JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 2008, p. 314–316 Vol. 46, No. 1
0095-1137/08/$08.00+0 doi:10.1128/JCM.01313-07

Specimen Dilution Improves Sensitivity of the Amplified *Mycobacterium tuberculosis* Direct Test for Smear Microscopy-Positive Respiratory Specimens▽

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Received 29 June 2007/Returned for modification 9 September 2007/Accepted 21 October 2007

Specimen dilution has been proposed as a strategy to minimize amplified *Mycobacterium tuberculosis* direct (MTD) test inhibition (N. Pollock, J. Westerling, and A. Sloutsky, Am. J. Clin. Pathol. 126:142–147, 2006; A. Sloutsky, L. L. Han, and B. G. Werner, J. Clin. Microbiol. 42:1547–1551, 2004). We evaluated the impact of respiratory specimen dilution on MTD test accuracy in a public health laboratory. The difference in MTD test sensitivity between the dilution and conventional methods was 15.9% ($P = 0.001$) for smear microscopy-positive specimens and −3.6% ($P = 0.38$) for smear microscopy-negative specimens.

For the diagnosis of pulmonary tuberculosis (TB), nucleic acid amplification tests, such as the amplified *Mycobacterium tuberculosis* direct (MTD) test (Gen-Probe, Inc, San Diego, CA), are potential adjuncts of smear microscopy and mycobacterial culture (1, 2, 4, 5). However, false-negative results can be caused by inhibitory substances present either in specimens or in reagents used for specimen decontamination and concentration (3, 8, 10). Specimen dilution has been proposed as a strategy to minimize the impact of inhibitory substances (9, 10).

In order to detect inhibitors in MTD test-negative specimens, the MTD test manufacturer's protocol recommends repeating the test using a 450-μl aliquot of the specimen sediment along with an internal amplification positive control (3). If inhibitors are present, the negative results for the previously tested specimen cannot be reliably interpreted and another specimen should be collected and evaluated. This approach requires additional processed specimen sediment, which may not be available. In addition, this approach may be costly if a substantial number of specimens have inhibitors and may not resolve the diagnostic question if subsequent specimens from the same patient also contain inhibitors.

The TB Laboratory of the Maryland Department of Health and Mental Hygiene serves as the primary mycobacteriology laboratory for all public TB control programs in Maryland. This laboratory routinely performs the MTD test on respiratory specimens submitted for mycobacterial smear microscopy and culture. In April 2004, the laboratory initiated the routine use of a testing strategy in which, for respiratory specimens, the MTD test was performed simultaneously on undiluted specimens and specimens diluted 1:10. The strategy was undertaken in an effort to minimize the impact of inhibitors. The objective of this retrospective study was to compare the results of MTD testing using undiluted (conventional) and diluted specimens. In addition, we sought to determine the impact of the specimen smear microscopy status on the results of conventional and dilution methods of MTD testing, since to our knowledge this had not been reported previously and an understanding of this issue may inform the development of optimally efficient testing strategies.

Laboratory records for consecutive respiratory specimens submitted between April 2004 and March 2006 were reviewed. For each patient, the first specimen for which both conventional and dilution MTD testing methods were run in parallel was included in the analysis. Specimens from patients who had received ≥7 days of TB treatment prior to specimen collection were excluded. Respiratory specimens were digested, decontaminated, and concentrated by the *N*-acetyl-L-cysteine–NaOH method (6). A smear of the processed sediment was stained by the Truant fluorescence acid-fast-bacillus (AFB) staining method using auramine O-rhodamine B. Microscopy and results reporting were according to published standards (6). A 0.5-ml portion of the decontaminated specimen was inoculated into BACTEC 12B liquid culture medium (Becton, Dickinson and Co., Franklin Lakes, NJ), and 0.2 ml was inoculated onto one Löwenstein-Jensen slant. Liquid cultures were incubated at 37°C for 6 weeks using a BACTEC 460 TB instrument (Becton, Dickinson and Co.). Löwenstein-Jensen slants were incubated at 35°C and examined weekly for 8 weeks. Mycobacterial isolates were identified using DNA probes (AccuProbe; Gen-Probe, Inc.), the BACTEC NAP (nitro-α-acetylamino-β-hydroxypropiophenone) test (Becton, Dickinson and Co.), and/or biochemical tests (7).

The MTD assay was performed and its results were interpreted according to the manufacturer's protocol (3). For each respiratory specimen, two tests were run in parallel—one using an undiluted aliquot of the concentrated processed specimen and the other using a 1:10 dilution of the concentrated pro-

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▽ Published ahead of print on 31 October 2007.

TABLE 1. Characteristics of respiratory specimens

AFB smear microscopy result	No. (%) of specimens with culture result of:			Total
	Negative	Positive for *M. tuberculosis* complex	Positive for NTM	
Negative	247 (84)	28 (9.5)	19 (6.5)	294 (100)
Positive	16 (8.1)	113 (57.4)	68 (34.5)	197 (100)
Total	263 (53.6)	141 (28.7)	87 (17.7)	491 (100)

cessed specimen. The diluted specimen was prepared by adding 450 μl of sterile distilled water to 50 μl of the concentrated processed specimen. The diluted specimen was briefly subjected to a vortex inside a biosafety cabinet.

The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the MTD test were estimated using mycobacterial culture results as the "gold standard." For sensitivity and specificity calculations, specimens with equivocal MTD test results were excluded from the numerators (the numbers of specimens with a given result) but included in the denominators (the total numbers of specimens). The McNemar test was used to analyze the differences in sensitivities, specificities, PPVs, and NPVs for paired diluted and undiluted specimens.

A total of 494 respiratory specimens (from 494 patients suspected of having pulmonary TB) were evaluated. Three specimens were excluded from the analysis because the source patients had received ≥7 days of TB treatment prior to specimen collection. Table 1 shows the mycobacterial smear microscopy and culture results for the remaining 491 specimens included in the analysis. Culture was positive for the *M. tuberculosis* complex for 141 specimens (28.7%), positive for nontuberculous mycobacteria (NTM) for 87 specimens (17.7%), and negative for 263 specimens (53.6%) (Table 1).

The performance of conventional and dilution MTD testing methods is summarized in Table 2. Among smear microscopy-positive specimens that were culture positive for the *M. tuberculosis* complex, 19 (16.8%) were either falsely negative or had equivocal results by the conventional MTD testing method while only 1 (0.8%) was falsely negative by the dilution MTD testing method. Therefore, for smear microscopy-positive specimens, the sensitivities of the dilution and conventional methods of MTD testing were 99.1% (95% confidence interval [95% CI], 97.8 to 100%) and 83.2% (95% CI, 78.0 to 88.4%), respectively (difference, 15.9% [95% CI, 10.5 to 21.3%]; $P = 0.001$). Among smear-negative specimens, there was no significant difference in MTD test sensitivity between the dilution and conventional testing methods (46.4% [95% CI, 40.7 to 52.1%] versus 50.0% [95% CI, 44.3 to 55.7%]; difference, −3.6% [95% CI, −4.5 to 11.7%]; $P = 0.38$). There was no significant difference in specificity between the dilution and conventional testing methods regardless of the specimen smear microscopy status.

For smear microscopy-negative specimens, the PPV of the conventional strategy was unexpectedly low at 82.4%. This result was driven by three specimens (from three patients) that were MTD test positive yet culture negative. However, the three patients had additional respiratory specimens with culture growth of the *M. tuberculosis* complex. Among smear microscopy-positive specimens, the two specimens with negative culture results and positive MTD test results by both methods were from patients who had additional respiratory specimens with culture growth of the *M. tuberculosis* complex. In

TABLE 2. Accuracy of conventional and 1:10 dilution methods of MTD testing

AFB smear microscopy result and MTD method	MTD result(s)	No. of specimens with culture result of:			Total no. of specimens	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	PPV (%) (95% CI)	NPV (%) (95% CI)
		Positive for *M. tuberculosis* complex	Positive for NTM	Negative					
Positive									
Conventional	Positive	94	2	2	98	83.2 (78.0–88.4)	95.2 (92.2–98.2)	95.9 (93.1–98.7)	84.2 (79.1–89.3)
	Negative	15	66	14	95				
	Equivocal	4	0	0	4				
	All	113	68	16	197				
1:10 dilution	Positive	112	1	2	115	99.1 (97.8–100)	95.2 (92.2–98.2)	97.4 (95.2–99.6)	98.8 (97.3–100)
	Negative	1	66	14	81				
	Equivocal	0	1	0	1				
	All	113	68	16	197				
Negative									
Conventional	Positive	14	0	3	17	50.0 (44.3–55.7)	97.7 (96.0–99.4)	82.4 (78.0–86.8)	96.3 (94.1–98.5)
	Negative	10	19	241	270				
	Equivocal	4	0	3	7				
	All	28	19	247	294				
1:10 dilution	Positive	13	0	1	14	46.4 (40.7–52.1)	99.6 (98.9–100)	92.9 (90.0–95.8)	95.3 (92.9–97.7)
	Negative	13	19	246	278				
	Equivocal	2	0	0	2				
	All	28	19	247	294				

addition, two smear microscopy-positive specimens with NTM culture growth had a positive MTD test result (by the conventional method). One specimen was from a patient who had additional respiratory specimens with culture growth of the *M. tuberculosis* complex, and the other specimen grew *M. celatum*, an organism previously shown to cause false-positive MTD test results (11).

In our study, 16 specimens were smear microscopy positive but culture negative for mycobacteria. The positive smear microscopy results were not rereviewed when the culture results were reported as negative. Six specimens were from six patients who had additional respiratory specimens yielding the growth of mycobacteria—the *M. tuberculosis* complex for two patients and NTM for four patients (*M. avium* in two cases and *M. gordonae* in two cases)–upon culture. Study specimens from the former two patients were MTD test positive and those from the latter four patients were MTD test negative. If the remaining 10 study specimens were actually smear microscopy negative, these results could have had an impact on the evaluation of MTD test performance for smear microscopy-negative specimens in terms of improved specificity, since all of these specimens were MTD test negative and culture negative. However, there would be no anticipated impact on MTD test sensitivity.

A total of 11 specimens (2.2%) had equivocal MTD test results by the conventional MTD testing method. Among these, eight specimens (all four of the smear microscopy-positive specimens and four of seven smear microscopy-negative specimens) with equivocal conventional MTD test results yielded dilution MTD test results that were concordant with culture results. Among the remaining three smear microscopy-negative specimens with equivocal conventional MTD test results, two also had equivocal dilution MTD test results and one gave false-negative MTD test results by the dilution method.

Overall, among 197 smear microscopy-positive respiratory specimens, MTD test results were concordant with the culture results for 174 specimens (88.3%) by the conventional method and for 192 specimens (97.5%) by the dilution method. This finding is in overall accordance with the results of a previous study by Pollock et al., although in that study MTD test results were not stratified by specimen smear microscopy status (9). However, in our study, for smear microscopy-negative specimens, dilution resulted in no benefit in terms of test sensitivity. A possible explanation is that the dilution of specimens containing few bacilli decreases even further the number of bacilli in the specimen, compromising *M. tuberculosis* detection by the MTD test.

We conclude that the dilution of respiratory specimens improves MTD test performance for smear microscopy-positive but not smear microscopy-negative specimens. This knowledge may aid in the development of efficient laboratory testing algorithms.

This work was supported by grants K23AI51528, U19AI45432, D43 TW05574-01, and U2R TW006883 from the National Institutes of Health.

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