

SHORT REPORT

The efficacy of the heat killing of *Mycobacterium tuberculosis*

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There is concern that current procedures for the heat inactivation of *Mycobacterium tuberculosis* may not be adequate. This raises serious safety issues for laboratory staff performing molecular investigations such as IS6110 restriction fragment length polymorphism typing. This paper confirms that the protocol of van Embden *et al*, as performed routinely in this laboratory, is safe and effective for the heat inactivation of *M tuberculosis*. This procedure involves complete immersion of a tube containing a suspension of one loopfull of growth in a water bath at 80°C for 20 minutes. Seventy four isolates were included in this investigation. Despite prolonged incubation for 20 weeks, none of the heat killed *M tuberculosis* suspensions produced visible colonies or gave a positive growth signal from liquid culture. This method did not affect the integrity of the DNA for subsequent molecular investigations.

IS6110 restriction fragment length polymorphism analysis is considered to be the "gold standard" typing method for DNA fingerprinting of *Mycobacterium tuberculosis*.¹ Before DNA extraction, *M tuberculosis* must be heat inactivated to render it safe for manipulation outwith a containment level 3 facility. Two reports have raised concerns that some heat killing procedures used for the inactivation of *M tuberculosis* are not reliably effective. This may put laboratory workers using molecular techniques at risk of laboratory acquired infection (P Bemer-Melchior *et al*. Transmission of *Mycobacterium tuberculosis* in a mycobacteriology laboratory. Presented at the 5th International Conference on the Prevention of Infection, 1998). Zwadyk and colleagues² first suggested that temperatures below 100°C do not consistently kill *M tuberculosis*. They showed survival of 50% and 25% of the organisms after heat inactivation at 95°C in a dry heat block for 20 and 30 minutes, respectively. These findings were confirmed by Bemer-Melchior and Drugeon,³ who investigated several different inactivation protocols involving heat killing at either 80°C for 20 minutes or 100°C for five minutes, followed by either lysozyme (0.5 mg/ml) or a combined proteinase K (0.4 mg/ml) and lysozyme (0.5 mg/ml) digestion. They reported the growth of *M tuberculosis* in 80% of subcultures on Löwenstein-Jensen (L-J) medium after heat inactivation at 80°C for 20 minutes and treatment with lysozyme and in 10% after heat inactivation at 80°C for 20 minutes and treatment with both lysozyme and proteinase K. In addition, Zwadyk and colleagues² showed that increasing exposure time did not always correlate with a decrease in viability. In view of these findings, we investigated the suitability of the heat killing procedure currently used in our laboratory. This assessment involved detailed attention to procedures used during heat inactivation and included extended viability checks before and after heat inactivation.

"Two reports have raised concerns that some heat killing procedures used for the inactivation of *Mycobacterium tuberculosis* are not reliably effective"

METHODS

A total of 74 *M tuberculosis* isolates were used in the heat inactivation experiments. For 50 isolates, viability checks were performed after heat inactivation only: a loopfull of organism was removed from a solid culture slope and added to 400 µl of Tris/EDTA (TE) buffer in a 1.5 ml eppendorf tube. This tube was submerged (using a lead weight) in a water bath preheated and maintained at 80°C for 20 minutes. To check that the bacteria had been inactivated, 100 µl of the heat killed suspension was used to inoculate each of two slants of modified L-J egg medium (one containing glycerol and one containing pyruvate as growth supplements) and incubated at 37°C for 20 weeks. The remaining 200 µl was used to inoculate a MB/BacT bottle, which was then incubated in a MB/BacT automated mycobacterial liquid culture system (BioMerieux UK Limited, Basingstoke, UK). The MB/BacT bottles were incubated at 37°C for six weeks in the first instance, as recommended, then re-loaded after this period to achieve a total incubation of 12 weeks. Although the recommended inoculum for this system is 500 µl of clinical sample, a reduced inoculum of concentrated organisms was considered to be acceptable.

The other 24 isolates were checked for viability before and after heat inactivation: a loopfull of organism was removed from a solid culture slope and resuspended in 500 µl of TE buffer. A 100 µl aliquot was removed immediately to inoculate glycerol containing egg culture medium. The residual suspension was then heat killed at 80°C for 20 minutes, as described previously. A 200 µl aliquot of the heat killed suspension was used to inoculate both glycerol and pyruvate containing egg media. All solid cultures were incubated at 37°C and checked for growth on a weekly basis for a total of 20 weeks.

RESULTS

None of the submerged heat killed samples produced growth on solid culture medium. All of the 24 mycobacteria inoculated on to solid egg culture medium before heat inactivation produced visible colonies after incubation, confirming their earlier viability.

Extended incubation of MB/BacT liquid cultures for 12 weeks yielded two positive signals. Liquid culture medium from both MB/BacT bottles was used to inoculate slopes of modified L-J egg media containing glycerol and pyruvate, which were incubated at 37°C for 20 weeks. In addition, a blood agar slope was inoculated to check for bacterial contamination. After overnight incubation at 37°C, the blood agar slope showed no visible growth and the egg media failed

Abbreviations: LJ, Löwenstein-Jensen; TE, Tris/EDTA

to show growth after extended incubation. Thus, the MB/BacT positive signals were deemed to be false positive results attributed to the breakdown of the medium, a phenomenon previously observed in our laboratory when MB/BacT bottles inoculated with cerebrospinal fluid are incubated beyond the normal six week period up to a total of 12 weeks (P Claxton, personal communication, 2001).

DISCUSSION

Our study has shown that heat inactivation performed at 80°C for 20 minutes using submerged suspensions of *M tuberculosis* in a water bath renders the samples safe for use by laboratory workers. However, our findings do not support those previously reported. First, Bemer-Melchior and Drugeon³ achieved complete inactivation of *M tuberculosis* in a boiling water bath with fully immersed glass bottles, but not in their experiments using a water bath set at 80°C for 20 minutes. However, it is not clear whether glass bottles were also used in this last experiment, or whether they were fully immersed in the water bath. If screw capped glass bottles were not fully immersed in the water bath, it is possible that some viable mycobacteria were trapped in the lids of the bottles and may have survived. Twenty one of the 40 cultures tested using 80°C for 20 minutes alone were found to be culture positive on L-J medium (after 21–62 days) and 65% were Bactec 12B positive (16–55 days). The growth of some heat killed cultures was reported as late as 90 days after inoculation. Second, Bemer-Melchior and Drugeon do not state the volume of suspension used in their heat inactivation experiments. Zwadyk and colleagues² showed that mycobacteria were more likely to survive heating in larger volumes and when there were higher concentrations of organisms. In summary, they concluded that consistent inactivation of mycobacteria could only be achieved using methods where the tubes were fully immersed in boiling water or in a forced dry air oven set at 100°C. The use of a dry heat block set at 95°C for heat killing allowed sporadic growth of various mycobacteria, although this was attributed to the sample temperature not reaching the set temperature during the 20 minute incubation period. The shape of the wells of a heat block is seldom a close fit to the shape of the sample tubes, and although oil is sometimes used to fill this gap, Zwadyk and colleagues² found this also to be ineffective for the heat inactivation of *M tuberculosis*. This issue is important because commercial molecular tests such as the MTD probe test (Gen-Probe Incorporated, San Diego, USA) use a dry heat block and so this method of inactivation should also be properly validated.

“We have found that it is possible to degrade DNA by excessive heat inactivation (95°C for two hours) so that it is no longer suitable as a polymerase chain reaction template”

Bemer-Melchior and Drugeon³ noted that heat inactivation at 80°C for 20 minutes had a deleterious effect on the isolated DNA, degrading it into small fragments, which electrophoresed as a smear of low molecular weight fragments in agarose gels, rather than as a high molecular weight band, whereas heat inactivation at 100°C for five minutes did not. In a small comparative study using 12 *M tuberculosis* isolates that were heat inactivated using both methods, the integrity of the

Take home messages

- The protocol of van Embden *et al*, which involves complete immersion of a tube containing a suspension of one loopfull of growth in a water bath at 80°C for 20 minutes, is safe and effective for the heat inactivation of *Mycobacterium tuberculosis*
- Despite prolonged incubation for 20 weeks, none of the heat killed *M tuberculosis* suspensions produced visible colonies or gave a positive growth signal from liquid culture
- This method did not affect the integrity of the DNA for subsequent molecular investigations

DNA for subsequent fingerprint analysis was found to be comparable, independent of the method of heat inactivation used. However, we have found that it is possible to degrade DNA by excessive heat inactivation (95°C for two hours) so that it is no longer suitable as a polymerase chain reaction template. Thus, it is clear that a balance is required between sample safety and the preservation of DNA for subsequent molecular investigations.

As a result of this work, we have revised our quality control procedures to incorporate a positive control test with every batch of isolates that are heat inactivated at 80°C for 20 minutes in a water bath. A control strain of *M tuberculosis* is inoculated on to solid egg medium before and after heat inactivation and these samples are incubated at 37°C for 20 weeks to assess viability.

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