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Research Note

RAPID PROCEDURE FOR MYCOBACTERIAL PLASMIDS VISUALIZATION

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The isolation of DNA plasmids from mycobacteria has been hindered by the thick lipid-rich cell wall and few copies of mycobacterial plasmids. A suitable lysis procedure for mycobacterial cells must be efficient enough to rupture the cell wall without any DNA damage. Also achievement of appropriate amount of extrachromosomal DNA for identification on agarose gel electrophoresis and further studies is essential.

To overcome the above mentioned difficulties, different laboratory techniques were compared in order to establish a final protocol for screening the mycobacterial strains that harbour plasmids.

Two strains of *Mycobacterium fortuitum* carrying plasmids with different sizes (7.25 and 32.0 Kb in a strain and 18.9 Kb in other) kindly suplied by Dr H. David from Institut Pasteur and 28 clinical isolates of *M. avium* complex were cultured in Middlebrook 7H9 broth or Loewenstein-Jensen. Filter-sterilized D-cycloserine to final concentration of 1 mg/ml was added to the liquid cultures in late exponential phase and the cultures were reincubated for an additional 24 to 48 h. Aproximatelly 80 mg of cells were recovered by centrifugation from 100 ml liquid medium or scraped off from the solid medium.

Three different plasmid DNA isolation procedures were used as described by the authors and are briefly described bellow:

C. I. Kado & S. T. Liu (1981, *J. Bacteriol., 145:* 1365-1373): cells were ressuspended in TE buffer (40 mM Tris-acetate pH 7.9; 2 mM EDTA) followed by the addition of lysis solution (3% SDS, 50 mM Tris-NaOh, pH 12.6). The mixture was kept at 65 °C for 60 min. Then, the bacterial lysate was extracted twice with phenol-chlorophorm (v/v) and centrifuged at 4,500 g for 15 min, at 4 °C. The upper aqueous phase was withdrew directly for eletrophoresis.

Lysis by alkali (J. Sambrook et al., 1989, *Molecular Cloning. A laboratory manual.* vol. 1. 2nd ed. Cold Spring Harbor Lab. Press. N. Y.): cells were ressuspended in solution I (50 mM glicose; 10 mM EDTA; 25 mM Tris, pH 8.0). Proteinase K containing freshly prepared solution II (0.2 N NaOH; 1% SDS, 2 mg/ml proteinase K) was added and the mixture was incubated at 65 °C for 20 min. Then, a neutralizing solution (5 M potassium acetate, pH 4.8) was added, shaken and incubated on ice for 20 min., followed by centrifugation (8,000 g for 20 min, at 4 °C). The supernatant was carefully transfered to a fresh tube and extracted twice with phenol-chlorophorm (v/v) and once with chlorophorm. The aqueous layer was saved, precipitated with 2 volumes of chilled ethanol and kept at –20 °C overnight. The plasmids DNA was recovered by centrifugation at 8,000 g for 30 min, at 0 °C, ressuspended in RNAase (0.1 µg/µl) containing TE buffer, pH 8.0 and incubated for 15 min, at 37 °C.

Mechanical lysis (S. S. Hurley et al., 1987, *J. Clin. Microbiol., 25:* 2227-2229; A. R. Santos et al., *J. Microbiol. Methods,* in press) bacterial cells were deposited in a microfuge tube (1.7 ml) containing 500 µl of Tris-equilibrated phenol. Approximately 750 µl of 0.1 mm glass beads and 400 µl of TE buffer (10 mM Tris; 1 mM EDTA, pH 8.0) were added. Then, the mixture was vigorously shaken in Vortex for 3 min, and centrifugated at 12,000 g for 2 min. The aqueous phase was transfered

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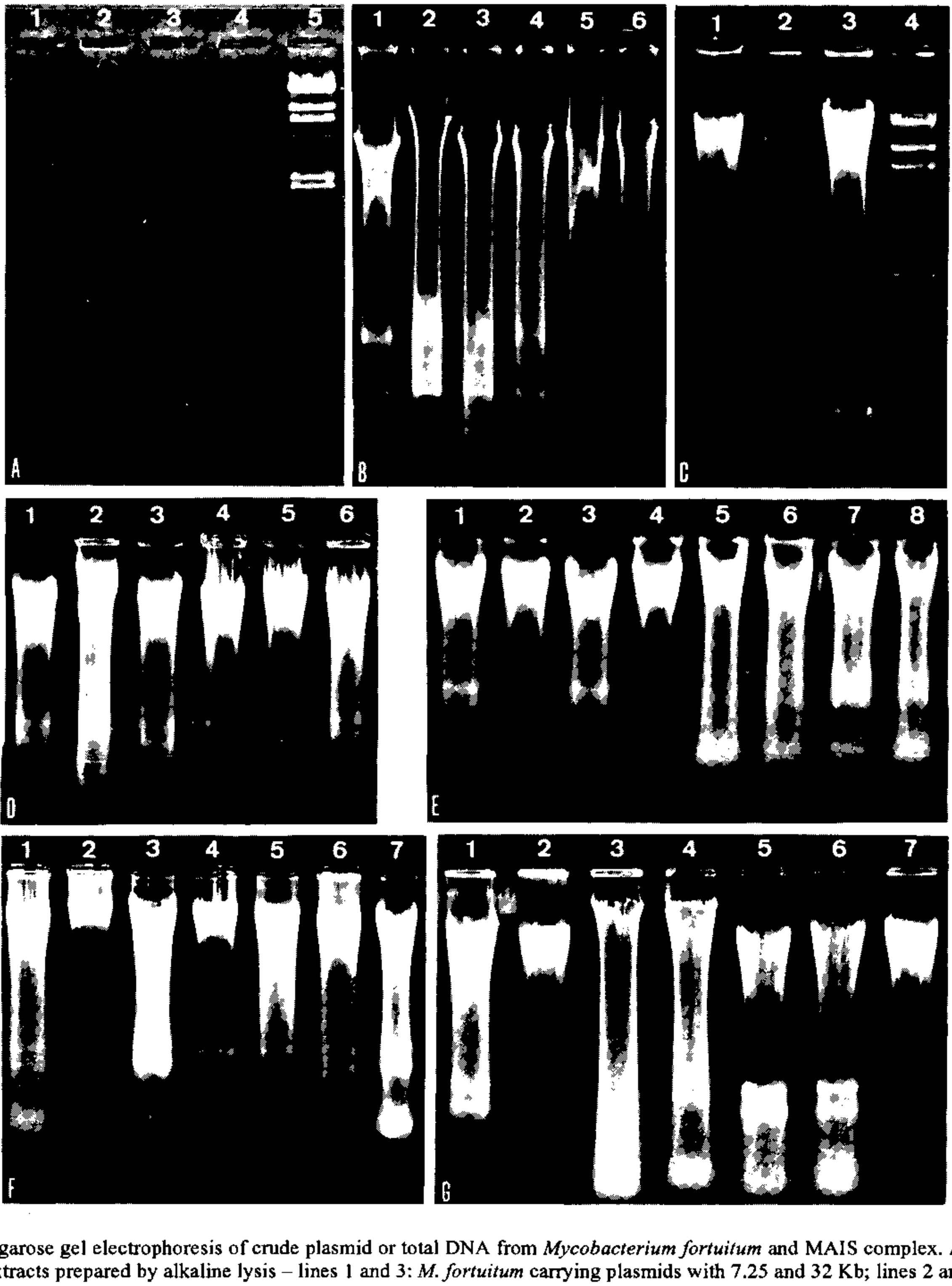

Agarose gel electrophoresis of crude plasmid or total DNA from *Mycobacterium fortuitum* and MAIS complex. A: extracts prepared by alkaline lysis – lines 1 and 3: *M. fortuitum* carrying plasmids with 7.25 and 32 Kb; lines 2 and 4: *M. fortuitum* carrying plasmid with 18.9 Kb; line 5: DNA of lambda/Hind III. B: extracts prepared by mechanical lysis (lines 1 to 4) and Kado and Liu's technique (lines 5 and 6) – lines 1, 3 and 5: *M. fortuitum* carrying plasmids with 7.25 and 32 Kb; lines 2, 4 and 6: *M. fortuitum* carrying plasmid with 18.9 Kb. C: extracts prepared by mechanical lysis – lines 1 to 3: different clinical isolates of MAIS complex; line 4: DNA lambda/HindIII. D, E, F, G: extracts prepared by mechanical lysis from different clinical isolates of MAIS complex.

to a fresh microfuge tube and treated with 1 M $NaClO_4$ and equal volume of phenol-chlorophorm-isoamyl alcohol (25:24:1). The layer phase was again recovered by spun (12,000 g), and precipited in 2 volumes of chilled ethanol. Before new centrifugation (12,000 g for 30 min, at 4 °C), the DNA pellet was resuspended in 150 µl of TE buffer.

Agarose gel electrophoresis: 2 µl of gel-loading buffer (0.25% bromophenol blue; 0.25% xylene cyanol FF; 30% glycerol in water) were diluted in 12 µl aliquots of DNA samples which contained aproximatelly 10, 5 and 2 µg of DNA when proceeding respectively from mechanical lysis, Kado and Liu's technique and lysis by alkali. Electrophoresis was performed with a horizontal apparatus, in 0.7% agarose gel dissolved in TBE buffer (0.045 mM Tris-borato; 1 mM EDTA pH 8.2) and mixed with ethidium bromide (0.5 µl/ml). Gels were run for 3 h at 20 mA, observed under U. V. illumination and photographed with a red filter.

The results showed as expected that chemical methods were less effective on mycobacterial lysis, although they are suitable for the majority of microorganisms. The procedure described by C. I. Kado & S. T. Liu (*loc. cit.*) yielded slight better lysis since a higher concentration of SDS and a long incubation time of the cells with SDS and NaOH were used. This method was employed by others (J. T. Crawfod & J. H. Bates, 1986, *Am. Rev. Resp. Dis., 134:* 659-661; R. S. Stormer & J. O. Falkinham III, 1989, *J. Clin. Microbiol., 27:* 2459-2465) in the screening of plasmids-containing mycobacteria, nevertheless our attemps to apply the method was disapoiting, the extrachromosomal DNA was denatured and it was not possible to visualize any DNA band. The lysis by alkali can be considered a gentle method for mycobacteria. The lysis was minimal and only faint plasmid bands were observed because of the insufficiente amount of available extrachromosomal DNA. The genomic DNA was not fragmented and could be completely eliminated, therefore pure plasmid bands, even though very faint, could be detected.

The physical method was more drastic and yielded very efficient lysis and adequate amount of total DNA: 1.5 µg DNA/mg cells, while the Kado and Liu's technique and lysis by alkali yielded respectively 0.75 and 0.3 µg DNA/mg cells. However the DNA was breaked into fragments of different sizes, generating smears along the gels. Despite the intense degradation of chromosomal DNA, this procedure preserved the plasmids that could be lost in alkaline lysis.

According this procedure we have found a high percentage of plasmids (96%) in clinical MAIS isolates. Also this was an easy to perform method and gave efficient lysis with reliable results. Figure shows the electrophoretic pattern of plasmids isolated with the described methods.

Even though the mechanical lysis showed suitable for rapid screening of plasmids-containing mycobacterial strains, it is necessary to find an additional treatment for total DNA lysate. For further studies it is essential to obtain a purified extract without chromosomal DNA contamination.