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Purification of *Mycobacterium leprae* RNA for gene expression analysis from leprosy biopsy specimens

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Gene expression analysis in Mycobacterium leprae, an obligate intracellular pathogen and the etiologic agent of leprosy, has been hampered by the lack of an efficient method to purify RNA from leprosy lesions. Therefore to date, transcripts for only a few genes have been identified. We report the use of a single-tube homogenization/RNA extraction method that produces enough RNA to study the expression of 30 genes from a single skin biopsy specimen of a multibacillary leprosy patient and demonstrate that RNA can be purified after fixation of biopsies in 70% ethanol for up to a year. This represents a major advancement in the ability to study M. leprae gene expression directly from biopsy material and should help to define genes that are associated with intracellular survival of this human pathogen.

INTRODUCTION

Mycobacterium leprae, the etiologic agent of leprosy, affects the skin, eyes, and mucosa of the upper respiratory tract and is unique among bacterial pathogens in its ability to invade the peripheral nervous system (1). Approximately 3 million leprosy patients in the world are physically disabled as a result of damage to peripheral nerves and the attendant sensorimotor loss. One-fourth of the 700,000 newly diagnosed patients each year (2) will suffer from irreversible nerve damage and are at risk of developing classic hand and foot deformities and associated disabilities as a long-term consequence of infection with *M. leprae* (3).

Fundamental to understanding the pathogenicity of *M. leprae* is to define the composite phenotype of the bacillus and to determine gene expression related to intracellular survival and virulence. A major obstacle impeding progress in this area has been our inability to culture *M. leprae* in vitro. Lacking sufficient quantities of viable *M. leprae* from biopsy material of leprosy lesions from which to study the biochemical basis of phenotype and genotype, most findings in this area have been of limited scope. In addition, gene expression analysis of *M. leprae* has been greatly

hampered by the lack of a suitable method to purify RNA from infected tissues in sufficient quantities to analyze a large number of genes from a single biopsy. To date, transcripts for only a few *M. leprae* genes have been identified (4,5). Therefore, there is a general lack of understanding of which genes are required for intracellular survival and virulence.

A major reason for the inability to purify *M. leprae* RNA from clinical specimens is that *M. leprae* possesses a complex mycobacterial surface structure consisting of a Gram-positive-like cell wall surrounded by a waxy, lipid, mycolic acid- and phenolic glycolipid-containing envelope (6). This dense coat makes lysis of *M. leprae* for subsequent purification of RNA extremely difficult. Therefore, conventional techniques for purification of mycobacterial RNA for gene expression analysis have not been successfully applied to generate sufficient quantities of *M. leprae* mRNA, even when large numbers of bacteria from infected mouse foot pads have been extracted (4,5). To complicate this issue, only low numbers of bacilli ($<10^9$) are recovered from skin biopsies of leprosy lesions (7). Recently, we have described a method to purify RNA from purified bacteria, which employs a single-tube homogenization/RNA

Table 1. Primers for RT-PCR Analysis of *Mycobacterium leprae* RNA Purification Methods

Gene Name		Primer Sequence
<i>hsp18</i>	MLhsp18-F	5'-ATTCGTCGTCGAGTTCGACCTTCCT-3'
	MLhsp18-R	5'-CTTAGCTTGTTGCGCAAACAACAGT-3'
<i>gap</i>	MLgap-F	5'-GGCTCTTGCGGTTTCG-3'
	MLgap-R	5'-GCAGGTCAGAATGCG-3'
<i>sodA</i>	MLSodA-F	5'-TGAGATCAACGAGATCCACC-3'
	MLSodA-R	5'-GCCCAGTTTACGACATTCC-3'
<i>gyrA</i>	MLgyrA-F	5'-GCATCTCCAATGTAGAAGACC-3'
	MLgyrA-R	5'-CAGTTATCGTGACAACGACC-3'
<i>rpoB</i>	MLrpoB-F	5'-CGTCGAGGCGATCACGCCGACGACGC-3'
	MLrpoB-R	5'-GACCTCTAGCCCGGCACGCTCACG-3'
<i>folP</i>	MLfolP-F	5'-GTCCAGCACGGCCTGGC-3'
	MLfolP-R	5'-CTGCTCGCCACCAG-3'

extraction method using a vertical angular motion homogenizer and standard chloroform extraction and RNA precipitation (8). Using this technique, microgram quantities of RNA, sufficient to perform >150 reverse transcription PCR (RT-PCR) assays and cross-species DNA array analysis, were obtained from 1×10^{10} purified nude mouse foot pad-derived *M. leprae*. This RNA provided the first comprehensive insight into transcriptionally active genes that are potentially essential for intracellular survival of *M. leprae*. How-

ever, when this procedure was applied to purify RNA from *M. leprae* obtained by standard Mickle homogenization of skin biopsy tissue of leprosy patients, only small amounts of *M. leprae* RNA were recovered (8). Therefore, to increase these RNA yields, we modified the above method by simply mincing biopsy material prior to the single-tube homogenization/RNA extraction method (direct method). In addition, since ethanol has been shown to preserve *M. leprae* DNA (9) and prokaryote RNA (10) for subsequent extraction method-

ologies, we determined the effect of ethanol fixation on the above procedure.

MATERIALS AND METHODS

Skin Biopsy Specimens

Diagnostic skin biopsies, obtained from multibacillary (MB) and paucibacillary (PB) leprosy patients prior to treatment, were bisected, and one-half was immersed in cryoprotective medium (O.C.T.; Tissue Tek, Columbus, OH, USA), snap-frozen in liquid nitrogen, and stored at -70°C . The other portion was fixed in 10% neutral-buffered formalin overnight and embedded in paraffin. Sections (4 μm) were stained with hematoxylin and eosin for histopathologic classification and with Fite's acid-fast stain for determination of the bacterial index (BI). Biopsy specimens were also obtained from MB lesions and stored in 1 mL of 70% ethanol for up to 1 year at room temperature. To conceal patient identification, as specified by approved protocols for research with archived material, identifying information for each specimen was blinded by assigning a random code number to each specimen.

RNA Extraction and Purification

Biopsies were thawed, removed from the O.C.T. medium, rinsed three times with 1 mL sterile phosphate-buffered saline (PBS; Sigma, St. Louis, MO, USA), and weighed. Ethanol-fixed biopsies were removed from ethanol, rehydrated for 20 min in 1 mL diethylpyrocarbonate (DEPC) water (Sigma), and then the water was decanted. Biopsies (10–12 mg) were then finely minced to a uniform consistency with sterile curved scissors in 50 μL PBS.

M. leprae RNA was extracted directly from minced biopsy tissue using a modification of a previously described single-tube homogenization/RNA extraction protocol (8) or bacteria-purified by the Mickle homogenization method (7). Briefly, 1 mL TRIzol[®] Reagent (Invitrogen, Carlsbad, CA, USA) was added to each minced biopsy or purified bacteria. Each suspension was then transferred to a FastRNA[™] Blue tube (FastRNA Kit-Blue; Qbiogene, Carlsbad, CA, USA).

Figure 1. RT-PCR analysis of Mickle and direct method for purifying *Mycobacterium leprae* RNA from biopsy specimens of lepromatous leprosy patients. (A) *M. leprae hsp18* RT-PCR, generating a 360-bp fragment of the *hsp18* gene transcript and (B) *M. leprae gap* RT-PCR, generating a 706-bp fragment of the *gap* gene transcript. (A and B) Lane M, 100-bp DNA ladder (Promega, Madison, WI, USA); lane 1, *M. leprae* cDNA from Mickle homogenization method; lane 2, RT-control of Mickle homogenization method; lane 3, *M. leprae* cDNA from the direct mincing method; lane 4, RT-control of the direct mincing method; lane 5, 1 ng *M. leprae* T-53 control DNA (positive control); (A) lane 6, human monocyte cDNA (negative control).

Table 2. RT-PCR Analysis of the Direct and Mickle Methods for Purification of *Mycobacterium leprae* RNA from Skin Biopsy Specimens from Multibacillary Leprosy Patients

Gene	RT-PCR	
	Direct Method	Mickle Method
<i>hsp18</i>	2/2 ^a	1/2
<i>gap</i>	2/2	0/2
<i>sodA</i>	2/2	1/2
<i>gyrA</i>	2/2	0/2
<i>rpoB</i>	2/2	0/2
<i>folP</i>	2/2	0/2

^aNumber of biopsies positive of two biopsies prepared by each method.

The tubes were processed in a vertical angular motion homogenizer (FastPrep[®] FP120 Instrument; Qbiogene) for 45 s at a speed setting of 6.5 and then allowed to sit for 5 min to cool. This procedure was repeated once. Tubes were held on ice for 5 min, and 200 μ L chloroform/isoamyl alcohol (CIA) (24:1) (Sigma) were added. The tubes were then vortex mixed for 1 min. The FastRNA Blue glass matrix was pelleted at 700 $\times$ *g* for 5 min at room temperature, and the entire liquid phase from each tube was transferred to a new 1.5-mL tube. Tubes were centrifuged at 14,000 $\times$ *g* for 10 min, and the aqueous phase from each tube was transferred to a new 1.5-mL tube. A 100- μ L aliquot of Cleanascite[™] HC (CPG Biotech, Lincoln Park, NJ, USA) was added to each tube, and the tubes were mixed on a rocker shaker for 10 min to remove excess lipid material. Cleanascite was pelleted at 14,000 $\times$ *g* for 1 min at room temperature, and each supernatant was added to a 1.5-mL tube containing 500 μ L CIA and vortex mixed for 10 s. The phases were separated by centrifugation at 14,000 $\times$ *g* for 2 min, and the aqueous phase was transferred to a new 1.5-mL tube, leaving 15% above the interface undisturbed. GlycoBlue[™] co-precipitant (Ambion, Austin, TX, USA) (1 μ L) and one-tenth volume of 5 M NH₄OAc were added, and the tubes were mixed. An equal volume of cold isopropanol was then added, and the tubes were mixed and then stored at -80°C for at least 16 h to precipitate RNA. Tubes were then

spun at 14,000 $\times$ *g* for 30 min at 4°C, and the RNA pellets were washed in 500 μ L of salt ethanol wash solution (SEWS) (from the FastRNA Kit-Blue), air-dried at room temperature for 5 min, and dissolved in 35 μ L of DEPC-treated water. DNA was removed from RNA preparations using the DNA-free[™] kit (Ambion), as specified by the manufacturer, and purified RNA was stored at -80°C.

To provide a positive control for all RT-PCR analyses, DNA was purified from 1 $\times$ 10¹⁰ *M. leprae* Thai-53 freshly harvested from infected nude mouse foot pad tissue using a previously described procedure (8).

Reverse Transcription

cDNA was made from 10 μ L of *M. leprae* RNA using the Advantage[™] cDNA Polymerase Mix, Advantage RT-for-PCR Kit, and random hexamers in a final volume of 50 μ L according to the manufacturer's recommendations (BD Biosciences Clontech, Palo Alto, CA, USA). Controls for DNA contamination consisted of: *M. leprae* RNA, incubated with the reverse transcription reaction reagents excluding the Moloney murine leukemia virus (MMLV)-RT (RT control); and cDNA from human monocyte RNA and *M. leprae* T-53 DNA (PCR specificity controls).

PCR and DNA Sequencing

PCR assays were initially developed using gene-specific primers (Table 1) and *M. leprae* T-53 DNA for six genes, representing several different *M. leprae* gene families (http://www.sanger.ac.uk/Projects/M_leprae/). These genes included: (i) *hsp18*, encoding the 18 kDa heat shock protein, HSP 16.7 (chaperones/heat shock); (ii) *gap*, encoding the glyceraldehyde 3-phosphate dehydrogenase (glycolysis); (iii) *sodA*, encoding the superoxide dismutase (detoxification); (iv) *gyrA*, encoding a putative DNA gyrase subunit A (DNA replication, repair, recombination, and restriction/modification); (v) *rpoB*, encoding the

β -subunit of RNA polymerase (RNA synthesis, RNA modification, and DNA transcription); and (vi) *folP*, encoding the dihydropteroate synthase (folic acid biosynthesis). Then, 5 μ L of each *M. leprae* cDNA preparation and RT controls were amplified in 50- μ L PCRs. The resultant PCR products were separated by agarose gel electrophoresis on 2% NuSieve[®] GTG/SeaKem[®] GTG (1:1) agarose gels (BioWhittaker, Rockland, ME, USA) in 1 $\times$ TAE buffer (4 M Tris-acetate, pH 8.0, 1 mM EDTA; Invitrogen). Ethidium bromide-stained gels were scanned using the GelDoc[®]2000 Instrument (Bio-Rad Laboratories, Hercules, CA, USA). The reproducibility and sensitivity of the direct homogenization/RNA extraction protocol were determined using *M. leprae gap* and *hsp18* PCR analysis with cDNA from 6 MB and 6 PB leprosy patients. PCR products were purified using QIAquick[™] PCR Purification Kit spin columns (Qiagen, Valencia, CA, USA) according to manufacturer's recommendations. The DNA sequence of each PCR fragment was obtained using 2 μ M of forward primer for each gene fragment, PCR product, and automated dideoxy DNA sequencing (GeneLab, SVM, LSU, Baton Rouge, LA, USA).

RESULTS AND DISCUSSION

Initial results of this study demonstrated that *M. leprae hsp18* and *gap* gene transcripts were detected in cDNA obtained from the direct single-tube homogenization/RNA extraction of a minced skin biopsy of a lepromatous leprosy patient but not from the RT control (Figure 1). In addition, lower levels of *hsp18* transcripts and no *gap* transcripts were detected using the Mickle method. When the expression of these and four other genes was further analyzed from two MB skin biopsies processed by each method, gene transcripts were detected for all of the genes in the cDNA obtained from the direct method (Table 2). However, transcripts were only detected for *hsp18* and *sodA* in the cDNA obtained from the Mickle method. These data suggested that there was a substantial loss of *M. leprae* RNA using the Mickle method. A plausible explanation for this is that large numbers of bacteria remain associated with the cellular debris, and

Figure 2. *Mycobacterium leprae* gap reverse transcription PCR (RT-PCR) analysis of *M. leprae* RNA purification from ethanol-fixed skin biopsies. Lane M, 100-bp DNA ladder (Promega); lane 1, cDNA from unfixed biopsy; lane 2, unfixed biopsy RT-control; lane 3, cDNA from 24 h ethanol-fixed biopsy; lane 4, 24 h ethanol-fixed biopsy RT-control; lane 5, cDNA from 48 h ethanol-fixed biopsy; lane 6, 48 h ethanol-fixed biopsy RT-control; lane 7, cDNA from 1 week ethanol-fixed biopsy; lane 8, 1 week ethanol-fixed biopsy RT-control; lane 9, cDNA from 1 year ethanol-fixed biopsy; lane 10, 1 year ethanol-fixed biopsy RT-control; lane 11, 1 ng *M. leprae* T-53 control DNA (positive control); lane 12, human monocyte cDNA (negative control).

since this debris is removed prior to RNA purification, many *M. leprae* are lost. We therefore concluded that direct homogenization/RNA extraction of finely minced biopsy tissues using an angular motion homogenizer and TRIzol

provides an improved method for simultaneously disrupting tissue, lysing both human and bacterial cells and extracting *M. leprae* RNA.

Using the direct method, *M. leprae* RNA was obtained from all MB skin

biopsies containing BIs > 2+ (Table 3), demonstrating the reproducibility of this method. Although this technique represents an improvement in *M. leprae* RNA yields from biopsies of MB leprosy patients compared to our previous procedure (8), no *M. leprae* RNA was detected in PB biopsies, (BI ≤ 2+), and therefore, gene expression analysis of *M. leprae* cannot be performed on PB biopsies, representing the tuberculoid end of the leprosy spectrum, using this technique.

While our earlier report demonstrated the feasibility of obtaining *M. leprae* RNA from biopsy tissue of leprosy patients and has used this RNA to determine the expression of a few genes (8), the direct method produced 150 µL cDNA, which is sufficient template to perform 30 individual 50-µL RT-PCRs. It is therefore anticipated that combining the RNA from several MB biopsies will be sufficient to produce enough cDNA to study >100 *M. leprae* genes using RT-PCR and potentially provide enough

Table 3. Purification of *Mycobacterium leprae* RNA from Skin Biopsies of Leprosy Lesions Using the Direct Single-Tube Homogenization/RNA Extraction Method

Biopsy No.	Bacterial Index	RT-PCR	
Multibacillary		<i>hsp18</i>	<i>gap</i>
10817	3–6+	+	+
16163	5–6+	+	+
72368	5+	+	+
71398	5+	+	+
75338	4–6+	+	+
49356	3–4+	+	+
Paucibacillary			
91547	2+	-	-
56187	2+	-	-
38572	2+	-	-
46111	2+	-	-
82599	2+	-	-
99837	1+	-	-

RNA for full genomic microarray analysis when this tool becomes available. Therefore, it is now possible to analyze the expression of a large number of *M. leprae* genes directly from biopsies obtained from MB leprosy patients. This represents a major advancement in the ability to study *M. leprae* gene expression directly from biopsy material and should help to define genes that are associated with intracellular survival of this human pathogen.

The present study also demonstrated that storage of skin biopsies in 70% ethanol efficiently preserved *M. leprae* RNA for up to 1 year for subsequent gene expression analysis (Figure 2). This is a significant finding, since there are very few tissue banks that contain well-characterized biopsies from leprosy patients, and therefore, ethanol-fixation of biopsies in the field and storage of these specimens at room temperature will provide a valuable source of specimens for *M. leprae* gene expression studies. In addition, since RNA transcription is terminated during ethanol-fixation of tissue, fixation of biopsies immediately after specimen acquisition should give an accurate assessment of

the specific gene transcripts required for intracellular survival of *M. leprae*.

In summary, this study describes the development and characterization of an improved method for the preservation and purification of *M. leprae* RNA directly from frozen or ethanol-fixed skin biopsies of leprosy lesions. It is anticipated that this method will provide highly purified *M. leprae* RNA to study gene expression from granulomatous lesions of leprosy patients and, thereby, help to identify the genes associated with intracellular survival and virulence of *M. leprae*. In addition, the availability of mRNA from *M. leprae* should assist to identify a suitable mRNA target for the development of a quantitative RT-PCR assay for *M. leprae* viability analysis.

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