Evaluation of Methods for Isolation of DNA from Slowly and Rapidly Growing Mycobacteria[1]

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At present, 71 recognized or proposed species meet the standards for inclusion in the genus *Mycobacterium*. These species are usually grouped into two major divisions, "rapidly growing" and "slowly growing," based on the time required for visible colonies to appear on a solid medium. The appearance of colonies requires less than 7 days for the rapidly growing species and more than 7 days for the slowly growing species. These divisions are very important clinically and in identification schemes. The slowly growing species may cause disease in humans and animals; whereas the rapidly growing species do not ([18, 19, 20]). More than 25 species in the *Mycobacterium* genus, including *M. leprae* and *M. tuberculosis*, may cause disease in humans, and more than six species may cause disease in animals.

Mycobacterial cell walls have been shown to contain type-specific antigenic glycolipids, such as phenolic glycolipids (PGL), glycopeptidolipids (GPL), and trehalose-containing lipo-oligosaccharides (LOS). The compositions of these waxy lipids, mycolic acid-containing cell walls vary depending upon the species of *Mycobacterium* ([1, 2, 7, 21]). Various methods have been described for the extraction of DNA from mycobacteria ([3, 6, 9, 22]). The critical step is lysis of the cells to release DNA, while avoiding its degradation or shearing. Due to the cell-wall thickness, mycobacteria are resistant to enzyme lysis by the commonly described methods. The ability to identify and to type mycobacteria from environmental and clinical sources is limited by the lack of a simple, rapid and efficient method of DNA preparation by which many samples can safely be processed simultaneously ([22]). Since the morphology and the biosynthetic capabilities of mycobacteria vary, depending on cultural conditions and strain differences within a species, the most reliable method of identification would be at the DNA level ([15, 19]).

Although research in molecular biology and recombinant DNA technology has advanced markedly in recent years, the lack of a simple and efficient method for extracting DNA from mycobacteria has lagged behind the need of research. During this study, five methods were used to extract DNA from slowly growing mycobacteria such as *M. leprae* and *M. lepraemurium*, the causative agents of human and murine leprosy, respectively, as well as *M. bovis* BCG, an etiologic agent of tuberculosis. *M. phlei*, a nonpathogen and rapidly growing mycobacterium, was used as a control. The quantity and the quality of these DNAs were analyzed by electrophoresis and restriction endonuclease digestion.

MATERIALS AND METHODS

Sources and growth of mycobacteria

M. bovis BCG substrain Laval was kindly provided by Dr. P. Rousseau (BioVac, Institut Armand-Frappier, Laval, QC, Canada), after being grown on Sauton medium. *M. phlei* was grown on the Lowenstein-Jensen medium at 37°C for a period of 5–7 days ([15]). *M. leprae* were obtained from infected foot pads of nude mice maintained in our laboratory ([12]). *M. leprae* from infected armadillo liver tissue was a gift kindly provided by Dr. A. M. Dhople, Florida Institute of Technology, Melbourne, Florida, U.S.A. *M. lepraemurium* Hawaiian strain used in this study was obtained from Dr. P. Greenburg (La Roche Research Laboratory, Nut-

ley, New Jersey, U.S.A.). The strain was maintained in C3H mice by subcutaneous serial passages every 4–6 months in our laboratory ([13]).

Reagents

Reagents used for isolation of *M. leprae* and *M. lepraemurium* cells from infected animals were those recommended by Draper ([10]) and Clark-Curtiss, *et al.* ([8]). Buffer A contained 150 mM NaCl, 1 mM MgSO$_4$ and 15mM HEPES (N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid), pH 7.2. Buffer B contained 150 mM NaCl, 200 mM Tris buffer, and 1 mM MgSO$_4$, pH 7.2. Buffer C contained 1 ml 10% Tween 80, 0.2 ml of 10% 2-(N-morpholino)ethane-sulfonic acid (MES), and 100 ml distilled water, pH was adjusted to 7.2. All three buffers contained 1 mM benzamidine as an inhibitor of animal cellular proteases. The 30% Percoll gradient contained 30 ml Percoll (Sigma Chemical Co., St. Louis, Missouri U.S.A.) and 70 ml buffer C.

Reagents for DNA extraction and preservation were described by Sambrook, *et al.* ([17]). NET buffer contained 100 mM NaCl, 10 mM EDTA and 10 mM Tris-HCl, pH 7.5. TE buffer contained 1 mM EDTA and 10 mM Tris-HCl, pH 7.5.

The enzymes, such as proteinase K, pronase, lysozyme, DNase, and RNase A were purchased from Boehringer Mannheim (Laval, Canada); the other enzymes, such as subtilisin (protease VIII) and collagenase were purchased from Sigma; *Pfu* DNA polymerase was purchased from Stratagene (La Jolla, California, U.S.A.); *Pst* I was purchased from Pharmacia Biotech, (Baie d'Urfé, Canada).

Isolation and preparation of mycobacterial bacillary suspensions

Isolation of *M. leprae* bacilli from infected nude mouse foot pads and preparation of bacillary suspensions. The method for quantitative recovery of *M. leprae* cells from infected nude mouse foot pads was adapted from a method developed by Franzblau and Hastings ([11]) with some modifications. Briefly 10 g of foot pads were surface decontaminated by using ultraviolet (UV) irradiation, 1% iodine and 70% ethanol. Foot pads were then cut into small pieces with a pair of scissors and triturated thoroughly in a mortar in buffer A. The mixture was passed through a sterile nylon filter into a sterile 250-ml beaker to remove tissue strands, and then the filtrate was centrifuged (Beckman J21 B) at 1200 × g × 10 min at 4°C to remove the tissue debris. The tissue debris was triturated and filtered through a nylon filter once more as described above to further recover the bacilli. The pooled filtrate was centrifuged at 10,000 × g × 15 min at 4°C. The resulting supernatant was carefully removed, without disturbing the pellets, and discarded. The pellets were suspended in 30 ml NET buffer.

Enzymatic digestion of foot-pad tissues and collagen was accomplished by adding 4 mg of collagenase to the flask containing 30 ml of the homogenized mixture in the NET buffer. The flask was incubated at 37°C with gentle shaking for 1 hr. A solution containing 0.5% SDS and 0.1 mg/ml of proteinase K was added and incubated at 56°C for 1 hr. Thereafter, 50 μg/ml of DNase was added to the mixture and further incubated for 1 hr to digest nude mouse DNA. The mixture was then transferred into sterile tubes and centrifuged at 10,000 × g × 15 min. The supernatant fractions were carefully removed. The pellets were washed once with buffer B by centrifugation at 10,000 × g × 15 min. The resulting pellets were suspended in 12 ml of buffer C using a Pyrex tissue grinder.

Further purification of the bacilli in 12 ml of buffer C was accomplished by 30% Percoll gradient density separation ([16]). The suspended cells in 12 ml of buffer C were equally transferred to two sterile Beckman screw-cap centrifuge tubes. To each tube, 24 ml of 30% Percoll were added. The tubes were centrifuged at 20,000 × g × 1 hr at 5°C. The bottom layers were taken and washed twice with buffer C, twice with 0.9% NaCl solution, and twice with PBS buffer. Finally, the pellets containing pure *M. leprae* were frozen at −80°C until used for DNA extraction.

Isolation of *M. leprae* bacilli from infected armadillo liver tissues and preparation of bacillary suspensions. The isolation of *M. leprae* bacilli from infected armadillo liver tissues and preparation of

bacillary suspensions were carried out as described by Clark-Curtiss, *et al.* ([8]).

Isolation of *M. lepraemurium* bacilli from infected C3H mice and preparation of bacillary suspensions. The isolation and preparation of *M. lepraemurium* bacilli from C3H mice lepromas was carried out as described in isolation of *M. leprae* bacilli from infected nude mouse foot pads.

Preparation of *M. bovis* BCG bacillary suspensions. *M. bovis* BCG was grown on the synthetic Sauton medium for 14 days. The bacilli were removed carefully from the surface of the medium. They were washed twice with PBS buffer by centrifugation at $10,000 \times g \times 10$ min, and the pellets were frozen at $-80°C$ until used for DNA extraction.

Preparation of *M. phlei* bacillary suspensions. Actively growing cells of *M. phlei* on the surface of Lowenstein-Jensen medium were carefully removed. They were washed twice with PBS buffer by centrifugation at $10,000 \times g \times 10$ min, and the pellets were frozen at $-80°C$ until used for DNA extraction.

DNA extraction methods

Five different DNA extraction methods were compared for their ability to provide quality template DNA for endonuclease digestion. Before the DNA extraction, the mycobacterial cells were incubated at $70°C$ for 1 hr to minimize any biological hazard. Cells (about 50 mg wet weight) of each mycobacterium were harvested from suspension by centrifugation at $12,000 \times g \times 5$ min, and resuspended in Eppendorf tubes containing 1 ml NET buffer to be used in the following methods.

Intensive enzymic digestion method (M1). The intensive enzymic digestion method was carried out according to Visuvanathan, *et al.* ([23]) with modifications. To 1 ml of the cell suspension, 5 mg of subtilicin was added and the mixture was incubated at $37°C$ for 18 hr. Then 20 mg of lysozyme was added and the mixture was incubated at $50°C$ for 5 hr. Lysis was completed by the addition of 3% SDS and 1.5 mg of pronase. The mixture was further incubated for 20 hr, with the addition of a further 1.5 mg of pronase after 12–15 hr.

Two-min mechanical glass-bead disruption method (M2). The 2-min mechanical glass-bead disruption method was carried out as described by Via and Falkinham ([22]) and Jacobs, *et al.* ([14]) with some modifications. Briefly, the cells (50 mg) were added in a 2-ml screw-cap microcentrifuge tube. One g of 0.1 mm glass beads (Glasperlan; B. Braun Diessel Biotech GmbH, Melsungen, Germany) was added, and the tube was agitated in a shaking bead mill (B. Braun Diessel Biotech) for 2 min.

Thermal shock method (M3). In the thermal shock method, the cells (50 mg) were resuspended in 400 μl of H_2O and subjected to a series of heat/cold shocks, alternately boiling (5 min, $100°C$) and snap freezing (5 min, $-196°C$ in liquid nitrogen). The procedure was repeated six times.

Modified conventional enzymic digestion method (M4). A method described by Ausubel, *et al.* ([5]) was used with some modifications. Briefly, lysozyme was added to a final concentration of 2 mg/ml of cell suspension, and the tube was incubated at $37°C$ for 1 hr. Then 0.3 mg proteinase K and SDS to a final concentration of 3% were added, and the tube was further incubated at $56°C$ for 1 hr.

Manual disruption with modified conventional enzymic digestion method (M5). In the manual disruption with modified conventional enzymic digestion method the cells (50 mg) were placed in a mortar which contained dry ice and glass beads (0.1 g, 0.11 mm, Glasperlan; B. Braun Diessel Biotech). The cells were triturated for approximately 30 min, then transferred to a sterile 1.5 ml Eppendorf tube. The mortar was warmed to room temperature, and the appropriate NET buffer was used to wash any residual cells out of the mortar into the Eppendorf tube. The remaining steps were as described in the M4 method.

DNA purification. RNA elimination and DNA purification in the above five methods were carried out as described below: The lysed sample was centrifuged at $12,000 \times g \times 10$ min in a benchtop microcentrifuge to precipitate the cell debris. The supernatant was recovered, and an equal volume of phenol : isoamyl alcohol : chloroform (25:1:24) was added. The mixture was shaken by hand, and the phases were

THE TABLE. *DNA yields from mycobacterial species by using five extraction methods.*[a]

Organisms	Yield (µg DNA/mg wet weight cells)				
	M1	M2	M3	M4	M5
M. phlei	0.16	1.52	0.11	0.38	0.69
M. bovis BCG	0.56	1.16	0.40	1.06	1.66
M. lepraemurium	0.25	2.82	0.01	0.30	0.58
M. leprae (armadillo)	0.30	1.25	0.06	1.37	1.66
M. leprae (nude mice)	0.06	0.66	0.03	0.27	1.43

[a] M1 = Intensive enzymic digestion method; M2 = 2-min mechanical glass-bead disruption method; M3 = thermal shock method; M4 = modified conventional enzymic digestion method; M5 = manual disruption with modified conventional enzymic digestion method. Each DNA yield was obtained in duplicate.

separated by centrifugation at 12,000 × g × 5 min. The aqueous phase was carefully recovered and an equal volume of chloroform was added. The mixture was shaken and treated as described above. Added to the pooled aqueous phase was 0.1 vol of 3 M sodium acetate, pH 5.2, and 2 vol of 100% ethanol. After thorough mixing, the DNA could often be spooled onto a glass rod, or could be precipitated by centrifugation at 12,000 × g × 20 min. The collected DNA was washed in 70% ethanol, dried briefly, and resuspended in 400 µl NET buffer containing 10 µg/ml RNase A. After a 30-min incubation at 37°C, 10 µg/ml proteinase K and 0.1% SDS were added and incubated at 56°C for another 30 min. The DNA was recovered by centrifugation and retreated with phenol, chloroform, sodium acetate, and ethanol as before. Finally, DNA was dissolved in 100 µl TE buffer and stored at 4°C for at least 24 hr before use to ensure that it had fully dissolved. DNA concentration was determined spectrophotometrically ([17]).

Electrophoresis and imaging

DNA samples. Approximately 500 ng DNA of each sample was electrophoresed in a 0.7% agarose gel with 1 × TBE (250 mM Tris, 0.5 mM EDTA, 25 mM boric acid). DNA fragments of known sizes of 1 kb DNA ladder marker (Gibco BRL) were loaded in the same agarose gel. The gels were stained with ethidium bromide (0.5 µg/ml) and photographed on a UV transilluminator.

Restriction endonuclease digestion of DNA. Each DNA sample (500 ng) was used in a 10-µl volume to perform the restriction endonuclease digestion, in accordance with the manufacturer's recommen-

dations. The other procedures were the same as described in DNA samples.

RESULTS

Isolation and restriction endonuclease digestion of DNA

The different mycobacteria used in these experiments and the respective DNA yields recovered by different methods are shown in The Table. The highest DNA yield of 2.82 µg/mg wet weight cells was obtained by the 2-min mechanical glass-bead disruption method (M2) from *M. lepraemuriu* while the thermal shock method (M3) ga· the lowest DNA yield of 0.01 µg/mg wet weight cells from *M. lepraemurium.* These results show that 282-fold more DNA was obtained by M2 compared to M3 for this species. M1 and M3 were the least effective methods for DNA extraction from all the mycobacteria tested. M2 was found to extract DNA most efficiently from *in vitro* grown *M. phlei.* Manual disruption with modified conventional enzymic digestion method (M5) yielded comparatively higher yields of DNA from all mycobacteria used than those obtained by the modified conventional enzymic digestion only (M4). It is interesting to note that it is relatively more difficult to extract DNA from *M. leprae* recovered from foot pads of nude mice than the bacilli isolated from armadillo liver tissues (The Table).

The purified DNAs were separated by electrophoresis on a 0.7% agarose gel to evaluate the quality and the size of DNA fragments (Fig. 1). Every DNA tested exhibited a major band larger that 12 kbp (Fig. 1), and could be completely digested by *Pst* I (Fig. 2).
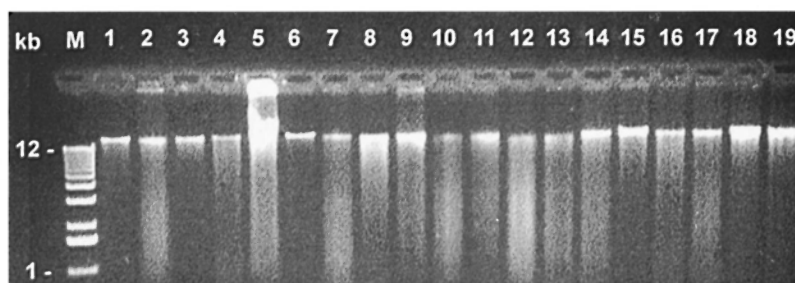
FIG. 1. DNA prepared from five mycobacterial species by using five DNA extraction methods. Five species: *M. phlei* = lanes 1–3; *M. bovis* BCG = lanes 4–8; *M. lepraemurium* = lanes 9–12; *M. leprae* (armadillo) = lanes 13–16; *M. leprae* (nude mice) = lanes 17–19. Five DNA extracting methods: Intensive enzymic digestion method (M1) = lanes 4, 9, 13; 2-min mechanical glass-bead disruption method (M2) = lanes 1, 5, 10, 14, 17; thermal shock method (M3) = lane 6; modified conventional enzymic digestion method (M4) = lanes 2, 7, 11, 15, 18; manual disruption with modified conventional enzymic digestion method (M5) = lanes 3, 8, 12, 16, 19.

DISCUSSION

The extraction of mycobacterial DNA has not been well exploited due to the thick, waxy lipid, and mycolic acid-containing mycobacterial cell walls that are extremely difficult to be lysed by methods routinely used. Visuvanathan, *et al.* ([23]) used an extensive enzymic digestion method (M1) in which subtilisin and pronase were used for isolation of DNA from different bacteria, and reported that this method was also useful for DNA extraction from mycobacteria. Our results have shown that this method (M1) gave much lower yields of DNA from all mycobacteria tested as compared to M2, M4 and M5. In our study, we found that a

modified enzymic digestion method (M4), in which the concentrated proteinase K and SDS were used, was quite effective for all rapidly and slowly growing mycobacteria tested (The Table). Contrary to the conclusion of Bollet, *et al.* ([6]), who indicated that proteinase K lacks efficacy on most mycobacterial species, we found that proteinase K is more effective than subtilisin and pronase to digest mycobacterial cell walls under the same conditions used in this study, especially for *M. leprae* derived from both armadillo liver tissues and foot pads of nude mice. It should be emphasized that this is the first study in which *M. leprae* purified from foot pads of nude mice and armadillo liver tissues have been used to in-
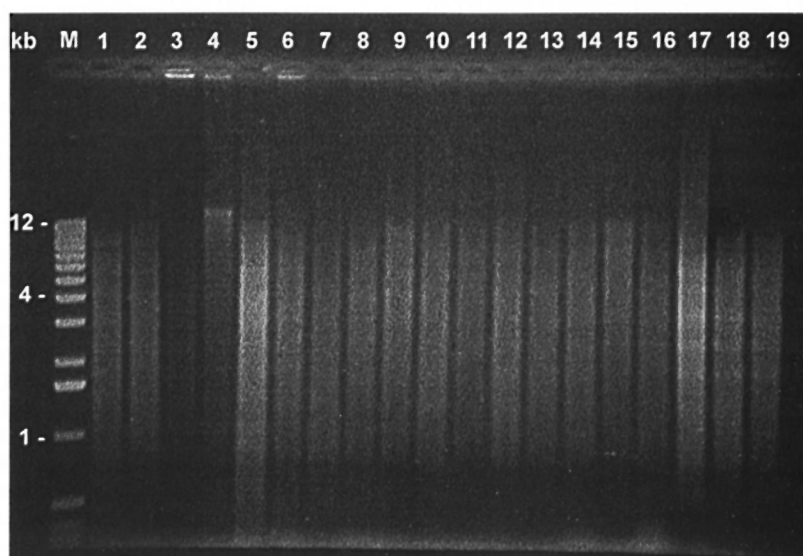

FIG. 2. Same as Figure 1, but DNA was digested by *Pst* I.

vestigate whether or not there could be differences in extracting DNA by various methods. M4 yielded more than three times higher DNA than M1 from both sources of *M. leprae*. Moreover, M4 takes less than 3 hr, while at least 40 hr are required for DNA extraction by M1.

Both 2-min mechanical glass-bead disruption (M2) and manual disruption with modified conventional enzymic digestion (M5) methods recovered more DNA than any other methods tested. The results in The Table show that M2 recovered the highest quantity of DNA; 2.82 μg of DNA/mg wet weight cells of *M. lepraemurium* (about 10^9 bacilli). This represents a theoretical yield of about 78% ([4, 23]). From a practical point of view, when M2 and M5 are compared the former needs special equipment and the latter demands more technical effort to produce reproducible results. Our experience has shown that M4 is the easiest, the least time-consuming, and is an effective method suitable for every laboratory for the extraction of DNA from rapidly and slowly growing mycobacteria. M3 gave the least DNA recovery from *M. lepraemurium*, yielding only 0.28% of the DNA theoretical yield.

Recently, de Lamballerie, *et al.* ([9]) used "Chelex 100" to extract DNA from *M. tuberculosis* and *M. avium*. We have also tried this method which seemed effective for DNA extraction from *M. phlei*, *M. bovis* BCG, and *M. lepraemurium*, but not from *M. leprae* either recovered from armadillo liver tissues or from the foot pads of nude mice (data not shown). It is possible that the chemical compositions of the cell walls of *M. leprae* differ from the other mycobacteria tested.

Our results have clearly shown that the yields of DNA of *M. leprae* isolated from the foot pads of nude mice are slightly lower than those isolated from armadillo liver tissues by all of the methods used in our study (The Table). We have observed from the stained preparations that the bacilli from the liver tissues of armadillos are generally thinner and longer than those from the foot pads of nude mice under the light microscope (data not shown). The biosynthetic capabilities of mycobacteria may vary depending upon cultural conditions and strain differences.

SUMMARY

Mycobacteria generally have thick cell walls and contain large amounts of lipid, making them resistant to DNA extraction. Five methods, namely, extensive enzymic digestion method (M1), 2-min mechanical glass-bead disruption method (M2), thermal shock method (M3), modified conventional enzymic digestion method (M4), and manual disruption with modified conventional enzymic digestion method (M5), were used to compare their effectiveness and simplicity in extracting DNA from slowly growing mycobacteria (*Mycobacterium leprae*, *M. lepraemurium* and *M. bovis* BCG), and a rapidly growing mycobacterium (*M. phlei*). The highest DNA yield was obtained by M2 from *M. lepraemurium* which produced 2.82 μg DNA/mg wet weight of cells, representing a theoretical yield of 78%. M3 gave the lowest DNA yield; 0.01 μg DNA/mg wet weight of cells of *M. lepraemurium* was obtained. M4, in which proteinase K was used, is more effective than M1, in which subtilisin and pronase were used. M5 yielded a higher amount of DNA, but it required more manipulations to extract DNA as compared to M4. Extraction of DNA of *M. leprae* from nude mice is more difficult than that of *M. leprae* from armadillos by all of the methods used. These results suggest that the biosynthetic capabilities of these two forms of *M. leprae* may vary, depending on their cultural conditions and/or strain differences. Our results have shown that both M2 and M4 are the simplest, most effective and time-saving methods which are suitable for every routine laboratory to extract DNA from slowly and rapidly growing mycobacteria.

RESUMEN

Las micobacterias en general, tienen gruesas paredes celulares y contienen grandes cantidades de lípidos que dificultan la extracción de su DNA. En este estudio se compararon 5 métodos de extracción del DNA de micobacterias de lento crecimiento (*Mycobacterium leprae, M. lepraemurium* y *M. bovis,* BCG) y de una micobacteria de crecimiento rápido (*M. phlei*). Los métodos fueron un método de digestión enzimática extensa (M1), un método de rompimiento mecánico por 2 min con perlas de vidrio (M2), un método de choque térmico (M3), un método modificado de digestión enzimática convencional (M4), y un método de rompimiento manual combinado con digestión enzimática (M5).

El mayor rendimiento de DNA se obtuvo con el M2 aplicado a *M. lepraemurium* (2.82 µg de DNA por mg de peso húmedo). Con esta bacteria, el M3 dio el menor rendimiento (0.01 µg por mg de peso húmedo). El M4, en el cual se usa proteinasa K, es más efectivo que el M1 en el que se usan subtilisina y pronasa. El M5 produjo una mayor cantidad de DNA pero se requiere de más manipulación que en el M4.

La extracción de DNA del *M. leprae* propagado en el ratón desnudo es más dificil que la extracción de DNA del *M. leprae* cultivado en el armadillo, por todos los métodos usados. Estos resultados sugieren que las capacidades biosintéticas de estas dos formas de *M. leprae* pueden variar dependiendo de sus condiciones de cultivo y de las diferencias entre las posibles cepas. Nuestros resultados han demostrado que tanto M2 como M4 son los métodos más simples, efectivos y cortos para la extracción de DNA de micobacterias tanto de crecimiento rápido como de crecimiento lento.

RÉSUMÉ

Les mycobactéries ont généralement des parois cellulaires épaisses et contiennent de grandes quantités de lipides, les rendant résistantes à l'extraction de l'ADN. Cinq méthodes—la digestion enzymatique extensive (M1), la dislocation mécanique durant deux minutes sur des perles de verre (M2), le choc thermique (M3), la digestion enzymatique conventionnelle modifiée (M4) et la dislocation manuelle avec une digestion enzymatique conventionnelle modifiée (M5)—ont été utilisées pour comparer leur efficacité et leur simplicité pour extraire l'ADN de mycobactéries à croissance lente (*Mycobacterium leprae, M. lepraemurium* et le BCG de *M. bovis*), et une mycobactérie à croissance rapide (*M. phlei*). La teneur la plus élevée en ADN a été obtenue par la M2 à partir de *M. lepraemurium* qui a produit 2,82 µg d'ADN/mg de poids humide des cellules, ce qui représente une teneur théorique de 78%. M3 a donné la teneur la plus faible en ADN; on a obtenu 0,01 µg d'ADN/mg de poids humide des cellules de *M. lepraemurium*. M4, dans laquelle la protéinase K fut utilisée, est plus efficace que M1, dans laquelle la subtilisine et la pronase furent utilisées. M5 a donné une quantité plus élevée d'ADN, mais a nécessité plus de manipulations pour extraire l'ADN, par comparaison avec M4. L'extraction de l'ADN de *M. leprae* provenant de souris nues est plus diffcile que son extraction à partir de *M. leprae* provenant de tatous, et ceci pour toutes les méthodes utilisées. Ces résultats suggèrent que les capacités biosynthétiques de ces deux formes de *M. leprae* peuvent varier en fonction de leurs conditions de culture et/ou de différences d'espèce. Nos résultats ont montré que M2 et M4 sont toutes les deux les méthodes les plus simples, les plus efficaces et les plus rapides qui soient adaptées à tout laboratoire de routine pour extraire l'ADN de mycobactéries à croissance lente et rapide.

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