Exposure of sputum to phenol disinfectant in conjunction with universal sample processing solution provides safety to laboratory workers during smear microscopy

Sputum smear microscopy is the backbone of any tuberculosis-control programme. Of paramount importance is the safety of laboratory workers, especially in programme settings, who are involved daily in handling a large number of *Mycobacterium tuberculosis*-infected samples. Increased incidence of tuberculosis amongst health-care workers, especially laboratory technicians, has been well documented (Baussano *et al.*, 2011). In developing countries, infectious samples are often handled on open benches or in hoods without biosafety certification. Moreover, sample concentration techniques are often used to increase the sensitivity of smear microscopy (Steingart *et al.*, 2006). The processing of samples containing live tubercle bacilli is associated with the risk of aerosol-mediated spread of infection and contamination of the laboratory environment. Early sample disinfection during processing, that does not compromise the performance of smear microscopy, would be highly welcome to laboratory personnel not having access to stringent biosafety laboratories. Unfortunately, mycobacteria are extremely resistant to disinfection processes. Importantly, heat fixing of slides has not proved to be effective in deactivating *M. tuberculosis*, and thus the process may potentially lead to the infection of laboratory workers (Allen, 1981; Chedore *et al.*, 2002). Various disinfectants have been evaluated for their ability to deactivate *M. tuberculosis* with the aim of rendering samples safe for handling and disposal (Chedore *et al.*, 2002; Best *et al.*, 1988, 1990; Rikimaru *et al.*, 2002; Selvakumar *et al.*, 2002; Hall *et al.*, 2007; Hegna, 1977). In our laboratory, we developed a sputum-processing methodology that employs universal sample processing (USP) solution and provides a concentrated sample that is suitable for smear

microscopy along with culture and PCR. USP solution consists of: 4–6 M guanidine hydrochloride; 50 mM Tris/HCl, pH 7.5; 25 mM EDTA; 0.5 % sarkosyl; and 0.1–0.2 M β -mercaptoethanol (Chakravorty & Tyagi, 2005). The purpose of the present study was to combine USP and a method of *M. tuberculosis* inactivation without compromising on the performance of smear microscopy. An important concern during USP solution-based processing was to test the efficacy of the disinfectant in penetrating organic matter present in samples such as sputum and its ability to remain effective as a bactericidal agent (Hegna, 1977).

In the first phase, various disinfectants selected based on earlier studies (Chedore *et al.*, 2002; Best *et al.*, 1988, 1990; Rikimaru *et al.*, 2002; Selvakumar *et al.*, 2002; Hegna, 1977), were tested on negative sputum samples that were spiked with 1×10^3 – 1×10^6 c.f.u. *M. tuberculosis* H37Rv ml⁻¹. Briefly, each disinfectant (5 % phenol in ethanol, 2.5 % phenol ammonium sulphate (PhAS), 7.5 % hydrogen peroxide, 2 % sodium or calcium hypochlorite), was individually added to 1 ml aliquots of spiked sputum, simultaneously with 2 ml USP solution, to achieve a final concentration of the disinfectant as mentioned above. After exposure of the

samples to the disinfectant for 30 min at room temperature, 10 ml sterile water was added and the samples were centrifuged at 3500 g for 15 min at room temperature. The resulting pellet was washed twice with 10 ml sterile water, pelleted again by centrifugation as above and the final pellet was resuspended in 500 μ l 0.05 % Tween 80. Two drops of this suspension were used to make a smear on a glass slide, and four to five drops were inoculated on Löwenstein–Jensen medium slants and one to two drops in Middlebrook 7H9 medium, which were incubated at 37 °C for 8 weeks. Aliquots of unspiked negative sputum samples were also processed similarly as negative controls. The Ziehl–Neelsen (ZN)-stained disinfectant + USP solution smears were compared to direct smears or USP solution smears with respect to their smear grade status and staining characteristics, including background staining and interference as per standard World Health Organization criteria (WHO, 2006).

Smears prepared after sputum treatment with USP solution in the presence of phenol, PhAS or hydrogen peroxide were found to be of an acceptable quality. Viability testing indicated that 5 % phenol was effective, as no growth of acid fast bacilli (AFB) was observed in any of the aliquots treated with this disinfectant. Hydrogen peroxide had a

Table 1. Comparison of sputum smear status

ZN-staining smear grade	Direct	USP*	USP + phenol
3 +	39	56	51
2 +	6	4	5
1 +	11	5	7
Scanty	6	4	7
Negative	8	1	0
Total	70	70	70
Background staining debris	Present	Absent	Absent

*ZN-stained smear by USP solution concentration method.

Fig. 1. Comparison between ZN-stained direct, USP solution and USP solution+phenol smears. (a) Direct smear showing background debris; (b) USP solution smear with clean background; (c) USP solution+phenol smear showing a clean background.

similar sterilizing effect, although one positive liquid culture was obtained. However, the inherent unstable nature of this chemical precludes its routine use for sample treatment. PhAS treatment for 30 min was not found to be effective in sterilizing the mycobacterial load as ~18 % of the sputum aliquots treated with this disinfectant showed growth of AFB. Treatment of the spiked sputum samples with sodium and calcium hypochlorite caused precipitates on the smear, and also reduced the grading of the smear as well as decreasing the staining intensity of the AFB. Moreover, the poor efficacy of short-term bleach treatment in killing *M. tuberculosis* present in sputum has been demonstrated recently (Githui *et al.*, 2007). Therefore, these disinfectants were not tested further.

Upon satisfactory completion of the first phase study wherein the sterilizing action of phenol did not compromise the performance of USP solution smear microscopy in spiked sputum samples, the study was extended to sputum specimens obtained from tuberculosis subjects. Seventy smear-positive sputum samples were obtained from New Delhi Tuberculosis Centre, India. Their direct smear grade status was decoded after completion of the study and found to vary from '3+' to 'scanty'. USP solution was added to sputum and the uniform slurry so obtained was distributed into two equal portions. One portion was processed by the standard USP solution methodology and the second portion was treated with USP

solution + phenol as described above. Two drops of the resuspended material were examined by ZN staining and the remaining portion was used for viability testing by plating serial dilutions on Middlebrook 7H11 agar. The viable counts obtained from USP solution versus USP solution + phenol-treated samples were compared.

The comparative smear grades obtained by the two methods are given in Table 1 and representative smears are shown in Fig. 1. All 70 USP solution-treated aliquots showed growth of AFB with loads ranging from 1×10^4 to 6×10^7 c.f.u. ml^{-1} , while none of the corresponding aliquots treated with USP solution + phenol yielded any growth of AFB at the end of 8 weeks of incubation (data not shown). Importantly, phenol-mediated disinfection did not compromise smear microscopy at all. This study demonstrates that phenol is tuberculocidal even in the presence of USP solution and organic matter in sputum. Standard safety precautions while handling phenol need to be observed (<http://www.osha.gov/SLTC/healthguidelines/phenol/recognition.html>). We recommend that sputum treatment with USP solution + phenol can be effectively used to deactivate *M. tuberculosis* and provide sensitive smear microscopy. Furthermore, because the USP solution method is compatible with all kinds of pulmonary and extrapulmonary samples (Chakravorty & Tyagi, 2005), its use in combination with phenol is expected to provide safety to laboratory personnel engaged in the handling of all types of

infectious material containing pathogenic mycobacteria in basic laboratory settings.

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Allen, B. W. (1981). Survival of tubercle bacilli in heat-fixed sputum smears. *J Clin Pathol* **34**, 719–722.

Baussano, I., Nunn, P., Williams, B., Pivetta, E., Bugiani, M. & Scano, F. (2011). Tuberculosis among health care workers. *Emerg Infect Dis* **17**, 488–494.

Best, M., Sattar, S. A., Springthorpe, V. S. & Kennedy, M. E. (1988). Comparative mycobactericidal efficacy of chemical disinfectants in suspension and carrier tests. *Appl Environ Microbiol* **54**, 2856–2858.

Best, M., Sattar, S. A., Springthorpe, V. S. & Kennedy, M. E. (1990). Efficacies of selected disinfectants against *Mycobacterium tuberculosis*. *J Clin Microbiol* **28**, 2234–2239.

Chakravorty, S. & Tyagi, J. S. (2005). Novel multipurpose methodology for detection of mycobacteria in pulmonary and extrapulmonary specimens by smear microscopy, culture, and PCR. *J Clin Microbiol* **43**, 2697–2702.

Chedore, P., Th'ng, C., Nolan, D. H., Churchwell, G. M., Sieffert, D. E., Hale, Y. M. & Jamieson, F. (2002). Method for inactivating and fixing unstained smear preparations of *Mycobacterium tuberculosis*

for improved laboratory safety. *J Clin Microbiol* **40**, 4077–4080.

Githui, W. A., Matu, S. W., Tunge, N. & Juma, E. (2007). Biocidal effect of bleach on *Mycobacterium tuberculosis*: a safety measure. *Int J Tuberc Lung Dis* **11**, 798–802.

Hall, L., Otter, J. A., Chewins, J. & Wengenack, N. L. (2007). Use of hydrogen peroxide vapor for deactivation of *Mycobacterium tuberculosis* in a biological safety cabinet and a room. *J Clin Microbiol* **45**, 810–815.

Hegna, I. K. (1977). An examination of the effect of three phenolic disinfectants on *Mycobacterium tuberculosis*. *J Appl Bacteriol* **43**, 183–187.

Rikimaru, T., Kondo, M., Kajimura, K., Hashimoto, K., Oyamada, K., Miyazaki, S., Sagawa, K., Aizawa, H. & Oizumi, K. (2002). Efficacy of common antiseptics against multidrug-resistant *Mycobacterium*

tuberculosis. *Int J Tuberc Lung Dis* **6**, 763–770.

Selvakumar, N., Rahman, F., Garg, R., Rajasekaran, S., Mohan, N. S., Thyagarajan, K., Sundaram, V., Santha, T., Frieden, T. R. & Narayanan, P. R. (2002). Evaluation of the phenol ammonium sulfate sedimentation smear microscopy method for diagnosis of pulmonary tuberculosis. *J Clin Microbiol* **40**, 3017–3020.

Steingart, K. R., Ng, V., Henry, M., Hopewell, P. C., Ramsay, A., Cunningham, J., Urbanczik, R., Perkins, M. D., Aziz, M. A. & Pai, M. (2006). Sputum processing methods to improve the sensitivity of smear microscopy for tuberculosis: a systematic review. *Lancet Infect Dis* **6**, 664–674.

WHO (2006). Tuberculosis – grading of microscopy smears. In *Guidelines on Standard Operating Procedures for Microbiology*, chapter 17. Geneva: World Health Organization. http://www.searo.who.int/en/Section10/Section17/Section53/Section482_1799.htm