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Rendering of Mycobacteria Safe for Molecular Diagnostic Studies and Development of a Lysis Method for Strand Displacement Amplification and PCR

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Two criteria must be met before mycobacterial specimens can be tested by DNA amplification methods: (i) the sample must be rendered noninfectious, and (ii) the organisms must be lysed to free the DNA. Previous publications reporting DNA amplification of mycobacteria have concentrated on lysis and amplification procedures and have not addressed the issue of sample safety. We have shown that heating of samples below 100°C may not consistently kill mycobacteria; however, heating at 100°C in a boiling-water bath or a forced-air oven for a minimum of 5 min kills mycobacteria, including *Mycobacterium thermoresistibile*. Furthermore, heating at 100°C for 30 min consistently lyses mycobacteria to produce short fragments of DNA that are suitable for amplification by PCR and strand displacement amplification. This procedure works with clinical samples digested by the *n*-acetyl cysteine–NaOH method as well as with suspensions of organisms in phosphate buffer. This paper also demonstrates the feasibility of using strand displacement amplification with clinical specimens.

Direct identification of mycobacteria in clinical samples by amplification of specific nucleic acid sequences offers the potential for same-day diagnosis of mycobacterial infections. Two methodologies used to amplify mycobacterial DNA are PCR (2–6, 11, 15, 18) and strand displacement amplification (SDA) (21–23). Because of the complex nature of the specimens submitted for mycobacterial culture and the difficulties associated with lysis of mycobacteria, most DNA amplification studies have focused on developing methods to process samples and improve the efficiency of DNA amplification. Studies using PCR have described lysis conditions of combinations of sonication (15), heating at 55 to 100°C (2, 6, 11, 15, 18, 19), detergents (2, 3, 6, 8, 18, 19), proteases (8, 15, 18, 19), lysozyme (6, 8, 11), freeze-thaw (19), sodium hydroxide (2, 6, 11), and hypertonic NaCl (2). In some cases, these methods have been followed by adsorption to silica (6, 11) or phenol-chloroform extraction (2, 3, 8) to isolate the DNA for amplification. However, the majority of studies of mycobacterium amplification have not confirmed that the organisms were dead. Phenol-chloroform extraction and heating of specimens should be lethal for mycobacteria since phenolic-based disinfectants have been shown to be tuberculocidal (12), and at least one medical text (25) states that pasteurization temperatures kill mycobacteria. However, our study shows that certain methods of heating may not render samples safe; that the heat killing of mycobacteria is dependent upon temperature, time, cell density, and sample volume; and that disinfectants interfere with PCR and SDA reactions. We describe a method by which mycobacteria are lysed to produce short DNA fragments suitable for amplification by PCR and SDA and which renders samples safe. This paper also demonstrates the feasibility of using SDA with clinical specimens.

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MATERIALS AND METHODS

Organism preparation and growth. Organisms were grown on Lowenstein-Jensen (LJ) slants (Becton Dickinson Microbiological Systems, Towson, Md.) and in BACTEC 12B vials (Becton Dickinson Instrument Systems, Sparks, Md.). Organism concentrations were adjusted to McFarland standards by dispersing organism clumps with glass tissue grinders and diluting with a 25 mM potassium phosphate buffer, pH 7.6. Organism viability was determined by culturing 0.2 ml of the suspensions in BACTEC 12B vials with PANTA solution for 6 to 8 weeks at 37°C. Positive BACTEC signals were confirmed by acid-fast stains (Kinyoun's stain and auramine O) and Gram stain. Vials with questionable results were subcultured onto blood agar and LJ slants. Growth on LJ slants incubated at 37°C in CO_2 was used as an adjunct to the BACTEC system as indicated below. Colony counts were determined by the pour plate method with Middlebrook 7H10 agar (Difco, Detroit, Mich.).

Clinical sample processing. Clinical samples were processed by the *N*-acetyl-L-cysteine (NALC)–NaOH (Remel, Lenexa, Kans.) method (9) and resuspended in 1 ml of 0.2% bovine albumin (Remel). The resulting suspension is referred to below as the NALC pellet.

Neutralization studies. Suspensions of mycobacteria in phosphate buffer and known positive and negative sputum samples were processed by the NALC method. The NALC pellets were divided into two aliquots, one that was neutralized with 1 N HCl to a pH between 7.0 and 7.5 and one that was not neutralized (pH > 9). Both aliquots were then refrigerated (0 to 4°C). Samples of both aliquots were then inoculated into BACTEC 12B vials and onto LJ slants at 0, 15, and 30 min and 1, 4, and 24 h.

Heat kill. Suspensions of mycobacteria were heated for various times at various temperatures in a dry-heat block (Thermolyne type 17600; Barnstead/Thermolyne, Dubuque, Iowa), a boiling-water bath, and a forced-dry-air oven (propri-

etary instrument; Becton Dickinson). Internal tube temperatures were measured with electronic probes (Fisher Scientific, Pittsburg, Pa.) immersed in phosphate buffer in a volume equal to that of the material being tested.

Effect of disinfectants on mycobacteria. Killing of mycobacteria by various concentrations of glutaraldehyde (Cidexplus; Surgikos, Arlington, Tex.), alkyldimethylbenzyl ammonium chloride (Roccal II; National Laboratories, Montvale, N.J.), and *O*-phenylphenol (O-SYL; National Laboratories) was determined by mixing disinfectant with 4.5 ml of a McFarland 2 suspension of organism to achieve the appropriate dilution of disinfectant. After 0, 15, and 30 min, 0.2 ml of the disinfectant-organism mixture was added to BACTEC 12B vials. To control for the continued inhibition or killing by residual disinfectant transferred to BACTEC vials, we inoculated 0.2 ml of a 1:1,000 dilution of a McFarland 2 suspension of *Mycobacterium tuberculosis* into BACTEC 12B vials containing disinfectants in comparable concentrations. These vials were then incubated at 37°C for 6 weeks. Negative controls consisted of BACTEC 12B vials with disinfectants and no organisms.

PCR. PCR amplification of *M. tuberculosis* target DNA was performed according to the standard protocol (13) by using primers specific for the IS*6110 M. tuberculosis* complex insertion element (primer 1, 5′-GACCCGCCAACAAGAAGGCG TACTC-3′; primer 2, 5′-ATGTGTACTGAGATCCCCTAT CCGT-3′) (20). PCR amplifications of mycobacterial genus DNA used primers specific for a region of the 65K antigen gene (17) (primer 1, 5′-TCGATCTCCTTGGCGATGGA-3′; primer 2, 5′-CGGAGGAATCACTTCGCAATG-3′) (primers were provided by P. Spears, Becton Dickinson). Each 0.5-ml microcentrifuge reaction tube contained 50 μl of PCR buffer composed of 20 mM Tris-HCl, pH 8.8; 100 mM KCl; 4.5 mM $MgCl_2$; 1.0 mM deoxynucleoside triphosphates; 20 mM 2-mercaptoethanol with freshly added primers (50 pM); 2.5 U of *Taq* polymerase (Perkin-Elmer Cetus, Branchburg, N.J.); and 50 μl of sample overlaid with approximately 50 μl of mineral oil (Sigma, St. Louis, Mo.). PCR amplification of IS*6110* was performed with a model 480 temperature cycler (Perkin-Elmer Cetus) set for 1 3-min cycle at 94°C; 30 cycles of 1 min at 94°C, 1 min at 62°C, and 1 min at 72°C; and then a 7-min cycle at 72°C. Amplification of the 65K antigen gene was performed with the temperature cycler set for 1 3-min cycle at 95°C; 27 cycles consisting of 1.5 min at 95°C, 1 min at 50°C, and 2 min at 72°C; and then a 7-min cycle at 72°C.

SDA. SDA of mycobacterial DNA was performed by the method of Walker et al. (21–23) with primers specific for the IS*6110* DNA insertion element to detect *M. tuberculosis* and primers specific for the 16S rRNA gene (19) to detect other *Mycobacterium* spp. Twenty-five microliters of sample was mixed with 20 μl of solution 1 in a 0.5-ml microcentrifuge tube, and then the mixture was denatured in a boiling-water bath for 2.5 min. After the mixture had been cooled to 41°C in a heat block (Thermal-Lok Dry Bath; USA/Scientific, Ocala, Fla.), 5 μl of solution 2 was added. After a 2-h incubation at 41°C, the SDA reaction was terminated by immersing the tubes in a boiling-water bath for 2 min.

(i) IS*6110* amplification. The following solutions were used for IS*6110* amplification. Solution 1 contained 0.25 mg of bovine serum albumin per ml; 22.5% glycerol; 90.75 mM KPO_4; 0.5 mM deoxynucleoside triphosphates (dGTP, dTTP, and dCTP); 0.5 mM deoxyadenosine 5′-[α-thio]triphosphate; 1,250 nM (each) the four primers B1 (5′-TGGACCCGCCA AC-3′), B2 (5′-CGCTGAACCGGAT-3′), S1 (5′-TTGAAT AGTCGGTTACTTGTTGACGGCGTACTCGACC-3′), and S2 (5′-TTGAAGTAACCGACTATTGTTGACACTGAGAT CCCCT-3′); 250 ng of ultrapure human placental DNA (Sigma) in H_2O; and 17.5 mM $MgCl_2$ (added last). To control for the effects of inhibitors, 25,000 copies of the synthetic sequence 5′-ACTGAGATCCCCTAGCGACGATGTCTGAGGCAAC TAGCAAAGCTGGTCGAGTACGCC-3′ were included in each reaction tube (23).

Solution 2 contained *Hin*cII restriction endonuclease (New England Biolabs, Beverly, Mass.) and the exo-Klenow fragment of DNA polymerase I (Gibco BRL, Gaithersburg, Md.) diluted with H_2O to the respective specific activities of 150 and 3 U/5 μl.

(ii) 16S amplification. Solution 1 contained 0.25 mg of bovine serum albumin per ml; 30% dimethyl sulfoxide; 81.25 mM KPO_4; 1.25 mM deoxynucleoside triphosphates (dGTP, dTTP, and dCTP); 0.5 mM deoxyadenosine 5′-[α-thio]triphosphate; 1,250 nM (each) the four primers B1 (5′-CGGAAT TACTGGG-3′), B2 (5′-AGTCTGCCCGTATC-3′), S1 (5′-TTGAATAGTCGGTTACTTGTTGACGGCGTACTCGA CC-3′), and S2 (5′-TTGAAGTAACCGACTATTGTTGAC ACTGAGATCCCCT-3′); and 250 ng of ultrapure human placental DNA (Sigma) in H_2O. Each reaction mixture also contained 500 copies of the synthetic sequence 5′-GACGGCG UACUCGACCAGCGACGAUGUCUGAGGCAACUAGC AAAGCUGAACAACGCGAC-3′ to control for the effects of inhibitors (23).

Solution 2 was the same as solution 2 used for IS*6110* amplification except that magnesium acetate was added to a concentration of 65 mM.

Detection of amplified DNA. The 65K antigen gene PCR products were quantified by densitometry of Polaroid photographs (type 57 film) of the ethidium bromide-stained polyacrylamide gels with a SciScan 5000 scanning densitometer (U.S. Biochemicals, Cleveland, Ohio).

PCR- and SDA-amplified *M. tuberculosis* IS*6110* DNA was hybridized in solution and detected by a solid-phase capture chemiluminescent assay (23). Hybridization and detection of the SDA-amplified mycobacterial 16S DNA were as described for IS*6110* except that a biotinylated capture probe (5′-ACT GTGAGCGTGGTC-3′) and two alkaline phosphatase-conjugated detector probes (5′-AAATCTCACGGCTTA-3′ and 5′-AAAACTCACAGCTTA-3′) were used and the amplified internal control was captured by a different set of capture (5′-CCGTTGATCGTTTCG-3′) and detector (5′-TCGCTGC TACAGACT-3′) probes.

Effects of disinfectants on amplification. To determine the effects of O-SYL, Roccal II, and Cidexplus on PCR amplification, 49 μl of the disinfectant diluted in 25 mM KPO_4, pH 7.6, was mixed with 1 μl of pure *M. tuberculosis* genomic DNA (10^5 genomes per μl) (courtesy of D. Shank, Becton Dickinson). Fifty microliters of PCR mixture was added to the DNA-disinfectant mixture and overlaid with mineral oil. PCR and detection of PCR products were performed as outlined above. For SDA, 24 μl of disinfectant dilution was mixed with 1 μl of pure *M. tuberculosis* genomic DNA (100 genomes per μl). This solution was mixed with 20 μl of SDA solution 1 and then heat denatured. After the addition of 5 μl of SDA solution 2, SDA was performed at 41°C and detection of products was performed as described above.

Effect of heat lysis on integrity of *M. tuberculosis* DNA. To determine the effects of heat on mycobacterial DNA, 10-μl aliquots containing approximately 1.8 μg of purified *M. tuberculosis* DNA in 10 μl of 25 mM KPO_4, pH 7.6, were heated at 100°C in a forced-air oven or were incubated at 20°C. After 30 min, 5-μl volumes of both samples were electrophoresed on a 1% agarose gel and visualized by ethidium bromide staining.

TABLE 1. Effect of heating at 95°C on *Mycobacterium* spp.

Organism	No. of expts	% Survival at exposure time (min)							
		0	5	10	15	20	25	30	40
M. tuberculosis Trudeau Society 201	4	100	100	0	25	50	0	25	0
M. bovis Trudeau Society 1011	1	100	100	100	0	100	100	0	0
M. avium ATCC 24291	3	100	100	100	33	0	66	33	0
M. intracellulare ATCC 13950	3	100	100	100	66	33	66	0	0
M. kansasii Trudeau Society 1201	3	100	100	100	33	33	33	0	100
M. chelonae Trudeau Society 1542	1	100	100	100	100	100	100	100	100
M. fortuitum Trudeau Society 1529	1	100	100	100	100	100	100	100	100
M. gordonae Trudeau Society 1318	1	100	100	0	0	0	0	0	100
M. thermoresistibile 1987 CAP Survey, sample E6	2	100	100	100	100	100	100	100	100
M. xenopi Trudeau Society 1482	1	100	100	100	100	100	100	100	100
Total	20	100	100	79	50	50	58	35	64

RESULTS

Survival at 95°C in dry-heat block. Table 1 depicts the results of multiple heat kill experiments using various species. At most temperatures, there was sporadic breakthrough growth of all mycobacterial species, including *M. tuberculosis*. When NALC pellets heated at 95°C were tested with the BACTEC system, similar sporadic results were obtained. Four of 11 heated NALC pellets containing *M. avium* complex grew, but none of the 9 samples containing *M. tuberculosis*, *M. kansasii*, or *M. fortuitum* were positive.

To determine if organism density and volume affected the killing efficiency at 95°C, McFarland 0.5, 1, 2, and 3 suspensions of *M. tuberculosis*, *M. chelonae*, *M. intracellulare*, and *M. kansasii* were prepared in 1.5- and 4.5-ml volumes. These suspensions were then heated at 95°C for 15 or 30 min. Table 2 shows that there was a tendency for mycobacteria to survive heating at higher volumes and concentrations of organisms.

One reason that the dry-heat block did not kill the mycobacteria is that the internal temperature of the sample tubes did not reach the set temperature quickly. The internal temperatures of the sample tubes were measured over time with a temperature probe immersed in 0.5, 1.0, 1.5, 2.0, and 4.5 ml of phosphate buffer. The 4.5-ml sample was also tested in wells filled with mineral oil. After 10 min, the internal tube temperatures were approximately 80°C in the 1.5-, 2.0-, and 4.5-ml tubes and approximately 90°C in the 0.5- and 4.5-ml tubes immersed in mineral oil. The internal temperature of no sample volume ever reached 95°C by 20 min in either the wet- or the dry-heat block. Setting the temperature of the heat block to 100°C had no significant effect on the results of the experiment.

Heating at 100°C. The killing experiments were repeated with a boiling-water bath with 1.5- and 4.5-ml volumes of McFarland 2 suspensions of all the organisms listed in Table 1 except *M. bovis*. Samples were taken at 0, 5, 10, 15, and 30 min. To ensure that the proper temperature was reached, timing was not begun until the internal temperature reached 100°C (approximately 9 to 13 min). In three separate experiments, no mycobacterial species grew after being heated at 100°C for at least 5 min. Because of the inherent safety problems associated with a boiling-water bath, the 100°C heating experiments were repeated by using a forced-air oven. The results obtained with the forced-air oven were identical to those obtained with the boiling-water bath. To ensure that the forced-air oven would work with clinical samples, seven positive and seven negative NALC pellets were heated at 100°C for 30 min. None of the heated NALC pellets were positive after 6 weeks of incubation.

Effect of disinfectants on killing of mycobacteria and DNA amplification. The bacteriocidal effects of O-SYL, Cidex, and Roccal II on *M. tuberculosis*, *M. kansasii*, *M. intracellulare*, and *M. chelonae* were determined after 15 and 30 min of exposure at both room temperature and 95°C. At a dilution of 1:510, O-SYL killed all species at both temperatures and both times. All species survived a 1:1,020 dilution of O-SYL at room

TABLE 2. Effect of organism density and volume on killing at 95°C

Organism	No. of expts	% Survival at indicated McFarland standard and exposure time												Vol (ml)
		0.5[a]			1			2			3			
		0[b]	15	30	0	15	30	0	15	30	0	15	30	
M. tuberculosis Trudeau Society 201	2	100	0	0	100	0	0	100	100	50	100	100	50	1.5
	2	100	100	50	100	0	0	100	100	50	100	100	100	4.5
M. kansasii Trudeau Society 1201	1	100	100	100	100	100	100	100	100	100	100	100	100	1.5
	1	100	100	100	100	100	100	100	100	100	100	100	100	4.5
M. intracellulare ATCC 13950	1	100	0	100	100	0	100	100	100	0	100	100	0	1.5
	1	100	0	0	100	100	100	100	100	100	100	100	0	4.5
M. chelonae Trudeau Society 1011	1	100	0	0	100	0	0	100	0	0	0	0	0	1.5
	1	100	0	0	100	0	0	100	0	0	100	100	100	4.5

[a] McFarland standard.
[b] Exposure time in minutes.

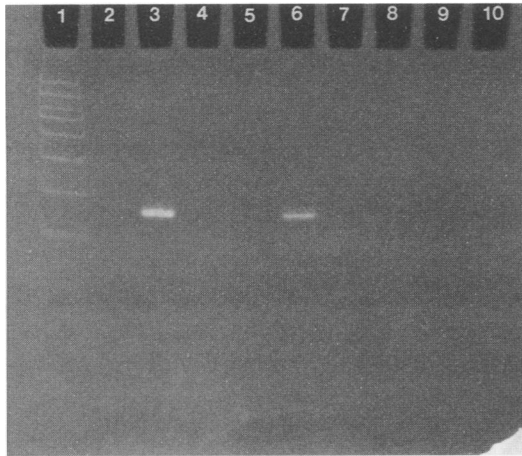

FIG. 1. Effects of disinfectants on PCR amplification of *M. tuberculosis* DNA. Lane 1, molecular size markers (50, 100, 200, 300, 400, 500, 700, and 1,000 bp); lane 2, negative control; lane 3, positive control containing *M. tuberculosis* DNA equivalent to 10^5 genomes; lanes 4 to 6, O-SYL dilutions of 1:510, 1:1,020, and 1:2,040, respectively; lanes 7 and 8, 1:125 and 1:510 dilutions of Roccal II, respectively; lane 9, 3.2% Cidexplus; lane 10, blank.

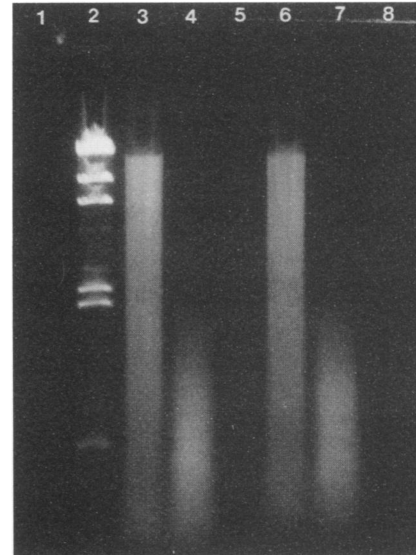

FIG. 2. Effect of heat lysis on integrity of *M. tuberculosis* DNA. Lane 1, blank; lane 2, lambda phage molecular size markers (23.1, 9.4, 6.6, 4.4, 2.3, 2.0, and 0.6 kb); lane 3, *M. tuberculosis* DNA after 30 min of incubation at 20°C; lane 4, *M. tuberculosis* DNA after 30 min of incubation at 100°C; lane 5, blank; lanes 6 and 7, same as lanes 3 and 4, respectively, except that lysis was done in the presence of 1 mM EDTA.

temperature after both 15- and 30-min exposures; however, all species were killed by the combination of heating to 95°C and disinfectant. Similar results were obtained at an O-SYL dilution of 1:2,040, except that *M. kansasii* survived a 30-min exposure to both heat and disinfectant.

Roccal II, diluted 1:125, killed *M. tuberculosis* and *M. chelonae* at both room temperature and 95°C after 15 and 30 min of exposure, but *M. intracellulare* and *M. kansasii* were killed only with the combination of heat and disinfectant. At a dilution of 1:510, Roccal II was still effective against *M. tuberculosis* at both temperatures. *M. chelonae* and *M. kansasii* were killed after a 15-min treatment at 95°C and a 1:510 dilution of Roccal II solution; however, *M. intracellulare* was killed only after exposure to both heat and a 1:510 dilution of Roccal II for 30 min.

M. tuberculosis was the only species killed by Cidexplus used at a concentration of 0.64% at room temperature. Heating to 95°C and exposure to 0.64% Cidexplus killed all species after 15 min.

All disinfectants at all concentrations tested interfered with the SDA reaction for both the *M. tuberculosis* target sequence and the internal control sequences. PCR amplification of purified *M. tuberculosis* DNA in the presence of disinfectants showed similar results (Fig. 1), with the exception that there was a slight amplification of target with the 1:2,040 dilution of O-SYL (Fig. 1, lane 6). Experiments in which disinfectants were washed away before PCR indicated that DNA from mycobacteria killed in this manner was compatible with amplification (data not shown).

Neutralization experiments. Since all amplification methods take several hours to overnight to produce results, we investigated how long the mycobacteria would remain viable after NALC processing. All NALC pellets of processed McFarland 2 suspensions of *M. tuberculosis*, *M. intracellulare*, *M. gordonae*, and *M. chelonae* held for 24 h at 4°C grew in BACTEC vials and had 4-plus growth on LJ slants. This experiment was repeated with a dilute suspension of *M. tuberculosis* made by processing the growth in four BACTEC 12B vials with the average growth index (GI) of 17.5. All samples taken at zero time and after 1, 2, 4, and 24 h had between one and three colonies growing on LJ slants after 8 weeks of incubation, and all grew in the BACTEC system. Unneutralized NALC pellets of 14 clinical specimens (7 *M. tuberculosis* positive and 7 negative) were tested for growth in BACTEC vials after 4 and 24 h at 4°C. All seven positive specimens were still viable after 24 h, and none of the seven negative specimens grew contaminating organisms.

Detection of mycobacterial DNA by PCR and SDA. Analysis of the effect of the heat lysis on the integrity of *M. tuberculosis* genomic DNA indicated that the DNA was sheared into pieces of approximately 2 kb and less (Fig. 2). Attempts to stop the breakdown by adding 1 mM EDTA were not successful (Fig. 2, lanes 6 and 7). However, the shearing of the DNA did not inhibit its ability to be amplified by SDA or PCR. Table 3 depicts the results of a typical experiment in which a 1:1,000

TABLE 3. SDA and PCR of DNA from heat-lysed *Mycobacterium* spp.

Organism	Value (RLU[a]) for:		PCR value (RDU[b])
	SDA	SDA internal control	
M. tuberculosis	13,058	1,601	239.7
M. avium	8,724	1,028	177.0
M. chelonae	1,306	1,544	78.1
M. fortuitum	3,033	1,083	192.7
M. gordonae	6,992	3,150	40.4
M. intracellulare	14,027	1,439	161.7
M. kansasii	13,947	942	149.5
M. thermoresistibile	1,706	2,247	92.5
M. xenopi	689	1,413	203.9
Negative control	27	3,275	4.7

[a] RLU, relative light units.
[b] RDU, relative density units.

TABLE 4. SDA and PCR amplification of DNA from heat-lysed *M. tuberculosis* in clinical specimens

Sample	Result[a] for:					
	AFB[b] smear	LJ slant	BACTEC	SDA[c]	Internal control[c]	PCR[c]
7405	+	+	+	20,000	6,608	20,000
11107	+	+	+	20,000	8,872	20,000
6548	+	+	+	20,000	2,266	20,000
2391	+	+	+	20,000	510	20,000
13372	+	+	+	20,000	9,085	20,000
11241	+	+	+	20,000	2,440	20,000
6604	+	+	+	20,000	7,154	20,000
415	+	−	−	2,306	8,204	186
13165	−	−	−	342	9,964	7
12395	−	−	−	721	12,575	3,009
12241	−	−	−	654	10,585	612
13240	−	−	−	974	11,078	889
12831	−	−	−	375	11,809	873
13904	−	−	−	766	5,943	14
Positive control				6,216	6,124	2,494
Negative control				630	7,328	129

[a] +, positive result; −, negative result.
[b] AFB, acid-fast bacillus.
[c] Numbers are relative light units.

dilution of a McFarland 2 suspension of various mycobacterial species was heated to 100°C for 30 min and amplified by PCR and SDA.

We also attempted to compare the sensitivities of the PCR and SDA methods with those of the BACTEC system and growth on solid media by using dilutions of *M. tuberculosis*. Several experiments using between 10^4 and 10^8 CFU indicated that the BACTEC method detected organisms at a level between 1 and 2 dilutions lower than did solid media. However, the amount of signal in both amplification methods far exceeded that expected by the number of organisms present in the starting dilutions. This may have been due to the large amounts of DNA present as a result of grinding the organisms to obtain a smooth suspension. Growing the organism in liquid media to obtain a smooth suspension and eliminating the grinding step gave similar results, indicating that there was free DNA in solution of growing organisms. To overcome these difficulties, *M. tuberculosis* grown in BACTEC vials to a GI of 100 was concentrated by centrifugation (13,800 × *g* for 2 min), washed 10 times, and diluted with 5 ml of 25 mM KPO_4. After the mixture had been heated for 30 min at 100°C, the DNA was amplified by PCR and SDA. Both SDA- and PCR-amplified products from the undiluted suspension, which had a plate count of 2×10^4 CFU/ml, produced light signals that were at the upper limit of detection of the luminometer (i.e., 20,000 relative light units). Positive signals were also obtained at dilutions of up to 10^6 or 100-fold lower than seen with plate counts or BACTEC vials.

The NALC pellets from the seven positive and seven negative clinical samples that were tested in neutralization experiments were lysed by 30 min of heating at 100°C in a forced-air oven, amplified by SDA and PCR, and detected by chemiluminescent assay. Cultures of the lysed samples demonstrated that *M. tuberculosis* had been killed. After PCR amplification or SDA, all culture and acid-fast-bacillus-smear-positive specimens yielded chemiluminescent signals that were above the limit of detection of the luminometer (20,000 relative light units) (Table 4). Signals obtained by SDA of negative samples were consistent with the negative control and were within the limits of variability of SDA. SDA internal control sequences were amplified and detected in all of the clinical specimens, indicating that sample components did not inhibit the reaction. The suppressed amplification of the internal control seen with *M. tuberculosis*-positive samples 6548, 2391, and 11241 was probably due to competition between the mycobacterial DNA and the synthetic control sequence for reactants rather than to an interference with the SDA reaction. One discrepancy was noted among the PCR amplifications of negative specimens. Sample 12395, which was *M. tuberculosis* culture and smear negative, produced a PCR signal that was near the value obtained for the positive control. The inconsistency between the SDA and PCR findings for this sample was not resolved.

DISCUSSION

Though various investigators have used heat as part of the lysis protocol (2, 6, 8, 10, 11, 18) or for pasteurization (15) of mycobacteria, none reported that the preparations were tested to confirm loss of viability after the heat treatment. We have demonstrated that the heating of mycobacteria in dry-heat blocks at temperatures below 100°C may not consistently kill the organisms and that certain procedures being used for DNA amplification may not be safe for laboratorians. There appeared to be a relationship between the volume of the suspension, the density of the organisms, and killing at temperatures below 100°C. It was surprising that under some conditions, heating at 95°C in a heat block was not adequate to kill suspensions of mycobacteria. This was found to be due to a lag time in the fluid samples' reaching the set temperature and the inability of the heating blocks to allow the experimental tubes to achieve the appropriate set temperature. Additional factors that may have also contributed to the inconsistency of killing were radiative heat loss, the size of the cell aggregates (16), and the volume of the samples. The breakthrough growth may have also been due to the adherence of organisms to the sides of the tubes, where they were not exposed to the liquid. We determined that the only methods which produced consistent killing of all the mycobacterial species tested were those by which the tubes were fully immersed in boiling water or within a forced-hot-air oven. At 100°C, there was boiling and condensation throughout the tubes. This may have washed the organisms from the sides of the tubes into liquid, and the turbulence in the liquid may have broken up aggregates of organisms. Like others (16), we observed that the mycobacterial species differed in their susceptibility to heat killing. On the basis of the numbers of cultures that survived the 95°C heat treatment, a ranking of heat resistance was estimated as follows: *M. xenopi*, *M. thermoresistible*, *M. fortuitum*, and *M. chelonae* (100% survival) > *M. bovis*, *M. intracellulare*, *M. avium*, and *M. kansasii* (approximately 60% survival) > *M. tuberculosis* (42% survival) > *M. gordonae* (25% survival). With the exception of the result for *M. fortuitum*, this estimate generally fits the ranking of heat susceptibilities of aquatic mycobacteria measured by Schulze-Röbbecke and Buchholtz (16).

Tests of heating at 95°C in the presence of disinfectant demonstrated a synergistic effect that, like heating alone, was species dependent. The suppression of the SDA signal was probably due to the inhibition of the SDA; however, surfactants interfere with chemiluminescent assays, and part of the suppression may have also been due to the interference with the detection assay. We did not pursue the use of disinfectants because of the need to perform washing or precipitation steps to remove the disinfectants prior to DNA amplification.

The BACTEC method was selected as the primary test for

viability for the rapidity of obtaining positive results and because previous unpublished data collected at the Veterans Affairs Medical Center laboratory indicated that this method was 1 to 3 log units more sensitive than growth on solid media. There was a consideration that the sporadic growth in the BACTEC vials might have been due to organism carryover, as reported in several bulletins from Becton Dickinson. However, the BACTEC 460 system used in these experiments was modified to increase the heating time of the needles per Becton Dickinson recommendations and has been extensively tested with blood culture vials seeded with *Bacillus subtilis* spores for evidence of organism carryover without detection of any cross-contamination. Furthermore, there was never any breakthrough growth with the 100°C experiments and there was no evidence of carryover of one mycobacterial species into another species bottle. Also, results of several experiments using LJ media confirmed the results obtained with the BACTEC method.

Numerous methods have been developed for isolation of mycobacterial DNA (1, 14, 24). The focus of these reports was purification of intact polynucleotide, and consequently the methods were necessarily cumbersome for the clinical laboratory. The DNA produced by these methods has sufficient quality to be used for nucleotide sequencing or molecular cloning. However, we found that PCR amplification or SDA of mycobacterial DNA does not require that the DNA be intact or as highly purified. Therefore, processing of clinical mycobacterial samples for DNA amplification can be done more simply and quickly. Lysis of mycobacteria to free DNA fragments of suitable size for PCR and SDA occurs at 100°C, without the need for mechanical disruption or lysis buffers containing EDTA, detergents, or proteases. This may explain previous suggestions that lysis of *M. tuberculosis* prior to PCR was not necessary for amplification to occur (7) and that addition of Nonidet P-40 or sonication did not improve the efficiency of PCR amplification of unlysed mycobacteria (10). It is probable that the heat cycles of the PCR were sufficient to promote lysis. However, this presupposes that extraneous mycobacterial DNA was not present.

Previous workers have used cultured *M. tuberculosis* to validate processing methods for DNA amplification by PCR (6, 7, 10, 18). Estimates of high-level sensitivity of PCR have been based on numbers derived from CFU. Our study suggests that direct correlation between CFU and *M. tuberculosis* genomes may not be possible and cautions against the use of live organisms for quantitation of DNA targets. For example, when *M. tuberculosis* cells were amplified after extensive washing to remove extraneous DNA, the value for detected SDA or PCR amplicons was three log units below the estimated number of organisms. That is, DNA amplification apparently detected 1/100 of an organism, if one assumes 10 copies of IS*6110* per cell (7). We suggest that for every live *M. tuberculosis* organism, there may be many dead organisms that contribute to the apparent sensitivity of DNA amplification. The presence of excess DNA may also account for a previous report that lysis of *M. tuberculosis* was not required for amplification of DNA to be observed (7). When *M. tuberculosis* was washed to remove the extraneous DNA, the amplification sensitivities of SDA and PCR were found to be on the order of single organisms.

Our results indicate that mycobacteria survive in a NALC pellet stored at 4°C for 24 h. This would allow one to process clinical samples in the usual manner, heat a portion of the NALC pellet at 100°C for 30 min, and perform amplification and detection methods. If the amplification and detection methods were of sufficient sensitivity, only the positive specimens would require culture for final identification and susceptibility testing, with a great savings of time and money. More importantly, the clinician would be able to eliminate mycobacterial disease in patients with negative samples. If the amplification and detection methods had a high level of specificity, a positive diagnosis would be available in 24 to 48 h. However, these results must be verified by using a wider variety of clinical samples containing different concentrations of organisms.

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