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Comparison of Single-Copy and Multicopy Real-Time PCR Targets for Detection of *Mycobacterium tuberculosis* in Paraffin-Embedded Tissue▽

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Real-time PCR can rapidly identify *Mycobacterium tuberculosis* in paraffin-embedded tissue in the absence of microbiological culture. In a comparison of single-copy and multicopy PCR targets in 70 tissue samples, the sensitivities were 26% and 54%, respectively, with 100% specificity. Sensitivity was 75% for newer samples and was not decreased for acid-fast bacillus (AFB) stain-negative specimens.

With a third of the world's population infected by *Mycobacterium tuberculosis* complex, accurate and timely diagnosis of tuberculosis is critical for management of this global epidemic (12). Although tuberculosis is often diagnosed by sputum smear and microbiological culture, the disease can also present as extrapulmonary mass lesions, which are frequently biopsied without clinical suspicion for infection (3). Under histopathologic examination, biopsy sections typically demonstrate necrotizing granulomas with or without acid-fast bacilli, neither of which is specific for tuberculosis (1, 5). Without an alternative method for diagnosis, a repeat biopsy is often necessary to procure tissue for mycobacterial culture.

More recently, real-time PCR has been utilized for detection of *M. tuberculosis* in culture and sputum (4, 6, 9, 10). Real-time PCR is faster than conventional PCR and does not require postamplification specimen handling (10). Several studies have also evaluated the usefulness of real-time PCR for *M. tuberculosis* in formalin-fixed, paraffin-embedded tissue, with sensitivities ranging from 67% to 100% (1, 3, 5, 8, 13). However, most of these studies did not have complete microbiological culture results to use as their reference standard and instead relied on histologic findings and/or clinical data to identify presumptive tuberculosis cases. Furthermore, none evaluated multiple tissue sites or other factors that may have impacted the sensitivity of real-time PCR in fixed tissue. An older study using conventional PCR suggested that IS*6110*, a multicopy PCR target, is more sensitive than a single-copy PCR target; however, this study also did not use culture as the reference standard, and the assays were compared across different laboratories using different instruments and reagents (11). Thus, the purpose of this study was to evaluate the sensitivity and specificity of real-time PCR for detection of culture-positive *M. tuberculosis* in formalin-fixed paraffin-embedded tissue and to identify factors that enhance or impede the sensitivity of this assay.

A retrospective study was performed on 70 formalin-fixed paraffin-embedded tissue biopsy specimens collected at a major academic hospital over a 10-year period. Biopsies of 35 consecutive specimens with *M. tuberculosis* complex, 23 specimens with nontuberculous mycobacteria, and 12 noninfectious specimens were included. Biopsy sites included lung ($n = 30$), lymph nodes ($n = 17$), bone ($n = 5$), skin ($n = 5$), and other sites ($n = 13$), such as soft tissue, with sample types evenly distributed over the study time period. Microbiological culture using reference methods (10) from tissue taken before fixation in formalin was the gold standard for diagnosis of *M. tuberculosis*. Acid-fast bacillus (AFB) tissue staining was done with 54 of the 58 specimens positive for mycobacteria by culture. Five 10-μm-thick sections were cut from each specimen using a new cryostat blade for each sample. DNA extraction was done on a Qiagen BioRobot EZ1 machine following the paraffin section protocol (Qiagen Inc., Valencia, CA), with an additional 10-min boiling step after overnight incubation with proteinase K (3).

Parallel real-time PCRs were performed for a single-copy internal transcribed spacer (ITS) target (10) and a multicopy IS*6110* target for *M. tuberculosis* complex (5), with primers designed in house (Table 1). *M. tuberculosis* H37Rv (ATCC 27294) was used as a positive control. A third, separate PCR with primers for human beta-actin was carried out to control for DNA extraction (2). All primer sequences and the expected melting temperatures are outlined in Table 1. Each PCR mixture contained 1.25 μl of 10 μM primer mix, 3.75 μl molecular-grade water, 12.5 μl 2× FastStart Sybr green master mix (Roche Diagnostics, Indianapolis, IN), and 7.5 μl of extracted mycobacterial DNA. PCR was carried out on a SmartCycler II instrument (Cepheid, Sunnyvale, CA) and consisted of a 5-min 95°C activation step followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. Melting-curve generation and analysis were done as previously described (10). Two sample tests of proportions were done to compare results using the Stata 8.0 software program (StataCorp LP, College Station, TX).

Overall, real-time PCR targeting the multicopy IS*6110* target demonstrated a sensitivity of 54% and specificity of 100% for all samples, regardless of the AFB stain result, compared to culture. The positive predictive value was 100%, and the negative predictive value was 69%. The IS*6110* target was superior to both the single-copy ITS target (26% sensitivity; $P = 0.0004$)

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TABLE 1. Primers used in this study

Primer target (description)[a]	Primer sequences (forward/reverse)	Amplicon size (bp)	T_m (°C)
MTC ITS (single copy)	GCGAGAGCCGGGTGCATG	47	75.8 ± 0.8
	AACAGTGTGTTGGTGGCCAA		
IS*6110* (multicopy)	GAACGGCTGATGACCAAACT	72	83.1 ± 1.0
	ATCAGCGATCGTGGTCCTG		
Beta-actin (control)	AGCGGGAAATCGTGCGTG	135	83.0 ± 0.4
	GGTGATGACCTGGCCGTC		

[a] MTC, *Mycobacterium tuberculosis* complex.

and acid-fast staining of tissue sections (29% sensitivity; $P = 0.01$). The sensitivity of the IS*6110* target (57%) was not significantly different in cases where the AFB stain was negative ($P > 0.05$). PCR sensitivity was negatively impacted, however, by the age of the block. For biopsy specimens less than 2 years old, the overall sensitivity of the IS*6110* PCR was 75%, while for samples older than 2 years, the sensitivity was 42% ($P = 0.04$). Although the sensitivity of PCR was higher for lung tissue (67%) than for all other tissue types (48%), the difference was not statistically significant ($P = 0.15$). PCR results for all culture-positive samples by tissue site and sample age are listed in Table 2. Since this assay was not quantitative, further studies may help elucidate if PCR sensitivity is higher for certain tissue samples due to a larger bacterial load or other factors.

Based on these findings, real-time PCR from paraffin-embedded tissue demonstrates up to 75% sensitivity and 100% specificity for detection of the multicopy IS*6110* target of *M. tuberculosis* complex. The sensitivity of PCR in this study is comparable to rates reported in other studies targeting IS*6110* (1, 5, 8). One study reported 100% sensitivity for real-time PCR, but this study tested only lung samples and demonstrated a specificity of 81% (13). The results of the current study suggest that paraffin-based assays should target multicopy sequences for maximal sensitivity. Although all the isolates in this study which were positive by ITS primers were also positive for IS*6110*, targeting of both sequences may help detect rare isolates, typically from Southeast Asia, which do not have IS*6110* (7).

The lack of a higher sensitivity for real-time PCR in this study is likely due in part to DNA damage and cross-linking during formalin fixation and tissue processing (11). This is supported by the fact that the sensitivity of PCR was significantly higher for recent biopsy specimens. Furthermore, this finding has implications for use of older tissues for retrospective diagnosis of tuberculosis by PCR. Since acid-fast staining of tissue sections shows low sensitivity for *M. tuberculosis*, real-time PCR can provide a rapid and more sensitive and specific method for the diagnosis of tuberculosis from paraffin-embedded tissue with a negative acid-fast stain. Thus, as with sputa, positive acid-fast staining should not be a prerequisite for PCR testing of tissue (5, 8).

In conclusion, real-time PCR targeting IS*6110* identified *M. tuberculosis* in a majority of paraffin-embedded tissue specimens with active tuberculosis across a wide range of tissue sites. Real-time PCR can prove particularly useful when the clinical lesion is difficult to rebiopsy and rapid diagnosis is crucial.

TABLE 2. Specimen characteristics and PCR results for culture-positive tuberculosis cases[a]

Tissue site (n[a])	Tissue age (yr)	PCR target[b]	
		IS*6110*	ITS
Lymph node (1), lung (1)	<0.5	Pos	Pos
Mediastinum (1)	<0.5	Pos	Neg
Lung (1), skin (1), endometrium (1)	<0.5	Neg	Neg
Lung (3)	1	Pos	Neg
Lymph node (2), bone (1)	1.5–2	Pos	Pos
Bone (1)	2.5	Pos	Neg
Lung (2)	2.5	Neg	Neg
Lymph node (1), lung (1)	3	Neg	Neg
Lung (1)	5	Pos	Pos
Lymph node (1), lung (1)	6–6.5	Pos	Neg
Lymph node (2), bone (1)	6–6.5	Neg	Neg
Lung (2)	6.5	Pos	Pos
Lymph node (1), bone (1)	7	Neg	Neg
Lymph node (2), bone (1)	8–8.5	Pos	Neg
Lymph node (2)	8.5	Neg	Neg
Lymph node (1)	9	Pos	Pos
Lymph node (2)	9	Neg	Neg

[a] n, no. of specimens.
[b] Pos, positive; Neg, negative.

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