The efficacy of the heat killing of Mycobacterium tuberculosis

C Doig, A L Seagar, B Watt, K J Forbes

SHORT REPORT

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 loopful 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.

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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 loopful 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 heat killed, 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 loopful 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 to produce visible colonies.

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 Drugeon 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 colleagues 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 colleagues 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 this method of inactivation should also be properly validated.

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Bemer-Melchior and Drugeon 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 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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