB and resistance to rifampicin,

**Abbreviations:** ETS, Enhanced Tuberculosis Surveillance; HPA, Health Protection Agency; MTB, *Mycobacterium tuberculosis*; NMRL, National Mycobacterium Reference Laboratory; PET, paraffin-embedded tissue; TB, tuberculosis.

and can be completed in about 12 h (DeBeenhouwer *et al.*, 1995). A retrospective analysis was undertaken to assess the clinical utility of the LiPA PCR assay for detection of MTB DNA in paraffin-embedded tissue (PET) specimens.

## METHODS

**Case definitions.** A case was considered as a laboratory-confirmed case of TB when MTB was isolated by culture or when the MTB complex was detected by PCR in tissue samples. A case was considered as a clinical case of TB when it was notified to the ETS database based on clinical or radiological findings.

**PET samples.** A total of 60 PET specimens from 44 local National Health Service laboratories in the UK were referred to the HPA National Mycobacterium Reference Laboratory (NMRL) for molecular detection of MTB and drug resistance.

**DNA extraction and amplification.** An 8 mm<sup>3</sup> block of tissue was cut and added to 100 µl proteinase K buffer (50 mM Tris pH 8.0, 0.1% Triton X-100, 0.1 mg proteinase K ml<sup>-1</sup>) and incubated at 65 °C for 60 min; then 100 µl chloroform was added. The sample was emulsified and heated to 80 °C for 20 min; after centrifugation (10 min at 12 000 g) the aqueous supernatant was used as a template. A second 8 mm<sup>3</sup> block of wax distant from the tissue was processed in the same way as a negative control. The LiPA PCR was performed as described by Sam *et al.* (2006), and a PCR inhibition control reaction was included for each sample.

**Clinical, microbiological and histopathological data.** The ETS database contains TB notification information that includes demographic, clinical and microbiological data, providing comprehensive analysis of current TB trends. The Mycobacterial Surveillance Network (MycobNet) database includes data on identification of mycobacterial species and drug sensitivity results. The ETS and MycobNet databases were reviewed to identify clinical and laboratory-confirmed cases of TB. A written request for histopathology reports was sent to the submitting laboratory in order to collect information regarding presence of caseating granulomas and whether acid-fast bacilli had been seen.

## RESULTS AND DISCUSSION

From January 2004 to August 2007, the HPA NMRL received 60 PET specimens for molecular detection of MTB. Table 1 summarizes data regarding the biopsy site and the site of disease collated from ETS, MycobNet and NMRL laboratory databases, and the positive LiPA PCR results. The response rate for the histopathology report request was 35 out of 60 cases (58%). A review of 35 histopathology reports showed evidence of caseating granulomas in 10 and necrotizing granulomas in the remaining 25 cases. Acid-fast bacilli were seen in 12 of the 35 PET samples (34%), and LiPA PCR detected MTB in 1 of the 12 cases. Failure of LiPA to identify acid-fast bacilli in the remaining 11 cases could be due to DNA damage at the time of fixation or DNA extraction, or the presence of atypical mycobacteria in the tissues that are not detected by the LiPA PCR assay.

A review of the ETS database showed that 29 cases were notified as TB based on clinical and radiological findings. The primary site of TB for the notified cases is shown in Table 1. Specimens from secondary sites such as sputum, pleural fluid, etc. were collected for culture in patients suspected with TB based on histology; 12 of 25 notified cases had a culture positive for MTB from other sites.

A total of 6 out of 60 (10%) PET specimens tested were PCR positive for MTB; 5 of them were true positives. One patient was not notified to the ETS database and had a false-positive PCR result based on our case definition. The

PCR-positive PET specimens were from the following sites: lung (five), lymph node (two) and central nervous system (one). None of the six cases had evidence of rifampicin resistance mutations. Two of the six cases were culture-confirmed TB cases.

Correlation of PCR results with histological findings was possible in two of the six PCR-positive cases. One patient with a true-positive PCR result had evidence of acid-fast bacilli-positive granulomas in a lung tissue sample. The other patient with a false-positive PCR result had evidence of acid-fast bacilli-negative necrotic granulomas in lymph node tissue.

Of the 29 cases that were notified to the ETS database and that fitted the case definition for clinical TB, 12 were laboratory-confirmed cases. The sensitivity of the LiPA PCR assay was calculated as the proportion of clinical TB cases that had a positive PCR result (5/29). The sensitivity of the LiPA PCR assay, therefore, was 17.2%. Two out of twelve (16.7%) laboratory-confirmed cases were PCR positive. It is important to note that cultures from other sites were used for defining patients as laboratory-confirmed TB cases, and therefore a negative PCR result does not necessarily indicate failure of the PCR amplification. The failure of PCR in 10 laboratory-confirmed cases could have been due to other factors, such as low bacterial load and DNA damage following fixation.

The LiPA PCR assay is a highly sensitive and specific test for the detection of rifampicin-resistant MTB in culture isolates (Rossau *et al.*, 1997). The sensitivity of the assay on acid-fast bacilli-positive respiratory specimens is lower than that of culture isolates (Skenders *et al.*, 2005). The sensitivities of LiPA PCR for acid-fast bacilli-negative and non-respiratory samples in a previous study at HPA NMRL were 29.4 and 61.1%, respectively (Sam *et al.*, 2006).

Several studies have demonstrated that PCR methods can detect MTB DNA from PET specimens (Rish *et al.*, 1996; Popper *et al.*, 1994). A recent study reported the use of the

**Table 1.** Clinical and laboratory diagnosis of TB based on ETS data, the nature of PETs tested and PCR results

Specimen site	No. (%) of PET specimens tested	No. (%) of ETS reported TB cases	Secondary site/no. of culture-confirmed TB cases	No. of positive LiPA PCR results
Lung	12 (20)	12 (41.4)	Sputum – 5	3
Lymph node	12 (20)	7 (24.1)	2	2
Pleural	4 (6.7)	2 (6.9)	Pleural fluid – 1	
Gastrointestinal	4 (6.7)	5 (17.2)	Caecal biopsy – 1	
Liver	4 (6.7)			
Skin	8 (13.3)			
Central nervous system	2 (3.3)	1 (3.4)	Cerebrospinal fluid – 1	1
Bone	1 (1.6)	1 (3.4)	1	
Eye	2 (3.3)	1 (3.4)		
Genitourinary	2 (3.3)		Urine – 1	
Unknown	9 (15)			
Total no.	60	29 (48.3)	12	6

INNO-LiPA MYCOBACTERIA v2 assay on PETs; it showed that mycobacterial DNA was detectable in 10 of 42 (24%) PET specimens (Alvarado-Esquivel *et al.*, 2009). The INNO-LiPA MYCOBACTERIA v2 assay uses the 16S–23S rRNA spacer region as a target, unlike the *rpoB* target used in our study. In that study, 37 (88%) PET specimens were derived from lymph node and 18 (43%) PET specimens showed acid-fast bacilli on staining.

Extrapulmonary TB accounts for up to 41% of reported TB cases and a significant proportion of extrapulmonary TB (55%) cases are diagnosed based on clinical, radiological and histological evidence (HPA, 2004). Although microbiological culture is the reference standard for assessing the performance of the PCR assay, not all extrapulmonary TB cases have positive culture results, and therefore use of clinical TB as a gold standard is justified in calculating the sensitivity of the LiPA PCR assay. However, the sensitivity calculated is not intrinsic sensitivity under ideal laboratory conditions. The intrinsic sensitivity of LiPA PCR assay can be affected by several variables such as the fixative used (Greer *et al.*, 1991), fixation time (Greer *et al.*, 1991), amplified target DNA size, repetitiveness of the target, concentration of the DNA extracted and DNA extraction procedures (Shibata, 1994). The sensitivity calculated in this study is based on the actual clinical setting rather than ideal laboratory conditions, and should take into account the quality of the gold-standard clinical diagnosis, which depends on the diagnostic skills of the TB clinician and variations in the mycobacterial load of different tissues.

Our study had several limitations. It was a retrospective study based on routine surveillance data. The number of clinical TB cases reported may be an underestimate or overestimate of the true number of cases. Nonetheless, it is very unlikely that 31 excluded cases would have been treated for TB by clinicians without reporting to the ETS database. However, there is a possibility that the patient with a false-positive result had evidence of clinical TB, but this was not notified to the ETS database. The sensitivity of the LiPA PCR assay reported in our study is based on primary results without adjustment of false-negative and false-positive results.

Our study involved specimens from multiple centres, and therefore the quality of tissue received could have varied depending on individual laboratory processing methods. Tissue samples tested were also heterogeneous and there may have been inter-tissue site variation in the mycobacterial load. In our study, five PCR-positive specimens were derived from either lung or lymph nodes. Previous studies have shown the presence of PCR inhibitors in extrapulmonary tissue specimens such as pleural fluid and tissue biopsies (Honoré-Bouakline *et al.*, 2003; Pfyffer *et al.*, 1996); 47% of tissues tested in our study were from sites other than lung or lymph node and this could have affected the overall sensitivity.

In this study, the LiPA PCR assay detected MTB DNA in only 17.2 and 16% of patients determined to have clinical- and laboratory-confirmed TB, respectively. Although the

sensitivity of the LiPA PCR assay on PETs is low, there is still a strong clinical need for a molecular diagnostic test to confirm TB in PET specimens and definitively exclude other serious diseases, each with its own different prognosis and treatment. There is also a need to differentiate MTB from non-tuberculous mycobacteria, especially from skin biopsies, which can affect treatment.

Clinicians requesting a molecular diagnostic test should understand the limitations of current molecular diagnostic assays in detecting MTB DNA in PET specimens. Clinicians should therefore save fresh tissue specimens at the time of collection for microbiological culture at a later date, depending on histological findings. A molecular diagnostic test should be used as a last resort in patients with a high clinical probability of TB and/or in presence of acid-fast bacilli in tissues.

## ACKNOWLEDGEMENTS

The authors thank Dr Ibrahim Abubakar, Consultant Epidemiologist, and Jonathan Moore at the HPA Centre for Infections, Colindale, London, for assistance with patient data from NOIDS (Notifications of Infectious Diseases) and MycobNet databases, and the consultant histopathologists for providing histopathology information for patients. We also thank the staff of the UK HPA NMRL for the laboratory testing of samples.

## REFERENCES

- Alvarado-Esquivel, C., García-Corral, N., Carrero-Dominguez, D., Enciso-Moreno, J. A., Gurrola-Morales, T., Portillo-Gómez, L., Rossau, R. & Mijs, W. (2009). Molecular analysis of *Mycobacterium* isolates from extrapulmonary specimens obtained from patients in Mexico. *BMC Clin Pathol* 9, 1.
- De Beenhouwer, H., Lhiang, Z., Jannes, W., Nfijs, L., Machtelincx, L., Rossau, R., Traore, H. & Portaels, F. (1995). Rapid detection of rifampin resistance in sputum and biopsy specimens from tuberculosis patients by PCR and line probe assay. *Tuber Lung Dis* 76, 425–430.
- Greer, C. E., Peterson, S. L., Kiviat, N. B. & Manos, M. M. (1991). PCR amplification from paraffin-embedded tissues – effects of fixative and fixation time. *Am J Clin Pathol* 95, 117–124.
- HPA (2004). *Annual Report on Tuberculosis Cases Reported in 2001 in England, Wales and Northern Ireland*. London: Health Protection Agency. [http://www.hpa.org.uk/web/HPAwebFile/HPAweb\\_C/1194947356221](http://www.hpa.org.uk/web/HPAwebFile/HPAweb_C/1194947356221)
- Honoré-Bouakline, S., Vincensini, J. P., Giacuzzo, V., Lagrange, P. H. & Herrman, J. L. (2003). Rapid diagnosis of extrapulmonary tuberculosis by PCR: impact of sample preparation and DNA extraction. *J Clin Microbiol* 41, 2323–2329.
- NICE (2006). *Tuberculosis: Clinical Diagnosis and Management of Tuberculosis, and Measures for its Prevention and Control*. London: National Institute for Health and Clinical Excellence. <http://www.nice.org.uk/nicemedia/pdf/CG033nicguideline.pdf>
- Pfyffer, G. E., Kissling, P., Jahn, E. M., Welscher, H. M., Salfinger, M. & Weber, R. (1996). Diagnostic performance of amplified *Mycobacterium tuberculosis* direct test with cerebrospinal fluid, other nonrespiratory, and respiratory specimens. *J Clin Microbiol* 34, 834–841.
- Popper, H. H., Winter, E. & Hofler, G. (1994). DNA of *Mycobacterium tuberculosis* in formalin-fixed, paraffin-embedded tissue in tuberculosis and sarcoidosis detected by polymerase chain reaction. *Am J Clin Pathol* 101, 738–741.

**Rish, J. A., Eisenach, K. D., Cave, M. D., Reddy, M. V., Gangadharam, P. R. J. & Bates, J. H. (1996).** Polymerase chain reaction of *Mycobacterium tuberculosis* in formalin-fixed tissue. *Am J Respir Crit Care Med* **153**, 1419–1423.

**Rossau, R., Traore, H., de Beenhouwer, H., Mijs, W., Jannes, G., de Rijk, P. & Portaels, F. (1997).** Evaluation of the INNO-LiPA Rif. TB assay, a reverse hybridization assay for the simultaneous detection of *Mycobacterium tuberculosis* complex and its resistance to rifampin. *Antimicrob Agents Chemother* **41**, 2093–2098.

**Sam, I. C., Drobniowski, F., More, P., Kemp, M. & Brown, T. (2006).** *Mycobacterium tuberculosis* and rifampin resistance, in the United Kingdom. *Emerg Infect Dis* **12**, 752–759.

**Shibata, D. (1994).** Extraction of DNA from paraffin-embedded tissue for analysis by polymerase chain reaction: new tricks from an old friend. *Hum Pathol* **25**, 561–563.

**Skenders, G., Fry, A. M., Prokopovica, I., Greckoseja, S., Broka, L., Metchock, B., Holtz, T. H., Wells, C. D. & Leimane, V. (2005).** Multidrug-resistant tuberculosis detection, Latvia. *Emerg Infect Dis* **11**, 1461–1463.