

Novel hypertonic saline–sodium hydroxide (HS–SH) method for decontamination and concentration of sputum samples for *Mycobacterium tuberculosis* microscopy and culture

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This study evaluated a new decontamination and concentration (DC) method for sputum microscopy and culture. Sputum samples from patients with suspected pulmonary tuberculosis (TB) ($n=106$) were tested using the proposed hypertonic saline–sodium hydroxide (HS–SH) DC method, the recommended *N*-acetyl-L-cysteine–sodium citrate–sodium hydroxide (NALC–NaOH) DC method and unconcentrated direct smear (Ziehl–Neelsen) techniques for the presence of mycobacteria using Löwenstein–Jensen culture and light microscopy. Of 94 valid specimens, 21 (22.3%) were positive in culture and were further characterized as *Mycobacterium tuberculosis*. The sensitivity for acid-fast bacilli (AFB) smears was increased from 28.6% using the direct method to 71.4% (HS–SH) and 66.7% (NALC–NaOH) using DC methods. Both concentration techniques were highly comparable for culture ($\kappa=0.794$) and smear ($\kappa=0.631$) for AFB. Thus the proposed HS–SH DC method improved the sensitivity of AFB microscopy compared with a routine unconcentrated direct smear; its performance was comparable to that of the NALC–NaOH DC method for AFB smears and culture, but it was methodologically simpler and less expensive, making it a promising candidate for evaluation by national TB control programmes in developing countries.

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INTRODUCTION

The social and economic burden of tuberculosis (TB) has been the subject of much study, and major efforts are under way to try to achieve its control. It is estimated that 2 billion people are infected with *Mycobacterium tuberculosis*; at least 10% of these people (200 million) will develop active TB in their lifetime. TB is one of the major health problems in Peru, where its incidence rate (192 per 100 000 of the population) is one of the highest in the

Americas; Peru was one of the high-burden countries until 2001 (Suarez *et al.*, 2001; WHO, 2002). The most accomplished TB control programmes in developing countries are reaching a 56% case detection rate, primarily by direct sputum microscopy (WHO, 2005). The simplest approach for improving the level of case detection is to upgrade the performance of existing inexpensive diagnostic tools, without making them too complicated.

The gold standard for pulmonary TB diagnosis remains culture, but in developing countries diagnosis relies on microscopy for acid-fast bacilli (AFB) in sputum smears because it is simple, inexpensive and provides rapid results. Unfortunately, this technique has a low sensitivity [22–43% for a single smear (Toman, 2004) and up to 60% under optimal conditions (Apers *et al.*, 2003; Siddiqi *et al.*, 2003)] when compared with that of cultures. This

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Abbreviations: AFB, acid-fast bacilli; DC, decontamination and concentration; HS–SH, hypertonic saline–sodium hydroxide; NALC–NaOH, *N*-acetyl-L-cysteine–sodium citrate–sodium hydroxide; NTP, National Tuberculosis Control Program; TB, tuberculosis.

sensitivity is even lower in paediatric and human immunodeficiency virus (HIV)/AIDS patients (Karstaedt *et al.*, 1998; Khan & Starke, 1995). The threshold for detection of AFB in sputum samples under optimal conditions is between 10^4 and 10^5 bacilli ml^{-1} ; TB programme conditions and technical constraints further decrease this yield (IUATLD, 2005).

Decontamination and concentration (DC) methods, such as using *N*-acetyl-L-cysteine–sodium citrate–sodium hydroxide (NALC–NaOH), improve the yield of AFB microscopy and are routinely used in industrialized countries. The high cost of the reagents and the need for additional specialized training prevent their widespread use in developing countries; the same considerations also hinder the adoption of currently available immunological and molecular diagnostic techniques (Palomino, 2006; Perkins & Kritski, 2002). Therefore, an inexpensive, straightforward DC method would improve case detection in a setting such as Peru.

We have developed a novel DC method that uses hypertonic saline (HS) for mucolysis and sodium hydroxide (SH) for decontamination (Ricaldi & Guerra, 2008), based on the observation that hypertonic saline has been used successfully for the symptomatic treatment of cystic fibrosis (King *et al.*, 1997). This new HS–SH method allowed better recovery of AFB in sputum than direct smears, could detect at least 10^4 bacilli $(\text{ml sputum})^{-1}$ and showed no significant difference when compared with NALC–NaOH *in vitro* in assays using AFB-spiked sputa (Ricaldi & Guerra, 2008). The present study evaluated the diagnostic accuracy and performance of the proposed DC technique in sputum and compared it with the standard NALC–NaOH method and with routine direct (unconcentrated) AFB smears used by the National Tuberculosis Control Program (NTP), in a hospital-based setting.

METHODS

Sputum samples. Samples were collected from the NTP Microbiology Laboratory at the Cayetano Heredia National Hospital in Lima, Peru, between August 2002 and February 2003; sputum samples were collected prospectively during times that the investigators were available. These samples were obtained from patients with suspected pulmonary TB (cough ≥ 14 days according to NTP guidelines), with or without previous diagnosis of pulmonary TB, and before starting appropriate anti-tuberculosis treatment.

The NTP Microbiology Laboratory performed AFB detection using the direct technique of Ziehl–Neelsen staining according to national and Pan American Health Organization guidelines under routine conditions; the results were recorded for this study. The other procedures were performed in the Biological Safety Level 3 facility at the Microbiology Laboratory of the Instituto de Medicina Tropical Alexander von Humboldt of the Universidad Peruana Cayetano Heredia, Peru.

Samples were either processed immediately on arrival at the laboratory or stored for less than 24 h at 4 °C before processing. Two 1 ml portions were taken from each homogenized sample. One portion was used for each method. Samples with a volume of less than 2 ml were excluded.

DC methods

HS–SH procedure. A 1 ml portion of sputum was mixed with 1 ml 7% (w/v) NaCl and 1 ml 4% (w/v) NaOH in a sterile 15 ml centrifuge tube (BD Falcon) and homogenized for 15–20 s using a vortex mixer [final concentrations (w/v) in 3 ml: 2.33% NaCl, 1.33% NaOH]. The tubes were then incubated at 37 °C for 30 min. After incubation, the mixture was neutralized with sterile PBS (pH 6.8), bringing the total volume to 15 ml. The mixture was vortexed for 5 s and then centrifuged at 3400 g for 15 min at 15 °C, using aerosol-proof shields (Thermo IEC Centra CL3-R, rotor 243; Thermo Scientific). The supernatant was discarded into a splash-proof container with a tuberculocidal solution. The pellet was then resuspended in 200 μl sterile PBS, homogenized for 5 s with a vortex mixer and inoculated into Löwenstein–Jensen (LJ) culture media; a smear was prepared for Ziehl–Neelsen staining.

NALC–NaOH procedure. The procedure used has been described previously (Kent & Kubica, 1985) and is recommended by the Centers for Disease Control and Prevention and the WHO/IUATLD (WHO, 1998). Briefly, 1 ml sputum was added to a 50 ml BD Falcon centrifuge tube with 1 ml of solution containing 0.5% (w/v) NALC, 2.67% (w/v) NaOH and 1.45% (w/v) sodium citrate and mixed well (final concentrations (w/v) in 2 ml: 0.25% NALC, 1.34% NaOH, 0.73% sodium citrate). The tubes were incubated at room temperature for 15 min. After incubation, the mixture was neutralized with PBS, bringing the total volume to 50 ml. The rest of the procedure was as described above.

Culture. Immediately after the DC process, 100 μl each resuspended pellet was used to inoculate a tube of LJ medium. The culture tubes were incubated at 37 °C for up to 8 weeks before being reported as negative. *Mycobacterium* sp. isolates were identified using the following criteria: time to visible growth, growth at 37 °C, colony pigmentation and morphology, AFB smear and niacin strip test (Remel) (Kent & Kubica, 1985).

AFB smears. One drop of each suspended pellet was used to prepare slides for AFB microscopy using the Ziehl–Neelsen stain. Each slide was coded, read blindly by a qualified technician and reported according to the NTP and WHO/IUATLD standards (WHO, 1998). Smears were reported as follows: <1+, 1–9 AFB in 100 microscopic fields (few bacilli); 1+, 10–99 AFB in 100 fields; 2+, 1–10 AFB per field in at least 50 fields; 3+, more than 10 AFB per field in at least 20 fields.

Statistical analysis. The values for sensitivity, specificity and efficiency were calculated using a 2×2 table; the degree of association (using the kappa statistic) and difference of proportions and their significance were calculated using McNemar's χ^2 and the Z test, using SPSS 11.0.3 for Macintosh (SPSS). When comparing the results of AFB smears, only their negativity or positivity for AFB was used because of the expected difference in quantitative results between the concentrated and unconcentrated portions of the same sample.

RESULTS AND DISCUSSION

The results of all of the tests are presented in Table 1. From the 106 samples included in the study, 12 were excluded because of contamination of their cultures [11 cultures contaminated with the NALC–NaOH method, one contaminated with both methods; 13/212 (6.13%) LJ tubes contaminated in total]. The remaining 94 samples were evaluated.

Table 1. Overall results of culture and AFB microscopy

Twelve samples with contaminated cultures were excluded; all 12 were contaminated using the NALC-NaOH method and one was also contaminated with the HS-SH method. Negative results are given in parentheses.

No. of specimens	Culture		AFB microscopy		
	NALC-NaOH	HS-SH	Direct NTP	NALC-NaOH	HS-SH
6	+	+	+	+	+
6	+	+	-	+	+
1	+	+	-	-	+
2	+	+	-	-	-
1	+	-	-	-	-
2	-	+	-	-	-
1	-	+	-	+	+
1	-	+	-	-	+
1	-	+	-	+	-
4	-	-	-	+	+
5	-	-	-	+	-
5	-	-	-	-	+
59	-	-	-	-	-
Total: 94	16 (78)	20 (74)	6 (88)	23 (71)	24 (70)

Performances of the HS-SH and NALC-NaOH DC methods for culture are comparable

Twenty-one samples (22.3%) were culture-positive by at least one of the DC methods used. All positive cultures were identified as *M. tuberculosis*. The sensitivity, specificity and efficiency of cultures processed by the HS-SH and NALC-NaOH methods were calculated by comparing their results with the total number of positive cases as determined by culture (Table 2). Agreement and reproducibility between the two DC methods in cultures was excellent (kappa index=0.794, 95% CI=0.638–0.951). DC using the HS-SH method yielded 4.3% more positive cultures than the NALC-NaOH method; this difference was

not significant (95% CI=-0.8% to +9.3%; McNemar's $\chi^2=2.67$, $P=0.1$) and did not produce a significant increase in sensitivity (Table 2).

There were 14 (14.9%) specimens positive in one or both AFB smears (NALC-NaOH or HS-SH) with very low AFB counts, which gave negative results by culture (Table 1). The AFB counts on the smears of these samples had a total of 1–9 AFB in 100 high-power fields or over the whole slide, suggesting that the bacterial load in these specimens was at or near the limit of sensitivity of the culture (<1+ in the NTP format; data not shown). Previous observations have indicated that, in samples with bacillary counts of less than 10^4 bacilli ml^{-1} , the sensitivity of culture for mycobacteria using LJ medium is suboptimal, ranging from 67% to a maximum of 95% (Levy *et al.*, 1989; Long, 2001). This may be due, for example, to the high toxicity of NaOH, the loss of bacilli when decanting the supernatant of centrifuged samples or the presence of damaged bacteria, all reducing the final recovery of bacilli, which has been estimated to be up to 60% of the initial total number under optimal conditions (Allen & Mitchison, 1992; Kent & Kubica, 1985). There were 10.4% more contaminated cultures by NALC-NaOH than by HS-SH (95% CI=4.6–16.2, McNemar's $\chi^2=11$, $P<0.05$); this statistically significant difference favoured the HS-SH method. The overall contamination rates (6.13%, total LJ cultures) are similar to those observed by others, ranging from 1.5 to 13.3% using LJ medium (Mirovic & Lepsanovic, 2002; Somoskovi & Magyar, 1999).

Sensitivity of AFB microscopy is improved by both DC methods

Of the 21 culture-positive samples, AFB were detected in 15 (71.4%) of the slides processed by the HS-SH DC method and 14 (66.7%) by the NALC-NaOH method (Table 2). In contrast, the direct method performed at the NTP Microbiology Laboratory detected AFB in only six (28.6%) of the culture-positive samples. The sensitivity,

Table 2. Sensitivity, specificity and efficiency of culture and AFB microscopy with each method, compared with the total number of positive cultures

95% CIs are given in parentheses; upper 95% CIs exceeding 100% were rounded to 100%.

	Culture		AFB microscopy		
	HS-SH	NALC-NaOH	HS-SH	NALC-NaOH	Direct (NTP)
Sensitivity	95.2% (86.1–100)	76.2% (58–94.4)	71.4% (52.1–90.8)	66.7% (46.5–86.8)	28.6% (9.2–47.9)
Specificity	100% (100–100)	100% (100–100)	87.7% (80.1–95.2)	87.7% (80.1–95.2)	100% (100–100)
Efficiency	99% (96.9–100)	95% (90.1–99.2)	84% (76.6–91.4)	83% (75.4–90.6)	84% (76.6–91.4)
Detected/non-detected	20/1*	17/4*	15/6†	14/7†	6/15†

*Culture: difference in the proportions for sensitivity (95% CI)=-0.023 to +0.404; $\chi^2=1.5$, d.f.=1, $P=0.22$.

†AFB microscopy: difference in the proportions for sensitivity (95% CI): HS-SH versus NTP's direct method=0.11–0.75 ($Z=2.47$, $P=0.014$), NALC-NaOH versus NTP's direct method=0.05–0.71 ($Z=2.16$, $P=0.031$), NALC-NaOH versus HS-SH=-0.3 to 0.28 ($Z=0$, $P=1$); $\chi^2=9.39$, d.f.=2, $P<0.01$.

specificity and efficiency of AFB microscopy after processing by the HS–SH and NALC–NaOH DC methods and for the direct AFB microscopy processed at the NTP Microbiology Laboratory were calculated by comparing their results with the total number of positive cases as determined by culture. The sensitivity of AFB microscopy was increased by the two DC methods compared with the direct microscopy method; this increase was statistically significant (Table 2).

The results (positivity or negativity) of the AFB smears prepared after the two DC methods had a high correlation: 81/94 (86.2%) were concordant and all of the 13 samples that were discordant had an AFB count of <1+ (data not shown). The kappa value of agreement of the two AFB microscopy methods was 0.631 (95% CI=0.445–0.817). DC using the HS–SH method for AFB microscopy yielded 1.1% more positive results than the NALC–NaOH method; this difference was not significant (95% CI=–6.5% to +8.6%; McNemar's $\chi^2=0.08$, $P=0.78$).

Of the 21 culture-positive samples, 16 (76.2%) had a positive AFB smear in one or both DC methods; the NTP's direct AFB smears missed 10 culture-positive samples. Microscopy using the NALC–NaOH and HS–SH DC methods detected 23 and 24 of the 30 samples that had AFB in at least one of the DC methods, respectively, whilst microscopy with the direct method used by the NTP detected only six AFB-positive samples; all smears were read by the same skilled technician. This indicated that 24 out of the 30 (80%) smear-positive samples detected by at least one DC method were missed by the NTP's direct microscopy method. Twelve of these missed samples had a low AFB count (<1+), even after concentration (data not shown); the rate of false-negatives with the direct AFB smear technique was greater in samples with low bacillary counts, a common observation also made by others (Marei *et al.*, 2003; Peterson *et al.*, 1999; Rattan *et al.*, 1994; Van Deun *et al.*, 2000). It should be noted that the gain in sensitivity using DC methods may also indicate poor routine direct AFB smear processing by the NTP, as a 29% yield for AFB microscopy is low in high-incidence settings.

Advantages of the HS–SH DC method

Accurate case detection is essential for the directly observed treatment short-course (DOTS) TB control strategy. All efforts to control TB depend on early detection to decrease transmission. The HS–SH technique substantially increased the sensitivity of AFB smears compared with the NTP's routine direct smear for AFB and correlated well with the NALC–NaOH DC method for sputum culture in suspected TB patients.

Many techniques have been developed to improve the sensitivity and timely detection of AFB in sputum. Among the procedures that one can find in the literature, most use concentration techniques and a chemical decontamination step. Some achieve sensitivities and specificities similar to

those of the standard NALC–NaOH method, but they use expensive reagents and equipment. Methods using bleach (NaOCl) and xylol show similar results for microscopic detection of AFB, but they both kill mycobacteria, which is a disadvantage because in specific populations at risk for multidrug-resistant TB, culture is mandatory (Steingart *et al.*, 2006b). An added advantage of the HS–SH method over the NALC–NaOH method is the much lower cost and the greater stability of NaCl solutions. The mixture containing NALC has to be prepared daily and must be discarded if unused (Kent & Kubica, 1985); in contrast, the NaCl solution can be kept at room temperature for several days without change. The proposed HS–SH method allows the use of 15 ml sterile centrifuge tubes, which are less expensive and more convenient to use in ordinary centrifuges than the 50 ml tubes recommended for the NALC–NaOH method. Another advantage of the proposed method is that the final pellet can also be stored for later use in drug-susceptibility testing.

The greatest limitation of the proposed method is the need for a high-speed centrifuge with aerosol-proof capped tube holders. This can be a problem in low-resource settings, but the considerable increase in patient detection may improve the efficiency of the overall control programme by reducing the number of undetected TB-positive carriers, who would otherwise continue spreading the disease.

This study was limited by a relatively small sample size and by a lack of information about the HIV status of the pulmonary TB suspects. Also, we only recovered *M. tuberculosis*, probably because of the low HIV prevalence (~2 per 100 000) (WHO, 2005) and a low non-TB mycobacterial prevalence in Peru (M. Cabello, personal communication). Despite these limitations, the advantages show that the HS–SH technique could improve diagnosis in difficult situations, such as in paediatric, HIV/AIDS and other paucibacillary pulmonary TB patients. Along with more sensitive techniques such as fluorescence microscopy (Steingart *et al.*, 2006a) and rapid culture methods, this DC procedure could improve the care and prognosis of TB patients by providing a more accurate and timely diagnosis.

The HS–SH DC method offers major improvements in sensitivity of microscopy for AFB compared with the direct AFB smear method and its performance for culture is comparable to that of the recommended NALC–NaOH method with fewer contaminated cultures. The proposed HS–SH method is simpler and less expensive than the recommended NALC–NaOH DC method and is therefore a promising candidate for evaluation and adoption by national TB control programmes in developing countries.

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