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Sterilization of *Mycobacterium tuberculosis* Erdman Samples by Antimicrobial Fixation in a Biosafety Level 3 Laboratory

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Incomplete sterilization of *Mycobacterium tuberculosis* Erdman cultures followed 1 h of incubation in low concentrations of glutaraldehyde (0.5 and 1.0%) or azide. In contrast, 2.5% glutaraldehyde, paraformaldehyde (2 or 4%), Vesphine IIse or 5% formalin sterilized these samples after 1 h. These results suggest caution in removing fixed *M. tuberculosis* samples from biosafety level 3.

Experimentation with *Mycobacterium tuberculosis* requires biosafety level 3 (BSL-3) containment because of the potential for human infection. General safety considerations for working with *M. tuberculosis* are described by B. W. Allen (1). Removal of *M. tuberculosis* suspensions from BSL-3 containment should be done only if there is certainty that all bacteria are killed. When preparing samples for electron microscopy (EM), the use of low concentrations of glutaraldehyde alone is believed to be less damaging to the *M. tuberculosis* envelope structures (7). *M. tuberculosis* cultures are fixed in a final concentration of 5% formalin before absorbance determination at 600 nm. *M. tuberculosis* is susceptible to phenolic disinfectants (6), and a commercial product such as Vesphine IIse can be used to sterilize *M. tuberculosis* samples in the BSL-3 biosafety cabinet. The efficacy of these methods for killing *M. tuberculosis* samples has not been reported, and it is unknown if bacterial clumping interferes with the activity of these chemicals. For these reasons, we evaluated the killing efficacy of several mixtures using *M. tuberculosis* Erdman, a common BSL-3 laboratory strain. *M. tuberculosis* Erdman was grown either as a pellicle or as a liquid suspension before fixation for 1 h. We believe the 1-h fixation period is optimal for safe and efficient sample processing. Our results suggest caution in assuming sterilization of *M. tuberculosis* samples after incubation in EM fixative and certain laboratory disinfectant solutions.

(The data in this paper are from a thesis to be submitted by J. Reid Schwebach in partial fulfillment of the requirements for a Ph.D. from the Sue Golding Graduate Division of Medical Sciences, Albert Einstein College of Medicine, Yeshiva University, Bronx, N.Y.)

Growth and preparation of *M. tuberculosis*. All work with *M. tuberculosis* was conducted in a BSL-3 safety cabinet at the Howard Hughes Medical Institute, Albert Einstein College of Medicine, Bronx, N.Y. A frozen vial of *M. tuberculosis* Erdman obtained from the laboratory of Barry Bloom was thawed and suspended in 25 ml of 7H9 Middlebrook medium (Difco, Detroit, Mich.) containing 0.5% glycerol (Sigma, St. Louis, Mo.) and 0.05% Tween 80 (Sigma). A 25-ml starter culture was grown in a 490-cm^2 roller bottle (Corning 430195; Corning, N.Y.) rotated at 1.25 rpm in a 5% CO_2 incubator at 37°C until the absorbance at 600 nm reached 0.3 (approximately 0.6 to 1.7×10^8 CFU per ml). One or 3 ml of this culture was then added to 50 ml of 7H9 medium either with 0.05% Tween 80 (7H9-T medium) or without Tween 80 (7H9 medium), respectively, and was grown in the same manner as the starter culture for 7 days. Entire cultures were then harvested by centrifugation at 2,000 × *g* for 8 min in a Sorvall RTH-750 swinging bucket rotor using a Sorvall RT7 centrifuge (Kendro Lab Products, Newtown, Conn.). The absorbance of the 7H9-T culture at this time was 1.1 at 600 nm. Each bacterial pellet was then washed twice in phosphate-buffered saline (PBS) (0.137 M NaCl, 0.003 M sodium phosphate, pH 7.4) before suspension in 15 ml of PBS. Bacteria grown in 7H9 medium were then sonicated for 15 s to disperse clumps using a Branson Sonifier 250 sonicator (Danbury, Conn.) with a cuphorn sonicator (Branson 102 Converter) set at 60% peak output constant-duty cycle prior to antimicrobial fixation.

Fixation of *M. tuberculosis* and counting of CFU. Fixatives contained EM-grade glutaraldehyde (Polysciences, Inc., Warrington, Pa.), paraformaldehyde (Electron Microscopy Sciences, Fort Washington, Pa.), Vesphine IIse (Calgon Vestal Vesphine IIse [EPA Reg. No. 1043-87] containing *o*-phenylphenol [9.09%], *p*-tertiary amylphenol [7.66%], and inert ingredients [83.25%]; Steris Corp., Calgon Vestal Division, St. Louis, Mo.) in tap water at a standard concentration (40 ml of Vesphine IIse in 1 gal of H_2O) or 10% formalin buffered in PBS (Biochemical Sciences, Swedesboro, N.J.). The final concentration of the fixatives in each solution after the addition of bacilli is indicated in Tables 1 and 2. The 0.5, 1.0, and 2.5% glutaraldehyde and the 2% paraformaldehyde solutions contained 100 mM HEPES (Gibco, Grand Island, N.Y.), while the solutions with both glutaraldehyde and paraformaldehyde contained 100 mM trihydrate sodium cacodylate at pH 7.4 (Electron Microscopy Sciences). All fixatives were prepared fresh and diluted with PBS. As an additional test, bacteria were incubated in PBS containing 1 or 10 mM sodium azide (Sigma). One milliliter of *M. tuberculosis* suspension in PBS was added to 1 ml of fixative. Based on the absorbance of the 7H9-T cultures at 600 nm, we estimate that 7 to 18×10^8 bacteria were fixed in samples containing 7H9-T *M. tuberculosis*, a similar number of organisms as would be useful for scanning an EM sample preparation.

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TABLE 1. Number of *M. tuberculosis* Erdman CFU after growth with Tween 80 and suspension in antimicrobial solution

Disinfectant[a]	CFU (1st expt/2nd expt) after week[b]:				
	1	2	3	4	5
0.5% glutaraldehyde	—	—	500/20	500/30	500/30
1% glutaraldehyde	—	—	—	—	—
2.5% glutaraldehyde	—	—	1/0	1/0	1/0
2% paraformaldehyde	—	—	—	—	—
2% paraformaldehyde–2% glutaraldehyde	—	—	—	—	—
4% paraformaldehyde–0.5% glutaraldehyde	—	—	—	—	—
1 mM azide	H/H	H/H	H/H	H/H	H/H
10 mM azide	H/H	H/H	H/H	H/H	H/H
5% formalin	—	—	—	—	—
Vesphine IIse	—	—	—	—	—

[a] Solutions with paraformaldehyde also contained 100 mM sodium cacodylate, pH 7.4.
[b] —, no CFU; H, heavy *M. tuberculosis* growth.

M. tuberculosis was incubated with the fixative solutions containing glutaraldehyde and/or paraformaldehyde for 1 h at 4°C, in conditions similar to and including those used for preserving the outermost material of the *M. tuberculosis* envelope when preparing samples for EM (7). For the other antimicrobial solutions, the incubation was 1 h at room temperature, conditions ideal for work in the BSL-3 safety hood. Following the incubation in the antimicrobial solutions, all samples were immediately washed 3 times in PBS and suspended in PBS. This suspension was then plated on Middlebrook 7H11 agar (Difco) containing 0.1 mg of cycloheximide/ml (Sigma), and the plates were examined weekly for colonies. In the first experiment, the entire *M. tuberculosis*-PBS suspension was plated, while in the second experiment 1/10 of the suspension was plated. The numbers in Tables 1 and 2 are the numbers of viable colonies in the entire sample after fixation. As suspected, colonies grown for more than 3 weeks on solid agar could have been contaminants; colonies were acid-fast stained to verify mycobacterial growth. After 5 weeks of growth or any sign of overgrowth or contamination, suspected colonies were removed from the plate, resuspended in 1 ml of PBS, and frozen at −80°C for storage. The frozen samples were then killed by heating for 2 h at 80°C. Killed samples were removed to BSL-2 for staining with an acid-fast kit (Difco).

Of the antimicrobial solutions tested, those containing only 0.5 or 1% glutaraldehyde or containing only 1 or 10 mM sodium azide were unable to sterilize the *M. tuberculosis* suspensions (Tables 1 and 2). Incubation of *M. tuberculosis* Erdman grown in 7H9-T medium with 0.05% glutaraldehyde resulted in less efficient killing than incubation of *M. tuberculosis* grown in 7H9 medium. Treating 7H9-grown *M. tuberculosis* with 1% glutaraldehyde did not sterilize the sample in one experiment, while this treatment sterilized 7H9-T-grown *M. tuberculosis*. These data indicate that 0.5 and 1.0% glutaraldehyde solutions do not consistently kill all *M. tuberculosis* in experimental samples. It is estimated that more than 7 to 18 $\times$ 10^8 bacteria were fixed and plated in each sample. Using this estimate of bacterial number, fewer than 1 in 10^6 bacteria survived fixing with 1.0% glutaraldehyde (Table 1). Incubation of *M. tuberculosis* in solutions containing 2 or 4% paraformaldehyde, the Vesphine IIse solution, or 5% formalin killed all bacteria. These substances achieved 100% killing regardless of whether the *M. tuberculosis* was grown with or without Tween 80.

This work demonstrates that glutaraldehyde, paraformaldehyde, and formalin fixatives are capable of killing *M. tuberculosis* Erdman samples after treatment for 1 h, as is Vesphine IIse, a commercial product that contains phenol. Previous experiments using 2% alkaline glutaraldehyde have shown that only 1 *M. tuberculosis* bacterium per 10^5 bacteria attached to the surface of porcelain penicylinders will survive treatment for 5 min at 18°C (4) and that *M. tuberculosis* in solution is usually sterilized after 20 min (3). These conditions are unlike the laboratory fixation methods used to prepare samples for mi-

TABLE 2. Number of *M. tuberculosis* Erdman CFU after growth in the absence of Tween 80 and suspension in antimicrobial solution

Disinfectant[a]	CFU (1st expt/2nd expt) after week[b]:				
	1	2	3	4	5
0.5% glutaraldehyde	—	—	50/0	50/0	50/0
1% glutaraldehyde	—	—	2/0	12/0	13/0
2.5% glutaraldehyde	—	—	—	—	—
2% paraformaldehyde	—	—	—	—	—
2% paraformaldehyde–2% glutaraldehyde	—	—	—	—	—
4% paraformaldehyde–0.5% glutaraldehyde	—	—	—	—	—
1 mM azide	H/H	H/H	H/H	H/H	H/H
10 mM azide	H/H	H/H	H/H	H/H	H/H
5% formalin	—	—	—	—	—
Vesphine IIse	—	—	—	—	—

[a] Solutions with paraformaldehyde also contained 100 mM sodium cacodylate, pH 7.4.
[b] —, no CFU; H, heavy *M. tuberculosis* growth.

croscopy or to measure culture turbidity. Glutaraldehyde is a slow mycobactericidal agent (5) and has been demonstrated to have variable efficacy in killing different species and strains of mycobacteria (2).

Our data indicate that *M. tuberculosis* Erdman samples grown as pellicles are more likely to be incompletely killed after fixation in 1% glutaraldehyde, perhaps because these aggregates contain compact clusters of cells that are not penetrated by the fixative. Higher concentrations of glutaraldehyde and the addition of paraformaldehyde to the fixation solutions ameliorated this problem. It is possible that in some conditions 1% glutaraldehyde is sufficient to sterilize *M. tuberculosis* samples for safe processing. However, if this low concentration of glutaraldehyde is used, we recommend testing to demonstrate that the particular samples are killed before removal from BSL-3. We urge caution in the use of glutaraldehyde alone, as it should be noted that in one sample (Table 1) a viable colony survived fixation in 2.5% glutaraldehyde. Since most experiments showed sterilization of *M. tuberculosis* Erdman suspensions with 2.5% glutaraldehyde, we regard fixation in 2.5% glutaraldehyde to be adequate for removal to the BSL-2 environment in our hands. We prefer to use fixatives not relying on glutaraldehyde alone and recommend that *M. tuberculosis* samples killed by fixation be handled in a biosafety cabinet after removal to the BSL-2 environment for added safety. Formalin fixation of *M. tuberculosis* cultures for determination of culture turbidity in the BSL-2 environment is a routine procedure, and the conditions described in this work are acceptable for rapid removal of *M. tuberculosis* samples from the BSL-3 laboratory. We strongly recommend testing the killing methods for all preparations of *M. tuberculosis* that deviate from these standard conditions, as different *M. tuberculosis* strains and growth conditions could influence sterilization with the antimicrobial agents used in this work.

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