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Pre-fixation of virulent *Mycobacterium tuberculosis* with glutaraldehyde preserves exquisite ultrastructure on transmission electron microscopy through cryofixation and freeze-substitution with osmium-acetone at ultralow temperature

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ABSTRACT

Sample preparations for transmission electron microscopy of virulent *Mycobacterium tuberculosis* are usually performed with chemical fixation using glutaraldehyde (GA) in a biosafety area followed by post-fixation with aqueous osmium tetroxide (OT) in a conventional laboratory outside the biosafety area. Freeze-substitution with osmium-acetone (OA) at ultralow temperature (−85 °C) has been shown to provide high quality final images and preserves cellular structures intact. However, some preparation procedures for freeze-substitution often require large fixed devices for freezing in a special laboratory. We have reported a novel freeze-substitution preparation method that can be performed using a portable device in a biosafety cabinet at biosafety level (BSL) 3 areas. Here, as a next step, we examined whether images obtained from rapid freeze-substitution (RFS) after fixation with glutaraldehyde (GA > RFS) are of comparable quality to those obtained using standard RFS. GA > RFS provided excellent preservation of mycobacterial cell ultrastructure, including visualization of cytoplasmic ribosomes, DNA fibers, and the outer membrane. The average number of ribosomes per cubic micrometer counted on RFS and GA > RFS was not significantly different (6987.8 ± 2181.0 and 6888.9 ± 1799.3, respectively). These values were higher, but not significantly so, than those obtained using conventional chemical fixation (5018.7 ± 2511.3). This procedure may be useful for RFS preparation of unculturable mycobacteria strains or virulent strains isolated in laboratories that cannot perform RFS.

1. Introduction

In the field of mycobacterial microscopy, novel procedures are being developed at an increasing pace. For example, microfluidic cultures and time-lapse microscopy have been developed to track the life cycle of a single live cell (Aldridge et al., 2012; Golchin et al., 2012; Wakamoto et al., 2013), and cryo-electron microscopy techniques such as specimen vitrification in ice followed by cryo-electron microscopy of vitreous sections (CEMOVIS), without specimen fixation or staining have been developed to observe fully hydrated bacterial cells (Matias and Beveridge, 2006; Hoffmann et al., 2008; Zuber et al., 2008; Couture-Tosi et al., 2010; Hurbain and Sachse, 2011; Perez-Cruz et al., 2013). Although both procedures provide live images of the intact ultrastructure of biological materials, they are performed without chemical fixation, which limits their use to nonpathogenic bacteria outside of strict biosafety facilities.

In several previous electron microscopy studies of mycobacteria, bacilli grown on solid media were embedded in melted agar and subjected to the impact freezing method (Amako et al., 2003; Paul and Beveridge, 1992, 1994; Takade et al., 2003). In our previous study, we reported a novel rapid freeze-substitution (RFS) procedure that was applicable to highly virulent *Mycobacterium tuberculosis* strains inside a class IIB biosafety cabinet at a biosafety level 3 facility and that met all biosafety requirements (Yamada et al., 2010). In this procedure, agar-embedding steps were excluded in order to preserve the ultrastructure intact, as far as possible. Instead, a small volume of highly concentrated bacillary solution was sandwiched between two single-holed copper grids and immediately immersed in melting propane. The bacilli were quickly fixed and substituted at ultralow temperature (−85 °C) with 2% osmium tetroxide (OsO_4, OT) dissolved in acetone (Yamada et al., 2010, 2012).

In this study, we report that the abovementioned pre-fixation procedure with glutaraldehyde (GA) followed by RFS at ultralow temperature with an osmium-acetone (OA) solution can preserve the exquisite ultrastructure of tubercle bacilli. It had been thought that GA fixation or chemical fixation (CF) performed with a combination of GA and OT

Abbreviations: BSL, biosafety level; CEMOVIS, cryo-electron microscopy of vitreous section; CF, chemical fixation; CF > RFS, rapid freeze-substitution after chemical fixation; GA, glutaraldehyde; GA > RFS, rapid freeze-substitution after glutaraldehyde fixation; MDR, multidrug-resistance; OA, osmium-acetone; OT, osmium tetroxide; PB, phosphate buffer; RFS, rapid freeze-substitution; TEM, transmission electron microscopy; XDR, extensively drug-resistant.

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could cause the ultrastructure to deteriorate. However, we demonstrate here that both pre-fixation of tubercle bacilli with GA before rapid freezing and cryofixation/substitution with OA substitution at ultralow temperature are crucial to preserving the ultrastructure. These results indicate that poor preservation of ultrastructure should be attributed to gradual freezing or OT fixation at a relatively higher temperature (even at 4 °C) than ultralow temperature. In the improved technique reported here, pathogenic bacilli were fixed with GA in a biosafety area, and could then be immediately transferred to a conventional laboratory desktop and subjected to rapid freezing and cryofixation/substitution with OA. This procedure is extremely useful for RFS sample preparation of highly biohazardous multidrug-resistant (MDR) or extensively drug-resistant (XDR) isolates, viable but nonculturable isolates, and samples isolated in locations where RFS cannot be performed in situ before transportation to an RFS-compatible laboratory.

2. Materials and methods

2.1. Bacteria

M. tuberculosis H37Rv (ATCC 25618) was cultured with Middlebrook 7H9 (Becton Dickinson, MD, USA) supplemented with oxalic acid, albumin (fraction V), dextrose and catalase (OADC) enrichment (Becton Dickinson, MD, USA), and 0.05% Tween 80 for 2 weeks. One-milliliter aliquots were transferred to sterile microcentrifuge tubes and centrifuged at 10,000 ×*g* for 1 min. Supernatants were discarded, and the remaining pellets were collected in two microcentrifuge tubes. One aliquot of the pellet was used for original RFS and another was either subjected to GA fixation alone or processed using conventional CF with GA and 1% OT and RFS after GA or CF as described below. All steps involving live virulent bacteria were performed in a biosafety cabinet at a biosafety level 3 facility.

2.2. Fixation

2.2.1. Pre-GA fixation and conventional CF

A highly concentrated bacillary pellet obtained as above was fixed with 2.5% GA in 0.1 M phosphate buffer (PB, pH 7.4) at 4 °C for at least 2 h. After centrifugation, the supernatant was discarded and rinsed with PB three times, after which the sample was divided into two microcentrifuge tubes. One part of the sample was subjected to RFS as described below (Fig. 1b) and the other was subjected to 1% aqueous OT fixation as conventional CF at 4 °C for 1 h. After OT fixation, the latter sample was rinsed with PB and one aliquot was subjected to rapid freezing in the same manner as the RFS described below (Fig. 1c) while the other was transferred to absolute acetone and stored in a freezer at −85 °C without rapid freezing. The remaining sample was prepared using conventional CF (Fig. 1d).

2.2.2. RFS

The sandwich method was performed as described (Yamada et al., 2010; Yamaguchi et al., 2003, 2009) and is summarized in Fig. 1a–d. Briefly, volumes of less than 1 μl of the highly concentrated bacillary pellets that were either not chemically fixed or were GA/CF fixed were applied to glow-discharge-treated single-hole copper grids (hole size 0.1 mm diameter; Veco, Eerbeek, Netherlands), then pellets were covered with another grid. Using tweezers, the grids were then immersed in melting propane for 20 s before being placed in the cooling device (Model VFZ-101 Multi Purpose Quick Freezing Device, Vacuum Device Inc., Mito, Ibaraki, Japan). The pair of grids was transferred, detached in liquid nitrogen, and immersed quickly into 2% OA solution and cooled in the abovementioned freezing device. Then, samples were transferred from the biosafety facility and placed in a freezer at −85 °C for several days, over which time the temperature was gradually raised to room temperature. Then, the OA solution was discarded and the samples were washed with absolute acetone three times at room temperature.

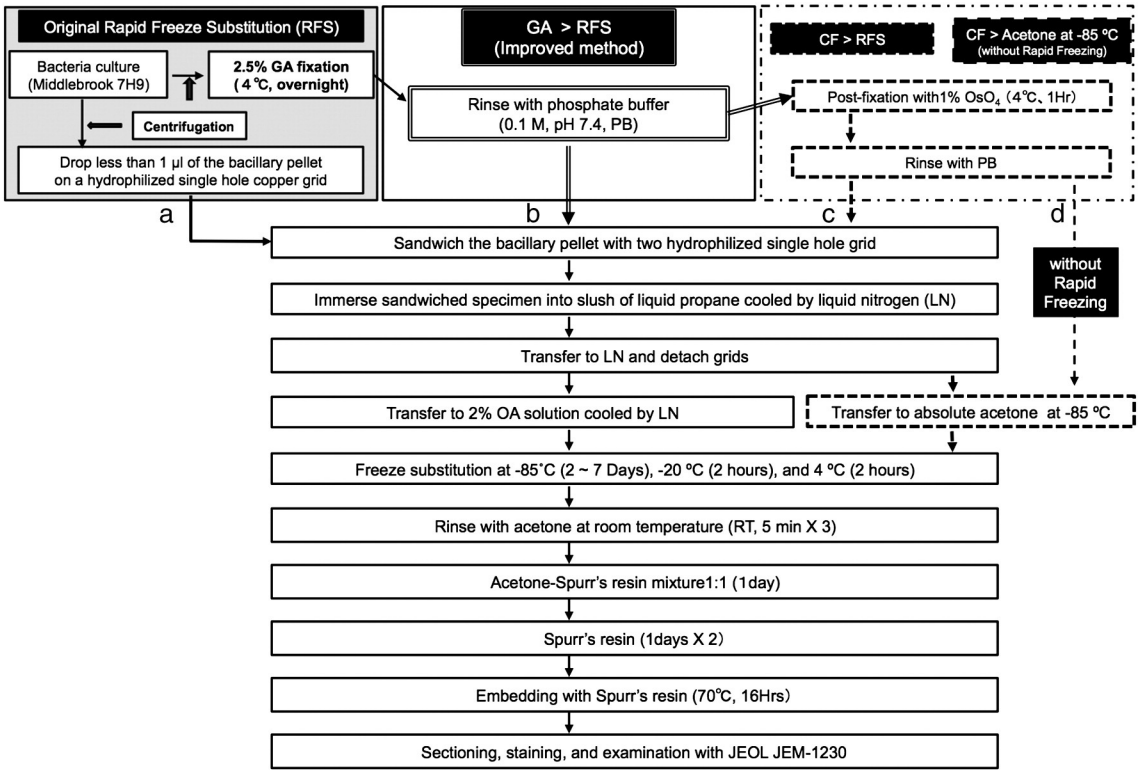

Fig. 1. Flowchart of the protocols for the four sample preparations used. (a) Original RFS, (b) GA > RFS, (c) CF > RFS, and (d) CF > acetone at −85 °C without rapid freezing. The steps contained in the shaded rectangle (top left) were performed in a type IIA biosafety cabinet at a biosafety level 3 facility. After fixation through exposure to 2% OA or 2.5% glutaraldehyde, the bacillary samples were transferred outside of the biosafety facility.