Optimized Method for Preparation of DNA from Pathogenic and Environmental Mycobacteria

Michael Käser,1* Marie-Thérèse Ruf,1 Julia Hauser,1 Laurent Marsollier,2 and Gerd Pluschke1
Swiss Tropical Institute, Socinstrasse 57, 4002 Basel, Switzerland, and Université d’Angers, Centre Hospitalier Universitaire, 4 Rue Larrey, 49033 Angers Cedex 1, France2

Received 17 June 2008/Accepted 11 November 2008

Genomic studies on pathogenic and environmental mycobacteria are of growing interest for understanding of their evolution, distribution, adaptation, and host-pathogen interaction. Since most mycobacteria are slow growers, material from in vitro cultures is usually scarce. The robust mycobacterial cell wall hinders both experimental cell lysis and efficient DNA extraction. Here, we compare elements of several DNA preparation protocols and describe a method that is economical and practical and reliably yields large amounts—usually 10-fold increased compared to earlier protocols—of highly pure genomic DNA for sophisticated downstream applications. This method was optimized for cultures of a variety of pathogenic and environmental mycobacterial species and proven to be suitable for direct mycobacterial DNA extraction from infected insect specimens.

Mycobacterial diseases are a major health concern for humans (i.e., Mycobacterium tuberculosis, M. leprae, M. ulcerans, M. avium, and M. paratuberculosis) (4, 13, 18, 29, 30), livestock (M. bovis and M. avium subsp. paratuberculosis) (1, 3), fisheries (M. marinum) (25), and wildlife (M. avium, M. pinnipedii, M. microti, M. caprae, and other species) (13, 20). Efficient methods for DNA preparation are required both for the identification and genotyping of such pathogens and for population genomics, which is developing into an important tool to study bacterial evolution, virulence, and epidemiology.

Extraction of mycobacterial genomic DNA is especially demanding since (i) many mycobacterial species are among the most extreme slow growers, accounting for small amounts of starting material, and (ii) a robust and waxy cell wall renders mycobacteria difficult to lyse. Published protocols for mycobacterial DNA preparations and commercially available extraction kits are mainly designed for the isolation of small amounts of genomic material suitable for conventional PCR application (2, 7, 9, 11, 14, 15, 23, 24, 27, 28, 33), such as for testing of potentially contaminated milk (6, 8, 17). However, such DNA quantities and qualities are usually not sufficient for more sophisticated molecular analyses.

M. ulcerans, the causative agent of the devastating human skin disease Buruli ulcer, is one of the slowest growers among mycobacterial species, and the development of molecular tools is crucial for studying its transmission and microepidemiology. The objective of this study was to develop an optimized extraction protocol for DNA of both high quantity and quality from scarce material of in vitro-cultivated M. ulcerans disease isolates. We compared elements of several protocols and developed a DNA preparation method that is optimized in each individual step and thus ready to use for virtually all mycobacterial species to yield a maximum of pure genetic material. In addition, we applied the established method to cultures of a variety of pathogenic and environmental mycobacterial species and tested it by isolating DNA from insects experimentally infected with M. ulcerans.

MATERIALS AND METHODS

Mycobacterial strains and preparation of mycobacterial cell suspensions. The strains used for this investigation and their origins are as follows: M. ulcerans Aggy99, Malaysia 1615, and Japan 753 (21); Ghana IFIK1066089, Ghana Nm50/04, Ghana Nm51/04, Ghana Nm53/04, Ghana Nm74/02, Ghana Nm97/02, Ghana Nm98/02, Ghana Nm103/02, and Mexico IFIK 973880 (this study); Ghana Nm18/02, Ghana Nm21/02, Ghana Nm31/04, Ghana Nm38/02, and Ghana Nm59/02 (10); and Japan ITM 8756, French Guiana ITM 7922, and Surinam ITM 842 (22); M. marinum M, ATCC 927, CC240299, and DL240490 and M. pseudohollis-sti L15 (21); M. lflavidus XT128 (32); Mycobacterium tuberculosis Pasteur 14001.0001 (5); and M. bovis BCG ATCC35734 (5). Mycobacteria were obtained from cultures as described earlier (19, 31), resuspended in phosphate-buffered saline (PBS; pH 7.4), and heat inactivated at 95°C for 60 min. Note that pathogenic mycobacteria need to be processed under appropriate biosafety containment. To avoid cross-contamination, 1.5-mL screw-cap tubes were used. Samples were centrifuged for 5 min at 2,500 × g to remove residual PBS.

Extraction of mycobacterial DNA from pellets. Mycobacterial pellets were resuspended in 300 μL of lysis buffer A, B, or C (buffer A contained 5% monosodium glutamate, 50 mM Tris [pH 8.5], and 25 mM EDTA; buffer B contained 15% sucrose, 50 mM Tris [pH 8.5], and 50 mM EDTA; and buffer C contained 4 mM guanidine isothiocyanate [GITC] and 50 mM Tris [pH 7.2]) and then incubated with different amounts of lysozyme. After incubation at 37°C for 1 h, sodium dodecyl sulfate (SDS) and protease K (PK) were added at different end concentrations and the mixture was further incubated at 37°C for 1 h, followed by enzyme inactivation at 70°C for 5 min. Some pellets were preincubated with chloroform-methanol (MeOH) at a 2:1 ratio for delipidation. Various matrix materials (200 μL of 0.1-mm zirconia beads [BioSpec Products, Bartlesville, OK] or 0.5-mm or 1.4-mm ceramic or glass beads [Bertin Technologies, Montigny-le-Bretonneux, France]) were used, and samples were homogenized with a mechanical bead beater device, the Mikro-Dismembrator S (B. Braun Biotech International, Melsungen, Germany) for 2 to 7 min at 2,000 to 3,000 rpm or the Precellys 24 (Bertin Technologies, Montigny-le-Bretonneux, France) at conditions ranging from 2 × 40 s at 5,000 rpm to 3 × 30 s at 6,300 rpm. Supernatants were transferred to new 1.5-mL reaction tubes and subjected to phenol-chloroform (Fluka, Buchs, Switzerland) extraction and chloroform purification (Fluka, Buchs, Switzerland). For this, addition of 500 μL of phenol-chloroform or chloroform was followed by a vortexing step and centrifugation at room temperature for 5 min at 4,000 rpm. After isopropanol or ethanol
Lysis buffer
Delipidation pretreatment
Purification
described for mycobacterial pellets. Insects were sacrificed with 70% cold EtOH, inoculated in the coelomic cavity with 10^6 CFU of Mycobacterium ulcerans (EtOH) precipitation at 70°C for 3 h. For details of the methodology, see Materials and Methods.

**TABLE 1. Comparative advantages and/or disadvantages of various elements of DNA extraction protocols**

<table>
<thead>
<tr>
<th>Reaction vol</th>
<th>Protocol step and option(s)</th>
<th>Advantage(s) and/or disadvantage(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5, 2, or 10 ml</td>
<td>Minimized vol reduces time and reagent consumption, round bottom of 2-ml tubes complicates separation of EtOH precipitate from supernatant</td>
<td></td>
</tr>
</tbody>
</table>

Starting material
5 to 40 mg (wet wt) pellets
Maximum DNA yield with 20 mg, no increase with more material in 1.5-ml reaction mixture vol

Delipidation pretreatment
Chloroform-MeOH treatment preceding lysis
Enhanced purity but substantial loss of material

Lysis buffer
A: 5% monosodium glutamate, 50 mM Tris (pH 8.5), 25 mM EDTA
B: 15% sucrose, 50 mM Tris (pH 8.5), 50 mM EDTA
C: 4 M GITC, 50 mM Tris (pH 7.2)

Disruption/digestion
1 vs 10 mg/ml lysozyme, 100 vs 200 μg/ml PK
No differences encountered
SDS concn, 0.05 to 4%
Highest yield concn yields best result
Mechanical vs chemical lysis (BD GeneOhm kit)
Higher yields with mechanical lysis
High-velocity mechanical treatment
Yields efficiently increased
Mikro-Dismembrator S vs Precellys 24 homogenization device
Slightly enhanced yields with Precellys 24

Homogenization conditions: 2–7 min at 2,000–3,000 rpm
(Mikro-Dismembrator S), 2 × 40 s at 5,000 rpm to 3 × 30 s at 6,800 rpm (Precellys 24)
Harshest conditions result in highest yields

Purification
Phenol-chloroform extraction steps followed by 1 chloroform purification step only
Purity efficiently increased
Phenol-chloroform vs column purification
Purity further increased
Isopropanol vs EtOH precipitation
Higher yields with isopropanol but better to handle pellet with EtOH

DNA quantification, amplification, and gel electrophoresis. DNA concentration was determined with the NanoDrop Spectrophotometer ND-1000 (NanoDrop, Wilmington, DE) by measuring the absorption at 260 nm, and the decontamination of DNA from solvents and proteins was estimated by measuring the absorption at 230 and 280 nm, respectively. Purity and fragmentation of the extracted genomic DNA were assessed by 1% agarose gel electrophoresis. Detection limits of purified genomic DNA materials were assessed by PCR with primers targeting unique regions in the mycobacterial genomes (primers MK810 [TCT GTCAAGACAAGCCGAG], MK811 [GACTCGTGTTGATCGAGGAT], MK60 [ATCGTTTAGGGCATGCTA], MK61 [CACAGGTCGACCCCAAAGT], MK63 [GTCGATGATCGCCTGTGGT], and MK35 [GTCGGCATCTGTTGCTCA]). The presence of *M. ulcerans* in environmental insect specimens was tested with primers MUS and MUS6 (26) for detection of IS2606 and MK289 (GTCGTAAGTGTGGCGGAAA). PCR was performed with FirePol 10× buffer and 0.5 μl FirePolTaq polymerase (Solis BioDyne, Tartu, Estonia), 10 ng genomic DNA, 0.6 μM each forward and reverse primer, 1.5 mM MgCl2, and 0.4 mM each deoxynucleoside triphosphate in a total volume of 30 μl. PCRs were run in a GeneAmp PCR System 9700 PCR machine (Perkin-Elmer, Schwerzenbach, Switzerland). The thermal profile for PCR amplification of *M. ulcerans* genomic DNA included an initial denaturation step of 95°C for 5 min, followed by 32 cycles of 95°C for 20 s, annealing at 58°C for 30 s, and elongation at 72°C for 45 s up to 2 min 20 s. The PCR was finalized by an extension step at 72°C for 10 min, followed by the analysis of the PCR products on 1% agarose gels by gel electrophoresis with ethidium bromide staining and the AlphaImager illuminator (Alpha Innotech, San Leandro, CA).

(ETOH) precipitation at −70°C for >30 min, DNA pellets were resuspended in 100 μl nuclease-free water. Alternatively, mycobacterial purification kits (Promega Wizard [Promega AG, Dubendorf, Switzerland], Sigma GeneElute [Sigma-Aldrich, Buchs, Switzerland], and the BD GeneOhm lysis kit [Becton Dickinson Biosciences, Allschwil, Switzerland]) were used according to the manufacturers’ protocols, without or in combination with mechanical treatment.

For further purification and quality control, extracted DNA was freed from residual RNA by incubation with 1.5 mg/ml RNase A (Fermentas, St. Leon-Rot, Germany) for 2 min at 37°C and purified from degradation products, residual solvent, and protein contaminants with the QIAamp DNA purification MiniKit (Qiagen AG, Hombrechtikon, Switzerland) according to the manufacturers’ protocols. For genome sequencing applications, DNA was concentrated with a Concentrator 5301 (Vaudaux-Eppendorf AG, Basel, Switzerland) according to the manufacturers’ protocols.

**Experimental infection of aquatic insects and DNA extraction.** Wild-caught insects belonging to the family Naucoridae (Naucoris cimicoides) were collected from swamps in western France. They were housed in an aquarium—filled with water of their natural environment—at 28°C and a photoperiod of 12 h each light and dark without any feeding for 7 days. These insects were then fed with a 15- to 20-day-old grub of Phormia terraenovae (Verminie’s du Ouest, Tremblay, France) that was infected by inoculation with 10^6 CFU of *M. ulcerans* in a volume of 30 μl with a 25-gauge needle. Alternatively, Naucoris insects were directly inoculated in the coelomic cavity with 10^6 CFU of *M. ulcerans* in a volume of 30 μl with a 25-gauge needle (16). Insects were sacrificed with 70% cold EtOH, transferred to a 1.5-ml screw-cap tube, and processed for DNA extraction as described for mycobacterial pellets.

These comparisons were made with *M. ulcerans* strain IFIK1066089. For details of the methodology, see Materials and Methods.
of the waxy lipid and mycolic acid-containing cell wall renders combination of mechanical disruption and chemical solubilization electrophoresis after purification. Figure 1 shows that a combination turned out to be very efficient in cell wall disruption, as the mycobacteria accessible for enzymatic lysis. Incubation with 4% SDS followed by mechanical disruption, a combination that was (to our knowledge) not used in any previous protocol, was here found to be essential for a high DNA yield (sample D). All protocols lacking one of these two treatments yielded strikingly smaller amounts of DNA (samples B and C, respectively). When both steps were omitted, no DNA was extracted at all (sample A).

For purification, the properties of DNA binding to silica in the presence of chaotrope salts are generally used to circumvent the use of phenol-chloroform. However, silica-based commercial purification kits reached neither our elevated quantity nor quality requirements, even when applied after mechanical solubilization (Table 1; Fig. 1, samples G and H). Thus, although column use facilitates handling, we decided to use conventional phenol-chloroform extraction. Two steps of phenol-chloroform extraction were found to be crucial for removing protein and lipid contaminations from the genomic DNA, and an additional purification step with only chloroform helped in removing residual phenol (Table 1). A second round of this three-step phenol-chloroform extraction after EtOH precipitation led to enhanced DNA purity but reduced the overall yield (Fig. 1, sample E). Although the highest DNA yield and purity were represented by sample F (Fig. 1), where DNA was precipitated with isopropanol instead of EtOH, this protocol resulted, in most cases, in a yellowish, slimy pellet that was difficult to resuspend and thus prone to material loss during handling. In conclusion, when a maximum of extracted genetic material is required, the protocol described for sample D should be applied, which had a 260/280-nm ratio (≈1.6) sufficient for most downstream applications. When a higher purity of DNA is required, a protocol including chloroform-MeOH pretreatment and/or a second round of phenol-chloroform extraction and EtOH precipitation is indicated, although it is associated with lower yields.

From these results, we derived an improved standard protocol (Table 2) that involves (i) the use of 20 mg (wet weight) of pellets; (ii) pretreatment with 10 mg/ml lysozyme, 200 μg/ml...
Phenol-chloroform extraction and EtOH precipitation; (iv) two phenol-chloroform extractions, followed by chloroform purification; and (v) EtOH precipitation with two EtOH washing steps, followed by resuspension of the DNA pellet in 100 μl of nuclease-free water.

We applied the established DNA extraction method to cell pellets of a panel of *M. ulcerans* strains and other mycobacterial species including *M. marinum*, *M. pseudoshottsii*, *M. liflandii*, *M. tuberculosis*, and *M. bovis* BCG (Table 3). A mean yield of 713 ng genomic DNA per mg (wet weight) cells with a 260/280-nm ratio of 1.58 (standard deviation, 0.09) was obtained. This represents a 10- to 20-fold increase in DNA yield compared to previous protocols, elements of which we combined for optimization. We performed detailed quality control for DNA extractions from two strains, *M. ulcerans* Agy99 and Japan ITM 8756, representing the two distinct lineages of *M. ulcerans* (Fig. 2). Genomic DNA yields from 20-mg (wet weight) cell pellets were sufficient in quantity (typically, >5 μg; Fig. 2) and quality for whole-genome microarray hybridization and whole-genome sequencing analyses. Single-copy gene sequences of >2 kb could be easily amplified by PCR with 10 ng as the template (Fig. 2). Subsequent RNase treatment and genomic DNA column purification decontaminated the samples from RNA and small DNA fragments resulting from shearing of genomic DNA (Fig. 2).

In addition, we applied the optimized DNA extraction method to aquatic insects experimentally infected with *M. ulcerans*. Genomic DNA was sufficient in quantity and purity to specifically detect *M. ulcerans* in infected insects (Table 4), showing that the established method is suitable for direct processing of environmental samples.

In conclusion, we envision this protocol to facilitate the processing of environmental samples.
investigation of pathogenic and non-pathogenic mycobacteria sampled from both infected tissue and the environment. In combining and optimizing crucial elements of established DNA extraction methods, our ready-to-use protocol meets the requirements of pathogens and nonpathogens and greatly enhances both the yield and the purity of mycobacterial DNA preparations.

ACKNOWLEDGMENTS

We are grateful to Pamela C. Small for provision of strains (M. marinum CC240299 and DL240490, M. pseudoshottii L15, and M. lipophilndii XT128), Thomas Bodmer for cultivation of strain M. ulcerans IFIK1066089, and Dorothy Yeboah-Manu for professional support. L. Marsollier was supported by the Institut National de la Santé et de la Recherche Médicale (INSERM) and the Foundation Raoul Follereau.

REFERENCES