Extraction of *Mycobacterium tuberculosis* DNA: a Question of Containment

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DNA fingerprinting of *Mycobacterium tuberculosis* by IS6110 restriction fragment length polymorphism analysis requires substantial high-quality DNA. We demonstrated that, despite extraction treatments that might be expected to inactivate this organism, *M. tuberculosis* remained viable during this process. These data suggest that the extraction of *M. tuberculosis* DNA should be performed within containment until complete.

The standard method employed for DNA fingerprinting of *Mycobacterium tuberculosis* is IS6110 restriction fragment length polymorphism. This modality requires a large quantity of high-molecular-weight DNA (5–7). The DNA extraction protocols currently used for this purpose are based on chemical and enzymatic lysis of the bacterial cells followed by a chloroform-isooamyl alcohol-based DNA extraction, a somewhat lengthy process that raises methodological and biosafety issues. The cultivation of *M. tuberculosis* requires a biosafety level 3 containment facility and up to 4 weeks of culture time. The extraction necessitates a 2- to 3-day protocol harsh enough to lyse the bacteria yet sufficiently gentle to prevent DNA shearing.

Due to the cumbersome nature of extraction protocols, there has been interest in determining at which point *M. tuberculosis* preparations can be considered inactivated and thus be safely removed from containment. Heating of the culture is widely used, and at least one report has deemed heating at 80°C as sufficient for inactivation (3). However, other reports have raised concerns as to the efficacy of heating (8) and even of further treatment with a combination of lysozyme and proteinase K (2). As such, the question of where and when these preparations may be safely manipulated remains unanswered. It is vital to address this issue, as improper inactivation and premature transfer of extraction mixtures from containment could result in the unnecessary exposure of laboratory personnel to *M. tuberculosis*.

Here, the DNA from clinical *M. tuberculosis* isolates was extracted using standard protocols (all reagents were from Sigma-Aldrich unless noted). In brief, isolates were inoculated onto Lowenstein-Jensen (L-J) slants, and when luxuriant growth was apparent, all visible colonies were collected into 500 μl Tris-EDTA buffer, pH 8.0, and heated for 20 min at 80°C. Lysozyme was then added to each tube (final concentration, 1 mg/ml), followed by incubation at 37°C for 2 h. Ten percent sodium dodecyl sulfate (final concentration, 1.1%) and proteinase K (final concentration, 0.2 mg/ml; Promega Inc.) were then added, and the tubes were vortexed gently and incubated for 20 min at 65°C. A mixture of N-acetyl-N,N,N-trimethyl ammonium bromide (CTAB; final concentration, 40 mM) and NaCl (final concentration, 0.1 M) was added, followed immediately by the addition of NaCl alone (final concentration, 0.6 M). The tubes were then vortexed until the suspension turned milky and were incubated for 10 min at 65°C. Seven hundred fifty microliters of chloroform-isooamyl alcohol (24:1) was added to each tube, and the tubes were vortexed and then centrifuged in a microcentrifuge at 13,000 rpm for 5 min at room temperature. The genomic DNA present in the resulting aqueous phase was then isolated by ethanol precipitation as previously described (1) and resuspended in 30 μl nuclease-free water.

To determine at which point inactivation of *M. tuberculosis* was achieved, 0.1 ml of the extraction mixture from 35 isolates obtained following treatment with 40 mM CTAB and 0.1 M NaCl-0.6 M NaCl (CTAB-NaCl) was inoculated directly onto both an L-J slant and into BACTEC 12B medium (BD Microbiology Systems). Additionally, in a second set of experiments, subsequent to chloroform-isooamyl alcohol extraction and centrifugation, a loopful of the interface obtained from 41 isolates was inoculated onto an L-J slant. L-J slants were incubated for 8 weeks at 36°C in an atmosphere of 5% CO2 and examined weekly for growth. BACTEC bottles were incubated for 8 weeks at 36°C, and radiometric readings were taken weekly.

Table 1 illustrates that, subsequent to treatment with CTAB-NaCl, 77% of extraction mixtures contained viable *M. tuberculosis* cells, as indicated by growth on L-J medium (median time to detection, 17.5 days). Notably, only 20% showed...
growth when incubated in BACTEC 12B medium (median time to detection, 35 days). We think it unlikely that our results are due to laboratory contamination, as we manipulated small volumes of cultures, samples were inoculated on five different days, all quality control parameters of all media used were met at the time of these experiments, and BACTEC readings included two uninoculated vials at the end that remained negative throughout. Contrary to previous reports (3, 8) these data indicate that neither heating at 80°C nor treatment with lysozyme and proteinase K reliably inactivates M. tuberculosis. In addition, this study confirms previously published data that subsequent to treatment with the chemicals used in this protocol, the growth of M. tuberculosis is better supported by traditional L-J medium than by BACTEC 12B medium (2). These data may seem counterintuitive, as most reports had previously indicated that the growth of M. tuberculosis is supported to a greater extent by BACTEC 12B medium than L-J slants (4). Without any direct evidence, we postulate that the chemicals used in this protocol may persist within the liquid milieu of the BACTEC 12B medium, thus inhibiting the growth and/or detection of viable M. tuberculosis cells. In contrast, the same chemicals might either evaporate from the surface of the solid L-J medium or be adsorbed away from the surface, reducing their impact on growth. As such, it would appear that in order to determine the viability of M. tuberculosis after treatment with the chemicals required for DNA extraction using this protocol, culture on traditional L-J medium is superior.

Despite treatment with organic solvents, 5% of isolates (2/41) remained viable in the interface obtained after treatment with chloroform-isooamyl alcohol. Growth was present at 4 weeks on L-J slants and confirmed by subculture to BACTEC 12B medium. That a few isolates retained the ability to grow after such extensive treatment may reflect the amount of inoculum extracted or, alternatively, be related to some innate capacity of those particular isolates. Regardless, the preparation of sufficient, high-quality genomic DNA suitable for restriction fragment length polymorphism analysis requires a large number of M. tuberculosis cells, and in our experience, viable bacteria were detected until the final step in the protocol.

In summary, these results indicate that standard DNA extraction protocols do not completely inactivate M. tuberculosis. To avoid the unnecessary exposure of laboratory personnel to this virulent organism, we suggest that the extraction of M. tuberculosis DNA be performed within containment until complete. In order to extract sufficient DNA for analysis, the options appear to be working from a large single culture where live bacteria may persist until the end of the protocol or extracting lesser amounts of DNA from repeated smaller cultures. From a risk assessment point of view, it can be argued that the probability of exposure increases with the number of occasions that one is exposed, so that the extraction of a large inoculum at one time would appear preferable.

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REFERENCES