Safe *Mycobacterium tuberculosis* DNA Extraction Method That Does Not Compromise Integrity

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Concern has been raised about the efficacy of the heat killing of mycobacteria during the isolation of DNA. We demonstrate a method that allows for the efficient killing of *Mycobacterium tuberculosis* without compromising DNA integrity for subsequent molecular investigation. This method reduces the risk of infection to the research scientist.

The development of an internationally standardized method for the DNA fingerprinting of clinical isolates of *Mycobacterium tuberculosis* (6) has enabled scientists to gain insight into the disease dynamics of the tuberculosis epidemic in different settings throughout the world. Furthermore, comparison of databases has allowed for the investigation of the global tuberculosis epidemic (2, 7). Since the development of the DNA fingerprinting technique, numerous reports have raised concern about the safety of the DNA extraction procedure (1, 3, 5). These reports have investigated the viability of *M. tuberculosis* bacilli during different stages of DNA extraction according to the standardized protocol. The findings are contradictory, with certain laboratories reporting that the standardized method is safe (1, 3), while others have reported that the mycobacteria may persist after heat killing and may remain viable even in the interphase during phenol extraction (5). This has raised the concern that the extraction procedure could put scientists at risk of infection (5). To limit such a possibility, it was suggested that the procedure should be conducted under biosafety level 3 conditions (5). Such a step would further complicate an already time-consuming and laborious method and would largely limit DNA fingerprinting to highly specialized laboratories, possibly compromising research in high-burden countries.

In our laboratory, we have developed an alternative method for the extraction of high-quality *M. tuberculosis* DNA for DNA fingerprinting in molecular epidemiological studies. In this procedure, each clinical isolate of *M. tuberculosis* was inoculated under biosafety level 3 conditions onto two Lowenstein-Jensen (L-J) slants (10 ml L-J medium in 25-ml Bijiou bottles) and incubated at 37°C with weekly aeration until confluent growth was observed. Thereafter, the outer surface of each L-J slant bottle was sterilized by swabbing with 5% Hy-

colin (William Pearson Chemicals, United Kingdom). Twenty-four L-J slants with tightly sealed caps were then placed in a stainless steel rack within an unsealed biosafety autoclave bag (305 by 660 mm; Sterilin, United Kingdom) and transferred to a prewarmed circulating fan oven (580 by 540 by 510 mm) at 80°C for 1 h to ensure heat killing. Three milliliters of extraction buffer (5% sodium glutamate, 50 mM Tris-HCl [pH 7.4], and 25 mM EDTA) was added to each slant in a biosafety class 2 laminar flow hood, and the colonies were gently scraped from the solid medium with a disposable 10- μ l loop. The suspension from both L-J slants was then transferred to a sterile 50-ml polypropylene Falcon tube containing approximately 20 glass beads (diameter, 4 mm) and vigorously vortexed to disrupt all colonies. Lysozyme (25 mg; Roche, Germany) and RNase A (50 μ g; Roche, Germany) (preheated to remove DNases) were added, and the suspension was incubated after gentle mixing for 2 h at 37°C. Thereafter, 600 μ l of 10 $\times$ proteinase K buffer (5% sodium dodecyl sulfate, 100 mM Tris-HCl [pH 7.8], 50 mM EDTA) and 1.5 mg of proteinase K (Roche, Germany) were added and the suspension was incubated at 45°C for 16 h. An equal volume of phenol-chloroform-isoamyl alcohol (25/24/1) was then added (standard fume cabinet) and intermittently mixed over a period of 2 h at room temperature. Following centrifugation at 3,000 $\times$ g for 20 min, the resultant aqueous phase was reextracted with an equal volume of chloroform-isoamyl alcohol (24/1) and centrifuged as described above. The resultant DNA was then precipitated with the addition of 3 M sodium acetate (pH 5.2) (600 μ l) and an equal volume of isopropanol and immediately collected on a fine glass rod. The DNA was washed in 70% ethanol and allowed to air dry at room temperature. The purified DNA was redissolved in 300 μ l TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). The DNA (6 μ g) was then subjected to PvuII restriction digestion (5 units per μ g DNA) in a volume of 100 μ l, according to the manufacturer's (New England Biolabs) instructions. Restricted DNA was then electrophoretically fractionated in 0.8% agarose (1 $\times$ Tris-borate-EDTA, pH 8.3) and Southern transferred to Hybond N+ (Amersham, United Kingdom) before hybridization with an enhanced-chemoluminescent IS6110 probe (8).

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FIG. 1. IS6110 DNA fingerprints of clinical *M. tuberculosis* isolates. *M. tuberculosis* cultures on L-J slants were subjected to heat killing at 80°C for either 1 h (lanes 2, 6, 7, 8, 9, 12, 13, 14, 16, and 18) or 2 h (lanes 1, 3, 4, 5, 10, 11, 15, and 17). Thereafter, genomic DNA was extracted using the described method, restricted with PvuII, and electrophoretically fractionated in 0.8% agarose (1× Tris-borate-EDTA, pH 8.3). The DNA was Southern transferred to Hybond N+, and the IS6110-containing fragments were visualized by autoradiography after hybridization with an enhanced-chemoluminescent IS6110 probe (8).

The heat-killing step is critical in ensuring safety and containment. The standardized method recommends the heat killing of *M. tuberculosis* suspended in TE by heating at 80°C for 20 min (1, 3, 5). In the procedure described above, we circumvent the transfer of infectious bacteria prior to heat killing by incubating the entire L-J slant at 80°C for 60 min. To test the efficiency of mycobacterial killing, duplicate L-J slant cultures from 48 patients (96 slants) were subjected to heat killing. An aliquot (60 µl) of the resuspended bacteria (pooled from two heat-killed L-J slants) was diluted in Tween 80 saline (0.0001% Tween 80, 0.084 M NaCl) and transferred onto a fresh L-J slant. The slants were incubated horizontally for 24 h, after which they were incubated in the upright position at 37°C with weekly aeration for up to 8 weeks. L-J slants were read weekly by two persons independently. Positive growth was scored if a single colony was visible and contained acid-fast bacilli upon staining with Ziehl-Neelsen. Two cultures were found to be positive after 1 week of incubation and were later confirmed to be *Mycobacterium fortuitum* by sequencing of the 16S ribosomal gene (4). This suggests that this species is resilient to heat killing at 80°C. In contrast, after 7 weeks of incubation, only three *M. tuberculosis* colonies were visible on a single L-J slant, suggesting that three bacilli had survived the heat-killing step in this culture. No further growth was observed on any of the remaining 45 L-J slants after 10 weeks of incubation.

The prolonged heating step used in this method did not

impede the integrity of the DNA, as demonstrated through subsequent molecular investigations using IS6110 DNA fingerprinting (Fig. 1). In support of this finding, we used a similar heat-killing step prior to the isolation of DNA of a sufficient length for the construction of a fosmid library for whole-genome sequencing of a clinical isolate (http://www.broad.mit.edu/annotation/microbes/mycobacterium_tuberculosis_f11). Heat killing for a period of 2 h did not grossly impact the integrity of the DNA, as background smears could not be detected on IS6110 DNA fingerprinting (Fig. 1).

In summary, we conclude that the described method significantly reduces the risk of infection to the research scientist without compromising DNA integrity.

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