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Quantitative Culture of *Mycobacterium tuberculosis* from Clinical Sputum Specimens and Dilution Endpoint of Its Detection by the Amplicor PCR Assay

DAVID M. YAJKO,[1]* CINDY WAGNER,[2] VINCENT J. TEVERE,[3] TANIL KOCAGÖZ,[4] W. KEITH HADLEY,[1] AND HENRY F. CHAMBERS[2]

Departments of Laboratory Medicine[1] and Medicine,[2] University of California, San Francisco General Hospital, San Francisco, California; Roche Molecular Systems, Somerville, New Jersey[3]; and Department of Laboratory Medicine, Hacettepe University, Ankara, Turkey[4]

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The minimum number of *Mycobacterium tuberculosis* CFU detectable in clinical sputum specimens by the Amplicor PCR test was estimated by performing the test on duplicate samples of quantitatively cultured serial dilutions of sputum. Positive PCR test results were obtained for all samples that contained ≥42 CFU of *M. tuberculosis*. The detection limits of the PCR assay for decontaminated (*N*-acetyl-L-cysteine [NALC]-NaOH) and nondecontaminated (NALC only) specimens were equivalent, even though the number of CFU cultured from decontaminated samples was only 11 to 20% of the number cultured from nondecontaminated samples. Thus, the 42 CFU that could be detected in nondecontaminated specimens by the Amplicor PCR test correspond to the approximately 8 CFU (0.20 × 42) that could be recovered in culture after decontamination with NALC-NaOH.

The use of PCR to detect the presence of *Mycobacterium tuberculosis* has been widely reported. The sensitivity of noncommercial PCR assays compared with that of culture ranged from 55.9 to 100% in various studies (1–3, 5, 7, 9, 11) and has been reported to be higher for specimens that are microscopically smear positive for acid-fast bacilli than for specimens that are acid-fast bacillus smear negative (1, 7).

The ultimate goal of PCR in the mycobacteriology laboratory is to be able to detect a single CFU of *M. tuberculosis* in a clinical sample. Estimates of the detection limits of PCR assays have been made, but they have usually been based on the addition of known numbers of *M. tuberculosis* CFU or known amounts of *M. tuberculosis* DNA to test samples (3, 5, 7–9, 11). A more relevant estimation of the detection limit of PCR can be obtained by comparing PCR results with the number of CFU of *M. tuberculosis* cultured from clinical sputum samples (9). Such methods allow one to estimate how close an assay comes to detecting a single CFU of *M. tuberculosis.*

The ability of PCR assays to detect small numbers of *M. tuberculosis* in sputum is influenced by a number of factors, including the efficiency of bacterial cell lysis, the presence of inhibitors, and the conditions used for the amplification and detection of target DNA. Likewise, the sensitivity of culture is influenced by factors which reduce the viability of *M. tuberculosis* in clinical sputum specimens, such as the harshness of sputum decontamination procedures and the use of antibiotic-containing selective media for culture. Sodium hydroxide, an agent that is commonly used to decontaminate sputum specimens, has been reported to cause the death of 28 to 70% of the mycobacteria in clinical specimens (4, 6). Studies which use decontaminated specimens to determine the detection limit of PCR assays may thus be confounded by factors which influence the viability of *M. tuberculosis* organisms.

It has been difficult to make comparisons of the sensitivity, specificity, and detection limits of various PCR assays because reagents and procedures vary considerably from one laboratory to the next. This was highlighted in a recent report of a blinded comparison study of PCR assays involving seven laboratories (8). Although each of the laboratories received the same samples for PCR assay, wide variation in assay sensitivity was reported, ranging from 2 to 90% among the laboratories for samples that contained 10^3 mycobacteria (8). The availability of commercially prepared reagents in kit form, along with specific procedural instructions and quality control tests, will be useful in helping to improve interlaboratory test agreement.

We report here on the use of a commercial PCR-based kit (Amplicor mycobacterium tuberculosis test; Roche Molecular Systems, Somerville, N.J.) to estimate the minimum number of *M. tuberculosis* organisms that can be detected in clinical sputum specimens by PCR. The study includes an evaluation of the impact of NaOH decontamination and the use of selective media on the recovery of *M. tuberculosis* organisms.

Processing and quantitative culturing of sputum specimens. The specimens used in this study were collected from patients whose initial sputum specimen was smear positive for acid-fast bacilli. All specimens were collected prior to initiation of antituberculosis therapy. A 2-ml portion of sputum specimen was digested and decontaminated by the *N*-acetyl-L-cysteine (NALC)-NaOH method described by Kent and Kubica (4). A stock solution of NALC-NaOH reagent was prepared by mixing 50 ml of 4% NaOH with 50 ml of 2.9% sodium citrate · $2H_2O$ and adding 0.5 g of NALC. Two milliliters of stock solution was mixed with 2 ml of sputum. A separate 2-ml portion of each sputum was treated with 2 ml of 0.5% NALC only. In other experiments some samples were processed with 4 ml of 6.5 mM dithiothreitol in 100 mM phosphate buffer, pH 7.0, instead of 0.5% NALC. After a 15-min incubation at room temperature, 0.067 M phosphate buffer (pH 6.8) was added to bring the volume to 50 ml, and then bacteria were sedimented by refrig-

* Corresponding author. Mailing address: Clinical Laboratories, Rm. 2M35, San Francisco General Hospital, 1001 Potrero Ave., San Francisco, CA 94110. Phone: (415) 206-8578. Fax: (415) 206-3045. Electronic mail address: yajko@labmed.ucsf.edu.

erated centrifugation at 3,000 × *g* for 15 min. Pellets were resuspended in 2 ml of saline containing 0.25% Tween 80. Serial 10-fold dilutions were prepared in saline-Tween 80 and 0.1-ml aliquots were plated onto nonselective Middlebrook 7H10 agar and Mitchison's selective Middlebrook 7H11 agar (for samples that were decontaminated with NaOH) or onto Mitchison's agar only (for nondecontaminated samples) (10). Plates were incubated for 4 weeks at 35°C before colonies were counted. Colony counts were rechecked after 6 weeks of incubation. The sensitivity of the PCR assay was determined by correlating the number of CFU of *M. tuberculosis* in serially diluted samples with the results of PCR assays performed on serially diluted samples.

PCR and *M. tuberculosis* DNA detection assays. PCR and *M. tuberculosis* DNA detection tests were performed with duplicate aliquots of NALC-NaOH-treated sputum specimens by using the Roche Amplicor mycobacterium tuberculosis test kit. Prior to amplification, duplicate 100-μl aliquots of sputum were washed by the addition of 500 μl of sputum wash solution and centrifugation at 12,500 × *g* for 10 min. Pellets were resuspended in 50 μl of mycobacterium lysis solution, heated to 55°C for 45 min, and neutralized by the addition of 50 μl of mycobacterium neutralization solution. The latter step returned the samples to their original volume. Serial 10-fold dilutions (10^{-1} to 10^{-7}) of the samples were then prepared in mycobacterium lysis solution-mycobacterium neutralization solution, and a 50-μl aliquot of each dilution was used in the PCR assay.

The DNA in duplicate 50-μl portions of the processed sputum samples was amplified in tubes containing 50 μl of master mix (AmpErase [uracil-*N*-glycosylase], *Taq* polymerase, dATP, dUTP, dCTP, dGTP, and biotinylated DNA primers). dUTP is included in place of dTTP in the master mix so that amplified DNA contains uracil in the place of thymidine. The inclusion of AmpErase causes uracil-containing strands to be destroyed prior to thermal cycling so that any contaminating amplicons that may be present are destroyed during the first denaturation cycle. Since AmpErase is inactivated at cycling temperatures, newly formed amplicons are unaffected.

Tubes were placed in a TC9600 thermal cycler (Perkin-Elmer, Norwalk, Conn.) and incubated at 50°C for 2 min; this was followed by 2 cycles of 98°C for 20 s (denaturation), 62°C for 20 s (annealing), and 72°C for 45 s (elongation) and then by 35 cycles of 94°C for 20 s, 62°C for 20 s, and 72°C for 45 s.

After amplification of mycobacterial DNA, 100 μl of denaturation solution was added to each PCR tube and tubes were incubated for 10 min at room temperature. Mycobacterium hybridization buffer (100 μl) was added to the wells of a microtiter tray containing a bound *M. tuberculosis*-specific DNA probe, and 25 μl of denatured amplicon was transferred to each well. After a 90-min incubation at 37°C to allow hybridization to occur, wells were washed five times with wash buffer, and 100 μl of avidin-horseradish peroxidase conjugate was added. Plates were incubated for 15 min at 37°C and washed five times with wash buffer, and then 100 μl of tetramethylbenzidine-peroxide detection solution was added. The tray was covered and incubated for 10 min at room temperature, 100 μl of stop reagent was added to stop color formation, and the A_{450} of each well was read. Absorbance values of ≥0.3 were scored as positive, and values of <0.3 were scored as negative.

The dilution endpoint of the PCR assay was defined as the greatest dilution that gave a positive PCR assay result on duplicate 50-μl samples. The number of *M. tuberculosis* CFU in these diluted specimens was estimated from the number of CFU that grew from aliquots of sputum that were diluted and plated.

TABLE 1. Recovery of *M. tuberculosis* from clinical sputum specimens treated and plated onto different agar media

Patient	Log_{10} CFU per ml recovered with:		
	NALC-NaOH on 7H10	NALC-NaOH on Mitchison's 7H11	NALC on Mitchison's 7H11
1	4.88	4.60	5.68
2	5.80	5.72	6.38
3	5.60	5.66	6.69
4	6.64	6.36	7.71
5	2.71	2.23	2.60
6	6.99	6.23	6.83
7	3.83	3.49	3.90
8	5.94	5.84	6.56
9	4.20	4.00	6.48
Mean ± SD[a]	5.16 ± 1.39 (A)	4.90 ± 1.42 (B)	5.87 ± 1.61 (C)

[a] $P < 0.002$ for C versus B, $0.05 < P < 0.075$ for C versus A, and $P > 0.05$ for A versus B by paired *t* test with Bonferroni's correction for multiple comparisons.

Effects of the use of nonselective versus selective media on recovery of *M. tuberculosis*. Shown in Table 1 is the number of CFU of *M. tuberculosis* recovered on nonselective (7H10) and selective (Mitchison's 7H11) media after decontamination of sputum samples with NALC-NaOH. The use of Mitchison's selective medium had a slight, and statistically insignificant, deleterious effect on the recovery of *M. tuberculosis* CFU from sputum specimens treated with NALC-NaOH. The mean number of CFU of *M. tuberculosis* that grew on Mitchison's medium after NALC-NaOH treatment was 54% of the number of CFU that grew on nonselective 7H10 medium after NALC-NaOH treatment. Additional studies suggest that this somewhat poorer recovery on Mitchison's medium was only indirectly related to the presence of antibiotics in this medium. When cultures of five of the strains were grown in broth and plated onto Mitchison's selective medium and onto nonselective 7H10 medium, omitting the decontamination step, the numbers of CFU recovered on the two media were virtually identical (mean number of CFU on Mitchison's agar/number of CFU on 7H10 agar = 0.98), suggesting that NaOH treatment of sputum renders *M. tuberculosis* organisms more susceptible to inhibition or killing by the antimicrobial agents present in Mitchison's medium.

Effect of NaOH treatment of sputum on recovery of *M. tuberculosis*. The numbers of CFU of *M. tuberculosis* recovered on agar media after treatment of sputum samples with NALC-NaOH and with NALC alone are shown in Table 1. The negative impact of NaOH decontamination on the survival of *M. tuberculosis* can be seen by comparing the number of CFU that grew on Mitchison's medium after NALC-NaOH treatment with the number of CFU that grew on the same medium after treatment with NALC alone. The mean (± standard deviation) number of *M. tuberculosis* CFU recovered on Mitchison's agar medium after NALC-NaOH treatment was 0.96 ± 0.76 $\log_{10}$ CFU less than the number recovered after treatment with NALC alone, corresponding to an average recovery rate of 11% for NaOH-treated samples. The recovery of CFU was also less for NaOH decontamination with plating onto nonselective 7H10 agar (the standard method used in many clinical microbiology laboratories for primary isolation of *M. tuberculosis*) than for treatment with NALC and plating on Mitchison's agar, with an average recovery of 20% and a difference of 0.69 ± 0.76 $\log_{10}$ CFU ($0.05 < P < 0.075$).

The results for four patients enrolled in an ongoing randomized, double-blind clinical trial, in which the rate of elimination

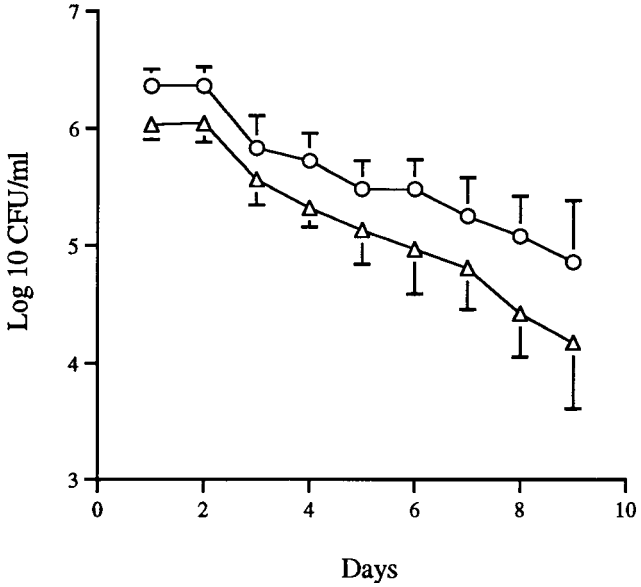

FIG. 1. Effect of NaOH decontamination on the recovery of *M. tuberculosis* CFU from sputum samples of patients. Samples were obtained from four patients enrolled in a double-blind, randomized trial comparing the elimination rates of *M. tuberculosis* from sputum by single-agent chemotherapy (with isoniazid, rifampin, amoxicillin-clavulanate, or ofloxacin). Treatment was initiated on day two for a total of 7 days. Because of the blinded nature of the study, the specific agent used for individual patients is not known, but the effect of NaOH decontamination on the recovery of *M. tuberculosis* CFU could be assessed. Samples were processed with either dithiothreitol (circles) or NALC-NaOH (triangles) (see text for details). Results are means ± standard errors of the means.

of *M. tuberculosis* from sputum during the first 7 days of single-drug antituberculous therapy is determined, graphically illustrate the deleterious effect of NaOH decontamination on the recovery of *M. tuberculosis* (Fig. 1). Samples decontaminated with NaOH and plated onto nonselective 7H10 agar had an average recovery rate of 38% (range, 20 to 54%) compared with paired samples processed with dithiothreitol and plated onto Mitchison's selective medium ($P < 0.002$ by analysis of variance).

Minimum number of *M. tuberculosis* CFU detectable in clinical sputum samples with the Amplicor mycobacterium tuberculosis test. The highest dilution of NALC-NaOH-treated sputum that gave a positive PCR test result on duplicate 50-μl samples was determined, and then the number of *M. tuberculosis* CFU in these 50-μl samples was calculated from the highest number of CFU recovered on plated media (typically from samples treated with NALC only). This number was taken to represent the minimum number of *M. tuberculosis* CFU detectable by PCR. The minimum number of CFU that could be detected in sputum varied among specimens from different patients, ranging from 4 to 42 CFU (Table 2). The PCR assay was positive for duplicate aliquots from all patient samples that contained ≥42 CFU. Twenty-five CFU were detectable by PCR in the sputum of patient 4, but 28 CFU and 24 CFU could not be reproducibly detected in sputum from patients 1 and patient 6, respectively. Interestingly, samples from patient 7, containing only 4 CFU of *M. tuberculosis*, were consistently positive by PCR.

TABLE 2. Minimum number of *M. tuberculosis* CFU detectable in duplicate 50-μl clinical sputum samples by the Amplicor PCR assay

Patient	No. of CFU[a] in sample with PCR result	
	Positive	Negative
1	≥280	28
2	≥150	15
3	≥150	15
4	≥25	2.5
5	≥42	4.2
6	≥240	24
7	≥4	0.4

[a] Based on CFU recovered from culture on agar media.

If the detection limit of the Amplicor PCR test is considered to be between 25 and 42 CFU, then the number of cultivable CFU in NALC-NaOH-decontaminated specimens that can be detected by this test can be calculated to be approximately 5 to 8 CFU. This calculation is based on the finding from Table 1 that the mean number of *M. tuberculosis* CFU recovered on nonselective solid media after NALC-NaOH treatment was 20% of the number of CFU recovered after treatment with NALC alone.

The PCR assays in this study were routinely performed on sputum specimens that had been treated with NALC-NaOH. To test whether NaOH treatment of sputum adversely affected the limit of detection of the PCR assay, aliquots from four sputum samples that were treated with NALC-NaOH or with NALC alone were diluted and assayed by PCR. In each case, the dilution endpoint for a positive PCR test was the same for NALC-NaOH-treated and NALC-treated samples (data not shown), suggesting that NALC-NaOH treatment does not greatly affect the sensitivity of the Amplicor test. This finding is of interest since three of the specimens treated with NALC alone contained numbers of CFU that were on the borderline of detection (25, 44, and 50 CFU) in the final dilution tube. Since NaOH treatment killed approximately 80 to 90% of the organisms, it might have been expected that NALC-NaOH-treated samples containing these numbers of CFU might be negative by PCR. The fact that the NALC-NaOH-treated samples from all three of these borderline specimens were positive by PCR suggests that *M. tuberculosis* DNA from organisms that are killed by NaOH treatment may still be detected by PCR. This hypothesis is consistent with our unpublished observations that patients on antituberculous medication can be culture negative for *M. tuberculosis* but still have a positive PCR for *M. tuberculosis* DNA in sputum.

The results of this study indicate that the Amplicor PCR assay can reproducibly detect approximately 25 to 42 cultivable CFU of *M. tuberculosis* in 50 μl of nondecontaminated sputum, which corresponds to approximately 5 to 8 cultivable CFU in 50 μl of NALC-NaOH-decontaminated sputum cultured onto nonselective medium. Given that the thresholds of detection for PCR and for culture after NaOH decontamination are very close, it is not surprising that some culture-negative samples test positive with PCR. The adverse effect of NaOH on the recovery of *M. tuberculosis* also should be borne in mind when a negative culture result from a patient with suspected tuberculosis is interpreted, particularly if the PCR is positive. Moreover, PCR must be employed in conjunction with culture for maximum sensitivity. NaOH treatment of sputum appears to have little impact on the ability of PCR to detect *M. tuberculosis* DNA in clinical sputum samples, suggesting that PCR may detect nonviable organisms in sputum.

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