Detection of Acid-Fast Bacilli in Postlysis Debris of Clinical Specimens
Improves the Reliability of PCR

Limitations in the sensitivity and speed of conventional methods have triggered the increased use of nucleic acid amplification technologies for diagnosing tuberculosis. We recently described the universal sample processing (USP) method, which utilizes PCR, smear microscopy, and culture for diagnosing tuberculosis (3). Here we compared the efficiency of the USP method for isolation of DNA (referred to herein as method 1 and described in reference 3) with that of other methods in contemporary use, namely, the guanidinium thiocyanate-silica method (referred to herein as method 2 and described in reference 2), the Chelex-detergent method (referred to herein as method 3 and described in reference 4), and a method involving boiling mycobacteria in the presence of 0.1% Triton X-100 (referred to herein as method 4). In method 1, the sputum sample bacterial pellet was treated with USP solution (4 to 6 M guanidinium hydrochloride, 50 mM Tris-Cl [pH 7.5], 25 mM EDTA, 0.5% sarcosyl, 0.1 to 0.2 M β-mercaptoethanol) as described previously, after which sterile water or 68 mM phosphate buffer (pH 6.8) was added and the sample was centrifuged at 5,000 to 6,000 × g at room temperature for 10 to 15 min. The USP treated sputum sediment/particle pellet was washed with water, and the DNA was isolated by boiling the washed sediment in the presence of a solution containing 10% Chelex 100 resin, 0.03% Triton X-100, and 0.3% Tween 20 as described previously (3a).

Serial dilutions (up to 10⁶-fold dilutions) of a Mycobacterium tuberculosis culture were prepared in five replicate sets. The prelysis bacterial load in each dilution (prelysis smear microscopy status) ranged from 3+ to negative. DNA was isolated from the remaining four dilution series according to methods 1 to 4, and its recovery was assessed by IS6110 PCR (5). The postlysis bacterial load was also determined by smear microscopy of equivalent volumes of processed material. The four methods were graded for PCR amplification efficiency: method 1 > method 2 > method 3 > method 4 (Fig. 1). Although exposure of tubercle bacilli to a chaotrope (in methods 1 and 2) resulted in a marginal reduction in the prelysis bacterial load (compare the prelysis smear status shown in panels A and B versus that shown in panels C and D of Fig. 1), maximal amplifiable DNA was also recovered by methods 1 and 2. The superior performance of the chaotrope-based procedures is attributed to the superior bacterial lysis and removal of PCR inhibitors by partial stripping of protein and glycopeptidolipid moieties from the bacterial surface. Surprisingly, postlysis debris smears were positive for acid-fast bacilli (AFB), and the smears’ grade status was inversely proportional to PCR efficiency. Detection of AFB in the leftover debris implied that the mycobacteria were only partially lysed, perhaps due to the presence of an exceedingly tough cell wall.

This novel observation was tested next in clinical settings. In a blinded study of sputum specimens (n = 120; number of tuberculosis cases = 91; number of controls = 29), samples were subjected to smear microscopy and PCR (20% and 80% of sample, respectively) according to the USP methodology (3). Postlysis pellets were also examined for AFB by smear microscopy. A total of 86 and 88 samples were positive by initial smear microscopy and PCR, respectively, and 78 postlysis pellets contained AFB. The postlysis smear test was evaluated next with samples of low bacterial load (21 pleural effusions from 18 tuberculous and 3 nontuberculous subjects), i.e., samples that presented more of a challenge. In this series, only one sample was positive by both routine smear microscopy and culture. Fifteen samples (71%) were PCR positive (all from tuberculous subjects); 7 of these postlysis debris samples were also AFB positive. One PCR-negative sample from a tuberculous subject was postlysis smear positive, indicating that postlysis smear microscopy helped to establish the diagnosis owing to the sheer amount of specimen used for microscopy.

The efficient removal of extraneous material by the USP method provides an unique, and hitherto unexplored, opportunity to use postlysis debris for smear microscopy, in contrast to other mycobacterial DNA isolation methods wherein postlysis debris cannot be examined either because of the retention of excessive amounts of counterstaining material or because the debris is traditionally discarded. This simple and inexpensive technique provides an additional test to the clinical microbiologist to confirm PCR-based reporting of tuberculosis, and we believe that detection of AFB in postlysis USP pellets can increase the reliability of PCR results (by minimizing PCR false positivity due to carryover/cross contamination by DNA),
particularly for those samples that are initially smear-negative and also negative by culture.

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REFERENCES


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