JCM
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Performance of the Roche LightCycler Real-Time PCR Assay for Diagnosing Extrapulmonary Tuberculosis

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The Roche LightCycler mycobacterium detection molecular assay for *Mycobacterium tuberculosis*, *M. avium*, and *M. kansasii*, was applied to tissue specimens. It performed well on lymph node and cerebrospinal fluid specimens and less well on lung, liver, and bone marrow core biopsy specimens, but used in conjunction with a clinical suspicion of tuberculosis, it could augment patient management.

The steady rise in pulmonary tuberculosis (TB) (35) and clinical extrapulmonary TB (EPTB) cases in South Africa can be attributed to HIV infection. At present, the national TB incidence rate is estimated at 971 cases per 100,000 (33), of which 58% of tested patients are HIV positive (33). Of the estimated 340,000 new TB cases diagnosed in South Africa in 2009, 16% were of extrapulmonary origin (33). EPTB accounts for approximately one-third of TB-related deaths in HIV-infected persons (1, 12, 21), of which only half are diagnosed before death (1, 21). TB lymphadenitis continues to be one of the most frequent presentations (28, 34), accounting for 30 to 50% of all EPTB cases (19).

Conventional diagnosis of EPTB hinges on identification of acid-fast bacilli, histopathological examination of tissues, or culture (6, 17). These methods are time-consuming, require expertise, can be nonspecific, and are frequently delayed. Difficulty in obtaining visceral and deep lymph node tissue and small numbers of bacilli at these sites make diagnosis even more difficult (22, 23). The shift is toward newer molecular diagnostic tools for detecting either RNA or DNA to improve sensitivity and speed up the diagnostic process (30). A number of studies have reported on performance of PCR-based assays for diagnosis of EPTB in formalin-fixed paraffin-embedded tissues (3, 9, 14, 18, 26), bone marrow, and lymph node (22, 23) and lung (17) tissue. The recent WHO-endorsed Xpert MTB/RIF assay (Cepheid, Sunnyvale, CA), has shown a sensitivity for nonrespiratory tissue specimens ranging from 53% to 100% and a specificity ranging from 97.3% to 100% (2, 10, 13, 31). However, with various degrees of sensitivity being reported for different assays (8), the clinical utility of PCR-based tests for diagnosing EPTB in tissue samples is still uncertain (17). We investigated another molecular assay for application in diagnosing EPTB, the LightCycler mycobacterium detection assay (LCTB) (Roche Diagnostics, GmbH, Germany), which has the added advantage of simultaneously diagnosing three species, *Mycobacterium tuberculosis*, *M. avium*, and *M. kansasii*. The assay is based on a combination of real-time PCR of a 200-bp mycobacterial fragment and fluorescent detection by HybProbe probes on the LightCycler version 2 instrument (24). Species differentiation is based on differences in melting curves of the species. This assay has been evaluated using sputum specimens ($n = 177$; sensitivity of 76%/specificity of 98%) (27) and bone marrow aspirates ($n = 60$; sensitivity of 50%/specificity of 73%) (6a), which showed a decreased specificity, probably due to the specimen preparation method employed for this study, in which bone marrow specimens were manually scraped off prestained slides. Our study extends this performance investigation to additional tissue biopsy specimens obtained postmortem to determine the clinical utility of the assay. Performance was compared to clinical diagnosis, which is often relied upon to make diagnoses and treatment decisions for patients awaiting laboratory results.

Patients older than 18 years of age, who died in the wards of the Charlotte Maxeke Academic Hospital (Johannesburg, South Africa), with known HIV infection, who either were on antiretroviral therapy (ART) or would have been eligible for ART if they had lived, were enrolled. The study was approved by the University of the Witwatersrand Human Ethics Review Committee (M081136; Medical/M090688 and M070826; Research). At postmortem, core biopsy specimens from liver, lung, bone marrow (BM), lymph nodes, and cerebrospinal fluid (CSF) were obtained from 39 cadavers (cases). Specimens were prepared for liquid mycobacterial culture (MGIT) (followed by species identification using the GenoType Mycobacterium CM assay [Hain Lifesciences, GmbH, Nehren, Germany]) and histologic examination by hematoxylin-and-eosin and Zhiel-Neelsen staining, as per standard protocols (5, 20). Clinicopathological causes of death were determined by an expert panel of pathologists, infectious disease and pulmonology specialists who based their decision on premortem clinical and microbiological data and postmortem clinical, microbiological, and histological data. Additional specimens from each source organ were prepared for molecular analysis. DNA was extracted with the MagNA Pure LC DNA isolation kit III (bacterial, fungi) (Roche Diagnostics, GmbH, Germany) using the recommended preisolation protocols for liquid and biopsy specimens (25). Briefly, this involved homogenization and extended lysis of ~1 to 10 mg of lung ($n = 28$), liver ($n = 34$), lymph node ($n = 17$), and

Received 26 January 2012 **Returned for modification** 29 February 2012
Accepted 4 April 2012
Published ahead of print 11 April 2012
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doi:10.1128/JCM.00252-12

TABLE 1 Overall sensitivities and specificities of the LCTB assay versus a clinical diagnosis, MGIT culture results, and smear microscopy[a]

Parameter	Value for LCTB assay compared with:		
	Clinical diagnosis	Culture	Histology smear
Sensitivity (%)	52.1	64.3	100
Specificity (%)	93.7	84	78.8

[a] Overall results, $n = 39$ cases.

BM core ($n = 32$) tissue using the MagNa Lyser instrument (Roche Diagnostics, GmbH, Germany) and centrifugation and resuspension of 500 μl of the CSF specimens ($n = 36$). All specimens were then extracted on the MagNA Pure LC instrument (Roche Diagnostics, GmbH, Germany), according to the manufacturer's instructions, with an input volume of 100 μl and a final elution volume of 50 μl. Eluates were stored at −70°C until amplification using the LCTB assay as per the manufacturer's instructions (27). In addition and at the end of the study, residual homogenized tissue specimens (stored at −70°C, ~18 months) ($n = 2$ lung, 3 liver, 3 lymph, 2 BM, and 4 CSF specimens) which had sufficient leftover volume were also tested in the Xpert MTB/RIF assay (G3 cartridge). Five hundred microliters of tissue lysate was deactivated with 1.5 ml Xpert SR buffer for 15 min, and the assay was performed as per the manufacturers' instructions.

Of the three methodologies employed, the percent positives detected in the total cases were as follows: (i) clinical case definition of disseminated mycobacterial infection to be either an immediate or contributing cause of death in 72% (28/39) of cases (24 cases defined as disseminated *M. tuberculosis*, 1 *M. avium* case, 3 undifferentiated [could not be defined to the species level] cases), (ii) culture positive in 35.9% (14/39) of cases (13 *M. tuberculosis* cases and 1 *M. avium* case), and (iii) LCTB positive in 33.3% (13/39) of cases (all *M. tuberculosis*). A comparison of LCTB assay versus different methodologies is shown in Table 1. A positive result for one patient/tissue type by clinical diagnosis, culture, or LCTB did not necessarily yield a positive result in the same patient/tissue type by the other methodologies. Of all specimens submitted for LCTB analysis, results were generated in 99.4% ($n = 1$ internal control "uncertain" result), whereas culture generated results in 86% of the biopsy specimens due to contamination ($n = 25$ specimens contaminated). Compared to a clinical diagnosis, the LCTB assay yielded a low, although slightly better, sensitivity than culture overall (52.1% versus 46.4%; $n = 39$), with best performance seen in lymph node specimens (Table 2).

Overall, the LCTB assay detected 4 more positive cases than did culture: for 2 cases, histology clearly showed TB infection present, thus demonstrating the increased sensitivity of PCR over culture; 2 cases had a history of TB treatment for more than 30 days and may represent PCR false positives. If all cases of patients on TB treatment for more than 30 days are excluded from analysis ($n = 12$), the overall performance (sensitivity/specificity) of the LCTB assay versus a clinical diagnosis was 50%/100%, and that versus culture was 58.3%/86.6% ($n = 27$). Comparison of different tissue types showed increased performance for lymph node and CSF specimens (Table 3).

TABLE 3 Comparison of LCTB assay with clinical diagnosis and with culture after exclusion of cases on TB treatment for more than 30 days

Comparison	Value when excluding cases with >30 days of TB treatment	
	% sensitivity (n[a])	% specificity
LCTB vs clinical diagnosis		
Lung	26.3 (27)	100
Liver	21 (27)	100
Lymph node	41.6 (14)	100
BM	5.3 (27)	100
CSF	10.5 (27)	100
LCTB vs culture		
Lung	37.5 (21)	84.6
Liver	42.8 (20)	94.4
Lymph node	100 (12)	70
BM	0 (22)	100
CSF	100 (25)	100

[a] n, no. of specimens.

The Xpert MTB/RIF assay showed performance similar to that of the LCTB assay with these preextracted tissues ($n = 14$ specimens tested using Xpert). Compared to culture, the Xpert MTB/RIF assay failed to detect three culture-positive specimens (lung, BM, and CSF), two of which were also negative by the LCTB assay. Two specimens (lymph and BM) were detected as positive by both the LCTB and Xpert MTB/RIF assays, which were culture negative (overall sensitivity of 73% and specificity of 33%), again demonstrating increased sensitivity of PCR-based assays for diagnosing EPTB over that of culture.

The use of lymph node biopsies is safe, well tolerated, and diagnostically relevant for tuberculous lymphadenitis due to the high yield of tissue obtained (11, 15, 32). Despite small sample numbers (for 19 patients, a lymph node biopsy specimen was not submitted due to there being no palpable lymph nodes to biopsy), our findings support the use of lymph tissue and CSF testing for active disease, since the LCTB molecular assay showed 100% specificity compared to clinical diagnosis

TABLE 2 Sensitivities and specificities for culture and LCTB assay versus a clinical diagnosis with individual tissue types

Parameter	Value for method										
	Culture results for tissue type[b]					Clinical diagnosis of EPTB[b]	LCTB results for tissue types[b]				
	Lung	Liver	Lymph	BM	CSF		Lung	Liver	Lymph	BM	CSF
% positivity (n[a])	28 (8)	31 (8)	18 (3)	16 (5)	14 (5)	72 (28)	23 (9)	13 (5)	30 (6)	8 (3)	8 (3)
Sensitivity (%) vs clinical diagnosis	38	33	21	23	19		32	18	35	11	11
Specificity (%) vs clinical diagnosis	100	100	100	100	100		100	100	100	100	100

[a] n, no. of positive specimens.
[b] Numbers of specimens are as follows. For culture results, $n = 29$ (lung), 34 (liver), 17 (lymph), 32 (BM), and 36 (CSF); for clinical diagnosis, $n = 39$; for LCTB results, $n = 39$ (lung, liver, BM, and CSF) or 20 (lymph).

and excellent sensitivity against culture in both these tissue types. Moreover, if a clinician suspects TB, the LCTB assay could assist in confirming the diagnosis and expedite patient management decisions.

Possibly, the poorer performance of the LCTB with other tissue types may be due to poor quality of tissues, uneven distribution of bacteria, difficulty in extracting *M. tuberculosis* DNA or loss of DNA during extraction, or the presence of PCR-inhibitory substances (6, 29). Similar studies have employed phenol-chloroform with ethanol precipitation (7, 22, 23) or manual commercial kit extraction (6, 17), which are labor intensive and time-consuming. Since an automated extraction method was used, a tissue homogenization step was performed preextraction to liquefy the solid tissue specimens and release any bacteria present. This step proved troublesome for the BM specimens, which were submitted as cores of bone containing BM.

This study used core biopsy material, but for optimal diagnostic efficiency, the performance of this assay with fine needle aspirates requires assessment. The LCTB assay has limited hands-on time and potential for automation, coupled with a total assay time of 1 h, 45 min and a cost per test of ~R78.40 (US$11.7), excluding extraction, implementation of such molecular methods within laboratories already under tremendous strain due to growing workloads, may help relieve many of the burdens associated with TB diagnosis. Although the performance of the Xpert MTB/RIF has recently been reported for EPTB diagnosis (2, 4, 10, 11, 16, 31), this study also shows its potential application for solid tissue and warrants further investigation.

ACKNOWLEDGMENTS

We have no conflict of interest to declare.

This study was funded through the U.S. Agency for International Development and PEPFAR. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

The contents are the responsibility of the authors and do not necessarily reflect the views of USAID or the U.S. government.

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