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Improved Laboratory Safety by Decontamination of Unstained Sputum Smears for Acid-Fast Microscopy

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Tubercle bacilli may survive in unstained heat-fixed sputum smears and may be an infection risk to laboratory staff. We compared the effectiveness of 1% and 5% sodium hypochlorite, 5% phenol, 2% glutaraldehyde, and 3.7% formalin in killing *Mycobacterium tuberculosis* present in smears prepared from 51 sputum samples. The smears were decontaminated by the tube and slide techniques. Phenol at 5%, glutaraldehyde at 2%, and buffered formalin at 3.7% for 1 min (tube technique) or for 10 min (slide technique) were effective in decontaminating sputum smears and preserved cell morphology and quantitative acid-fast microscopy results.

Direct microscopy for detection of acid-fast bacilli (AFB) in sputum specimens remains the main tool for presumptive diagnosis of pulmonary tuberculosis in many countries (3). In Brazil, 70% to 80% of all pulmonary tuberculosis cases are identified by this method (3, 4, 5, 12, 13). In health units with inadequate facilities, sputum specimens are normally collected and sent as heat-fixed smears to a reference laboratory for microscopic examination. Depending on the distance and available means of transport, analysis of the sputum smears may be delayed for days. Some studies have shown that tubercle bacilli may survive in unstained heat-fixed sputum smears for at least 7 days (1, 2, 6, 11, 14) and may therefore present a potential risk of transmission of laboratory-acquired infections (9, 17, 19).

The use of decontamination agents such as phenol (7), formaldehyde (2, 9), sodium hypochlorite (17), and glutaraldehyde (10, 11) to kill the tubercle bacilli present in smears has been documented. Decontamination of slides with these agents has improved the biosafety in transport, storage, or manipulation of slides containing sputa from patients suspected of having pulmonary tuberculosis.

The decontaminating agent should kill the tubercle bacilli present in sputum smears without impacting the quantitative acid-fast microscopic results, i.e., by altering the original characteristics of the specimen or by destroying the structure of the tubercle bacilli.

We compared the effectiveness of (i) 5% phenol (Merck, Darmstadt, Germany), (ii) 3.7% buffered formalin (Miyako do Brazil Indústria e Comércio Ltda., São Paulo, Brazil), (iii) 2% glutaraldehyde (Johnson & Johnson, São Paulo, Brazil), and (iv) 1% and 5% sodium hypochlorite (Miyako) for decontaminating unstained heat-fixed smears of sputum from patients suspected of having pulmonary tuberculosis. The effects of

these decontaminating agents on cell morphology and on quantitative acid-fast microscopy were also studied.

Sputa. Fifty-one specimens containing high numbers (grade 3+) (3) of viable *Mycobacterium tuberculosis* bacilli obtained from outpatients who attended a health care unit (Centro de Referência Professor Hélio Fraga, Rio de Janeiro, RJ, Brazil) were studied. The specimens were transported by air to Maringá City (Universidade Estadual de Maringá, PR, Brazil) and tested within 3 days after collection. One hundred twenty-four smears from each sample (total, 6,324) were prepared by spreading the sputum specimen over a 25 by 10 mm area of a 76 by 13 mm glass microscope slide using a 10- μ l disposable loop (Difco Laboratories, Sparks, Md.) (Fig. 1). The smears were air dried in a safety cabinet and heat fixed by passing the slide (three times), smear side away from the flame, through the flame of a gas burner. Two pairs of slides from each sample were used as controls (growth control and quantitative AFB control).

Forty smears from each sample (total, 2,040) were decontaminated by the tube technique. Briefly, the slide was placed in a 16 by 150 mm tube containing 8 ml of the decontaminating agent to be tested and incubated for 1, 5, 10, or 15 min. Slides were then aseptically transferred to 16 by 150 mm tubes containing 10 ml of sterile distilled water, rinsed briefly, and cultured in selective lysed blood (SLB) medium. Our previous studies showed better results using this medium (6).

Another series of 40 smears from each sputum specimen (total, 2,040) were decontaminated using a slide technique. Briefly, the slide was covered with the decontaminating agent; incubated for 1, 5, 10, or 15 min; rinsed briefly in 10 ml of sterile distilled water; and cultured in SLB medium.

Control of decontamination. To determine bacillary viability, duplicates of decontaminated slides (tests) and controls (slides with no decontamination) were cultured by placing each slide in 16 by 150 mm tubes containing 8 ml of SLB medium (1, 6, 18) and incubated at 37°C for 7 days. The slides were then removed from the SLB medium, rinsed briefly in 10 ml distilled water, decontaminated with 5% sodium hypochlorite for 10

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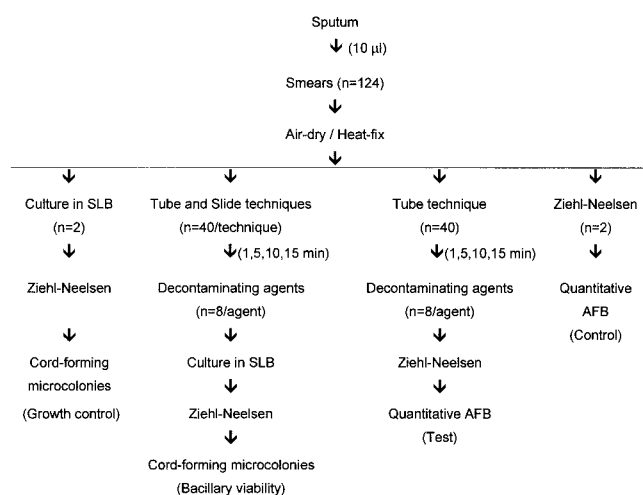


FIG. 1. Flow diagram of decontamination of unstained sputum smears for acid-fast microscopy.

min, stained by the Ziehl-Neelsen method (3, 16), and examined with a compound microscope. The entire surface of the smear was scanned using $\times 100$ magnification, and the presence of cord-forming microcolonies, i.e., positive tests, was confirmed using $\times 200$ magnification.

Quantitative acid-fast microscopy. To verify the effects of decontaminating agents on cell morphology and quantitative reduction of AFB, another series of 40 smears was prepared from each of 51 sputum specimens. Each slide was aseptically decontaminated by the tube technique, rinsed briefly in distilled water, air dried, heat fixed again, stained by the Ziehl-Neelsen method (3, 16), and examined with a compound microscope to detect AFB. The following semiquantitative scale was used: 1+, fewer than 1 AFB per immersion field; 2+, 1 to 10 AFB/field; 3+, more than 10 AFB/field (3). Statistical analysis was performed by Kruskal-Wallis analysis and Mann-Whitney test (U test), Statistica for Windows, version 6.0, 2001 (Stat Soft Inc., Tulsa, Okla.).

Additional bacteriologic data. All sputum specimens studied were cultured on Loewenstein-Jensen slants, and incubated at 37°C for 8 weeks. Positive cultures were identified as *M. tuberculosis* using the following tests: growth rate, pigment production, niacin accumulation, nitrate reduction, heat-stable catalase (at pH 7.0 and 68°C), and ability to grow in the presence of *p*-nitrobenzoic acid (500 µg/ml) and thiophen-2-carboxylic acid hydrazide (2 µg/ml) (3, 20).

As expected, all sputum specimens collected from tuberculosis outpatients analyzed in our study yielded positive cultures that were confirmed by identification methods as *M. tuberculosis*. In this sense, we can consider that all cord-forming growth of bacilli in slide cultures detected in this study was produced by tubercle bacilli.

The results of the decontamination assay showed that in the tube decontamination technique, all agents (phenol, formalin, glutaraldehyde, and sodium hypochlorite) were effective within 1 min. These decontaminating agents are traditionally used as disinfectants in mycobacteriology laboratories (8, 9). However, of 1,020 slide cultures in which slides were treated with the decontaminating agents using the slide technique for 1 and 5

TABLE 1. Effectiveness of 5% phenol, 3.7% buffered formalin, 2% glutaraldehyde, and 1% and 5% sodium hypochlorite in decontaminating 2,040 heat-fixed, unstained sputum smears by the slide technique

Decontaminating agent	Survival ^a of tubercle bacilli in slide smears decontaminated for:			
	1 min	5 min	10 min	15 min
5% phenol	3/102 ^b	1/102	0/102	0/102
3.7% buffered formalin	9/102	2/102	0/102	0/102
2% glutaraldehyde	3/102	2/102	0/102	0/102
1% sodium hypochlorite	3/102	5/102	0/102	0/102
5% sodium hypochlorite	6/102	3/102	0/102	0/102

^a Survival of tubercle bacilli was evidenced by the production of cord-forming microcolonies on slide smears cultured in SLB medium.

^b Number of survivors/total.

min, 37 (3.62%) were found to have cord-forming growth (Table 1). Our results showed that technicians who handle slides fixed only by heat are at risk because 3.62% of the heat-fixed smears, other than those decontaminated for 5 min, showed viable tubercle bacilli. The resistance of mycobacteria to disinfectants is considered to be due to the high lipid content of their complex cell wall (15).

The superior effectiveness of the tube technique may be due to a larger contact volume between the disinfectant and the slide. The survival of tubercle bacilli in the slides treated with germicides for 1 and 5 min was most likely due to failure of the slide decontamination technique; i.e., the germicide may not have been in contact with the smear throughout the decontamination period although additional agent was added as needed. On the other hand, for 10- and 15-min exposures it was easier to maintain confluence of the chemical agents on the slides. The slide technique was effective within 10 min and may also be considered as an alternative for routine laboratory use because of its simplicity, speed, and low cost. In addition, the difficulty of making smears with a uniform thickness varies with the physical characteristics of the sputum; i.e., mucus viscosity may also contribute to the lack of reproducibility in the smears ($n = 37$) that resisted disinfection.

The mean numbers (\pm the standard deviation) of AFB found per immersion field (magnification, $\times 1,000$) on slide smears treated with decontaminating agents were 69.57 ± 153.58 (5% phenol), 69.43 ± 142.54 (3.7% buffered formalin), 66.27 ± 157.96 (2% glutaraldehyde), 10 ± 90.77 (1% sodium hypochlorite), and 6.14 ± 39.32 (5% sodium hypochlorite). The quantitative acid-fast microscopy analysis showed clearly that neither phenol, nor formalin, nor glutaraldehyde interfered with the quantitative results (Fig. 2). Conversely, sodium hypochlorite containing 1% or 5% chlorine altered the number of AFB significantly in the smears ($P < 0.01$), decreasing the grade from 3+ to 1+, suggesting disintegration of the bacterial cell within 1 min of contact. This deleterious effect on the tubercle bacillus was also observed in other studies (16, 17).

Smithwick and Stratigos (17) proposed a safe method for preparing sputum smears for AFB studies, e.g., in clinical laboratories for proficiency tests or quality control. Briefly, the method consists of mixing AFB smear-positive sputum that has been decontaminated with sodium hypochlorite with AFB-negative sputum previously treated with sodium hypochlorite.

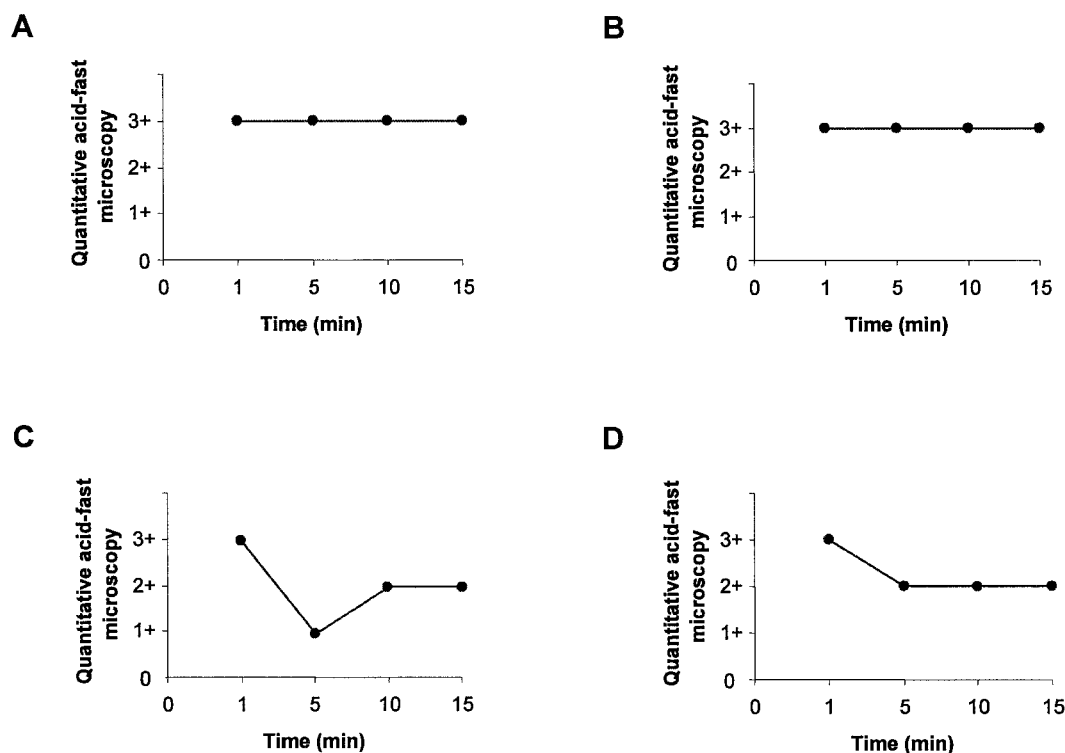


FIG. 2. Quantitative acid-fast microscopy of slide smears treated with decontaminating agents. Panels: A, control; B, 5% phenol, 3.7% buffered formalin, or 2% glutaraldehyde; C, 1% sodium hypochlorite; D, 5% sodium hypochlorite.

The germicide killed the bacilli but preserved acid fastness. However, the authors did not determine the reduction in the number of AFB. According to the authors, a solution of sodium hypochlorite containing 5% chloride sterilizes the smear in 10 min and can destroy AFB with additional contact time. In our study, perhaps due to our thinner films in slides, the hypochlorite destroyed the AFB within 1 min of contact, resulting in a reduction in the bacillus grade from 3+ to 2+ or 1+ (Fig. 2).

Goldfogel and Sewell (11) described the use of 1% glutaraldehyde for 15 min for decontaminating sputum smears. Our study showed that 2% glutaraldehyde killed *M. tuberculosis*, but in about half of the treated smears we observed a red background after Ziehl-Neelsen staining. This red background makes visualization of AFB difficult, but it did not decrease the results of quantitative acid-fast microscopy. The glutaraldehyde used in our study could have reacted with constituents of the sputum or with the Ziehl-Neelsen staining reagents.

In England, treatment with 3.7% buffered formalin for 5 min is a biosafety indication for routine decontamination of sputum smears, primarily when smears cannot be stained immediately by the Ziehl-Neelsen or fluorescence technique (9). Our results confirmed the effectiveness of formalin in killing tubercle bacilli and showed that it did not affect the original microscopic characteristics of the sputum smear, which remained 3+ (mean) quantitative graduation.

Recently, Chedore et al. (7) recommended 5% phenol in ethanol for 5 min to be used as a rapid and effective method to fix the smear and kill tubercle bacilli. In our study, the slides treated with phenol did not present growth in cord formation

and the treatment did not alter the cellular morphology of the bacillus or reduce the quantitative acid-fast microscopy.

The results of the quantitative acid-fast microscopy in our study demonstrated that, except for sodium hypochlorite, the decontaminating agents tested did not interfere with AFB counts. This finding may be an important aspect of microscopic diagnosis and control of pulmonary tuberculosis.

In conclusion, the results of this study suggest that treatment with 5% phenol, 2% glutaraldehyde, or 3.7% buffered formalin for 1 min (tube technique) or for 10 min (slide technique) renders sputum smears safe for storage, shipment, or handling prior to staining.

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