

# Development of a Single-Tube, Cell Lysis-Based, Genus-Specific PCR Method for Rapid Identification of Mycobacteria: Optimization of Cell Lysis, PCR Primers and Conditions, and Restriction Pattern Analysis

Izhar U. H. Khan and Jagjit S. Yadav\*

Molecular Toxicology Division, Department of Environmental Health, University of Cincinnati Medical Center, Cincinnati, Ohio 45267-0056

Received 5 May 2003/Returned for modification 4 June 2003/Accepted 29 September 2003

**A single-tube PCR method was developed for efficient identification of nontuberculous mycobacteria (NTM) and their environmental isolates in about 3 h without conventional DNA isolation. The following three steps were optimized or developed: (i) a simple, 6-min direct cell lysis protocol as a PCR prestep for generation of DNA-template, (ii) an improved *Mycobacterium*-specific PCR amplification protocol with a broader species specificity using newly designed primers targeting a 228-bp region of the 65-kDa heat shock protein (*hsp*) gene and optimal PCR amplification conditions, and (iii) a genus-specific restriction analysis of the PCR product for conclusive identification of the unknown NTM isolates.**

Nontuberculous mycobacteria (NTM) are important causes of nosocomial infections and occupational illnesses. These organisms are commonly associated with natural ecosystems such as water supplies, aerosols, food, and soil (3, 4, 6, 7, 17). NTM that cause nosocomial infections are frequently associated with hospital water supplies and washing equipment. From an occupational health standpoint, NTM are considered causal agents for hypersensitivity pneumonitis, asthma, and bronchitis in machine workers exposed to metalworking fluids (MWF) and their aerosols, which are used in metalworking industries for cooling and lubrication (8, 10, 12, 14, 18). A method for early and reliable detection of mycobacteria from these environments might help minimize these illnesses. The existing practice of identification of mycobacteria from clinical and environmental sources includes isolation using enrichment and selective agar media, a method which often results in the collection of a large number of putative isolates for subsequent screening and confirmation by morphological, culture, and biochemical methods (20). Molecular methods such as PCR offer a significant alternative for rapid screening and identification of these bacteria (11). In practical terms, the major limiting steps of the PCR approach for the rapid screening are the extraction of amplifiable-quality genomic DNA and the availability of genus-specific primers with broad specificity for different species. Due to the complexity and rigidity of the cell walls of these acid-fast bacteria, several efforts have been reported for the rapid isolation of amplifiable genomic DNA by using physical, chemical, or enzymatic strategies or combinations thereof (1, 5, 9, 13, 16, 22). However, these cumbersome and/or time-consuming protocols failed to yield effective cell lysis, thereby preventing their use in routine screening and

diagnosis of diverse environmental species of *Mycobacterium*. The two existing genus-specific PCR-protocols, one based on a 16S rRNA gene (15) and the other based on *Mycobacterium*-specific 65-kDa heat shock protein (*hsp*), are applied for identification of mycobacteria (16). The latter protocol has received wider acceptance. However, this protocol fails to reliably amplify the target *hsp* sequence in some environmental species such as *Mycobacterium immunogenum*, which is an important newly identified NTM species widely associated with both clinical infections and pseudo-outbreaks (19) and with MWF implicated in hypersensitivity pneumonitis (18). Considering the above limitations, we designed the present study to develop a simple and rapid cell lysis protocol that would be usable as a prestep in PCR; we also sought to develop an improved genus-specific *hsp* gene-based PCR protocol applicable to a broader range of *Mycobacterium* species coupled with a genus-specific restriction analysis of the amplicon for more reliable detection and identification.

**Bacterial strains and isolates.** Four reference strains of the genus *Mycobacterium*, *M. chelonae* (ATCC 35752), *M. smegmatis* (ATCC 19420), *M. immunogenum* (ATCC 700506), and *M. bovis* BCG (ATCC 35741), originating from both clinical and environmental sources, and a total of eight isolates obtained from different types of used MWF originating from different industrial plants at diverse geographical locations were used for methods development and comparisons. In ad-

TABLE 1. Different lysis reagents used for cell lysis

Reagent type	Composition (%) of:	
	SDS	Triton X-100
1	10	10
2	10	1
3	5	2
4	2	5
5	2	10

\* Corresponding author. Mailing address: Molecular Toxicology Division, Department of Environmental Health, University of Cincinnati Medical Center, Kettering Laboratory, 3223 Eden Ave., Room 138, Cincinnati, OH 45267-0056. Phone: (513) 558-4806. Fax: (513) 558-4397. E-mail: Jagjit.Yadav@uc.edu.

TABLE 2. Different thermal regimes used for cell lysis

Step no.	Parameters for thermal regime <sup>a</sup> :									
	I		II		III		IV		V	
	H	T	H	T	H	T	H	T	H	T
1	80	1	80	1	80	1	95	1	98	5
2	8	0.5	4	0.5	4	0.5	4	0.5	4	1
3	95	5	95	8	95	5	95	5		
4	8	1	4	1	4	1	4	1		
5	95	3								
6	4	1								

<sup>a</sup> H, heating-cooling temperature (°C); T, time (minutes).

dition, *Bacillus* sp. (B-22), *Escherichia coli* (DH 5 $\alpha$ ), and *Pseudomonas fluorescens* (ATCC 13525) were included as negative controls. For isolation of mycobacteria, samples were plated on Middlebrook 7H10 agar supplemented with oleic acid-albumin-dextrose-catalase enrichment and Lowenstein-Jensen agar slants and incubated at 37°C for up to 10 days. Putative *Mycobacterium* colonies selected based on growth rate, colony morphology, and acid-fast staining reactions were pursued further for confirmation by using the PCR method.

**Cell lysis optimization.** Five different cell lysis reagents were prepared using various concentrations of sodium dodecyl sulfate (SDS) and Triton X-100 (Sigma, St. Louis, Mo.) in Tris-EDTA buffer (pH 8.0) as presented in Table 1. Initially, a six-step thermal regime involving different heating-cooling temperatures and incubation times was used in combination with each of the formulated lysis reagents using the GeneAmp PCR system model 9700 (Applied Biosystems, Foster City, Calif.). In order to develop a simpler thermal regime, four other combinations involving shorter heating-cooling regimes were examined, as shown in Table 2. By using a sterile autopipette tip, we transferred an isolated mycobacterial colony to an amplification tube (0.2 ml) containing 5  $\mu$ l of a selected lysis reagent and resuspended by gentle mixing. The contents were subjected to lysis under the appropriate combinations of chemical lysis reagents and thermal regimes listed in Tables 1 and 2 by using the GeneAmp PCR system model 9700. The tube containing the crude DNA-containing lysate was used directly for the subsequent PCR.

**Genus-specific PCR.** We evaluated two distinct protocols for their applicability to diverse mycobacterial species. The first was an existing genus-specific PCR protocol based on the *hsp* gene and involving amplification of a 439-bp region using the recommended primer pair (16); the second was its modified version which uses newly designed broad-spectrum genus-specific PCR primers and optimal amplification conditions (Table 3). Direct cell lysis was used to generate the DNA template.

The PCR amplification reaction with either of the primer pairs was performed by using Ex-*Taq* DNA polymerase and the compatible PCR reagents (Panvera, Madison, Wis.) in the GeneAmp PCR system model 9700. The reaction mixture (50  $\mu$ l) consisted of 5  $\mu$ l of DNA template (cell lysate), 1 $\times$  Ex-*Taq* buffer with MgCl<sub>2</sub>, 200  $\mu$ M of each of the four deoxynucleoside triphosphates, 100 ng of both the forward and reverse primers, and 1.25 U of ex-*Taq* DNA polymerase. The presence of PCR products was determined by electrophoresing 10  $\mu$ l of the reaction product on a 1% Trevigel gel matrix (Trevigen, Gaithersburg, Md.) with 1 $\times$  Tris-acetate-EDTA buffer containing ethidium bromide (0.5  $\mu$ g/ml) and using 5  $\mu$ l of a 100-bp DNA size marker (PGC Scientifics [Frederick, Md.] and Invitrogen [Carlsbad, Calif.]). Each PCR product was further quantitated and photographed by using the Kodak EDAS 290 gel documentation system (Kodak, Rochester, N.Y.).

**Amplicon identification by restriction analysis and DNA sequencing.** The mycobacterial origin of the PCR products was confirmed by restriction analysis. A protocol was optimized based on the unique *NarI* restriction site in these amplicons as determined by aligning the available *hsp* gene sequences for this region. A randomly selected PCR amplicon (corresponding to the isolate M-JY3) was also sequenced at the University of Cincinnati's DNA core facility for confirmation. When analyzed with a BLAST search against available gene database, the sequence showed homology with *hsp* gene sequences for mycobacteria. The sequence showed closest homology (99%) with the *M. chelonae hsp* sequence.

Of the five lysis reagents tested with a six-step heating regime (Tables 1 and 2), only reagent 4 (containing 2% SDS and 5% Triton X-100) and reagent 5 (2% SDS and 10% Triton X-100) yielded an effective cell lysis result as a prestep to PCR amplification (Fig. 1A). However, for some mycobacterial isolates such as M-JY4, lysis reagent 4 was not as effective as lysis reagent 5 (data not shown). The results showed that the proportional ratio of the two chemicals (SDS and Triton X-100) is important for achieving optimal cell lysis without interfering with the subsequent amplification reaction step. Hence, reagent 5 was selected as the optimum lysis reagent and was used for subsequent thermal regime optimizations. The six-step thermal regime was further modified and shortened by examining four other heating-cooling regimes (Fig. 1B). The five regimes tested gave nearly comparable cell lysis results for all reference strains and culture isolates when used with the optimal lysis reagent 5. Hence, the shortest thermal regime (V) involving heating at 98°C for 5 min followed by cooling at 4°C for 1 min was selected for the optimized protocol. This shows that the heating regime is not as critical as the chemical composition of the lysis reagent in achieving the desired cell lysis.

TABLE 3. Oligonucleotide primers and conditions used for *hsp*-based PCRs for mycobacteria

Amplicon length (bp)	Direction and sequence of primer		PCR program
	Forward (5'-3')	Reverse (5'-3')	
439	ACCAACGATGGTGTGTCCAT	CTTGTGCAACCGCATACCCT	A <sup>a</sup>
228	CTGGTCAAGGAAGTCTGCG	GATGACACCCTCGTTGCCAAC	B <sup>b</sup>

<sup>a</sup> Program A consisted of 35 cycles of 94°C for 60 s, 60°C for 60 s, and 72°C for 60 s.

<sup>b</sup> Program B consisted of 30 cycles of 95°C for 15 s, 58°C for 15 s, and 72°C for 30 s.

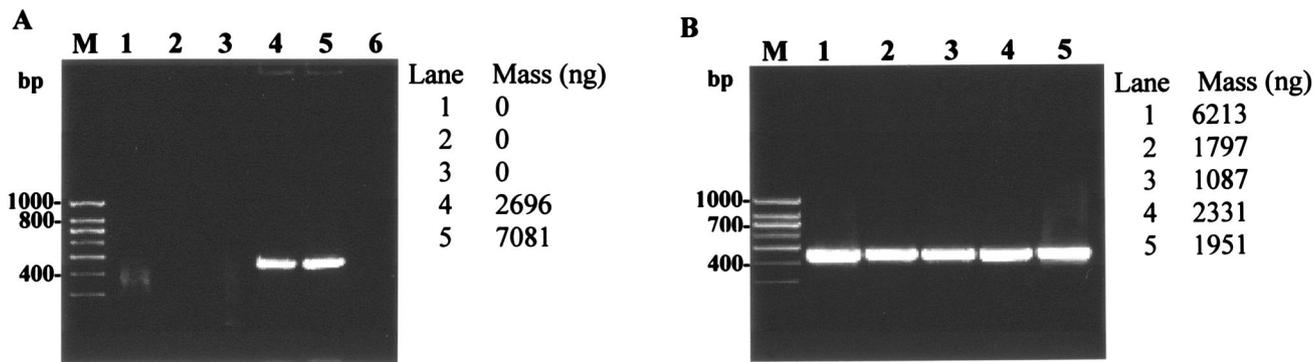


FIG. 1. Optimization of cell lysis protocol for direct PCR-based detection of mycobacteria. (A) Optimization of chemical reagent for cell lysis as a prestep in *hsp*-based PCR amplification of *M. smegmatis*. Lanes 1 to 5, comparison of five different lysis reagents (reagents 1 through 5) containing various concentrations of SDS and Triton X-100 in Tris-EDTA buffer used in combination with thermal regime I (Table 1); lane 6, negative control (no template DNA). (B) Optimization of heating regimes for cell lysis of *M. smegmatis* using lysis reagent 5 (Table 1) in the *hsp*-based PCR. Lanes 1 to 5, comparison of thermal regimes 1 through 5 (Table 2); PCR amplification was based on mycobacterium-specific *hsp* gene with an expected 439-bp amplicon as described in the text. Lane M, 100-bp DNA size marker (PGC Scientific).

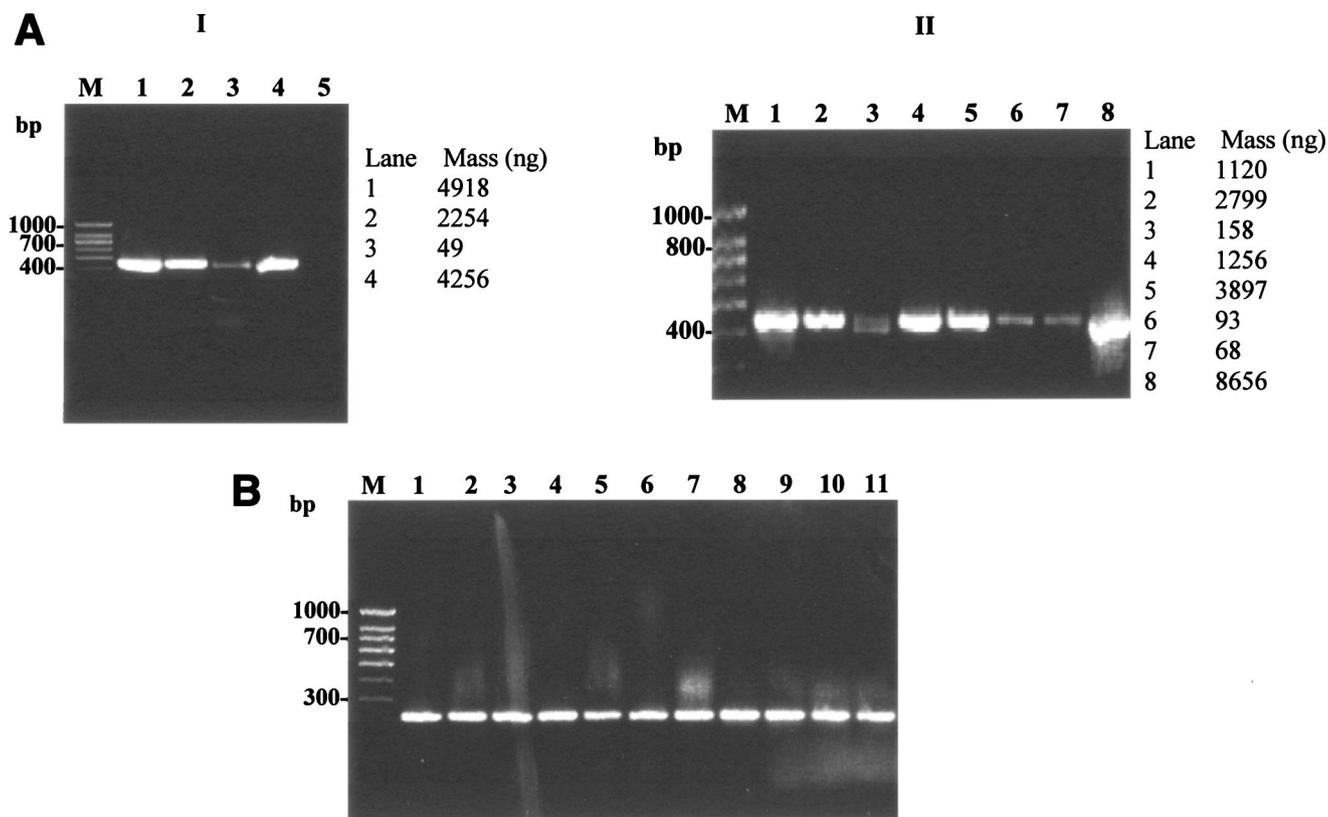


FIG. 2. Evaluation of the optimized protocols for cell lysis and *hsp*-based PCR for mycobacteria. (A) Evaluation of the optimized cell lysis protocol (lysis reagent 5, thermal regime V) for *hsp*-based PCR amplification (439 bp) of mycobacterial reference strains and MWF isolates. Panel I: lanes 1 to 4, *M. smegmatis*, *M. chelonae*, *M. immunogenum*, and *M. bovis*; lane 5, no-template control. Panel II: lanes 1 to 8, *M. smegmatis*, *M. chelonae*, *M. immunogenum*, and *Mycobacterium* isolates M-JY1, M-JY2, M-JY3, M-JY4 and M-JY5; lane M, 100-bp DNA size marker (PGC Scientific). PCR primers and conditions were the same as described in the legend of Fig. 1. (B) Evaluation of new *hsp*-based PCR primers and modified PCR conditions for genus-specific PCR amplification (228 bp) of mycobacteria. Lanes 1 to 3, *M. smegmatis*, *M. chelonae*, and *M. immunogenum*; lanes 4 to 11, *Mycobacterium* isolates M-JY1, M-JY2, M-JY3, M-JY4, M-JY5, M-JY6, M-JY7 and M-JY8; lane M, 100-bp DNA size marker (PGC Scientific). Cells were lysed using the optimized direct cell lysis method as described for panel A, and the lysates were amplified using new PCR primers and conditions described in the text for amplification of a 228-bp PCR product of the *hsp* gene.

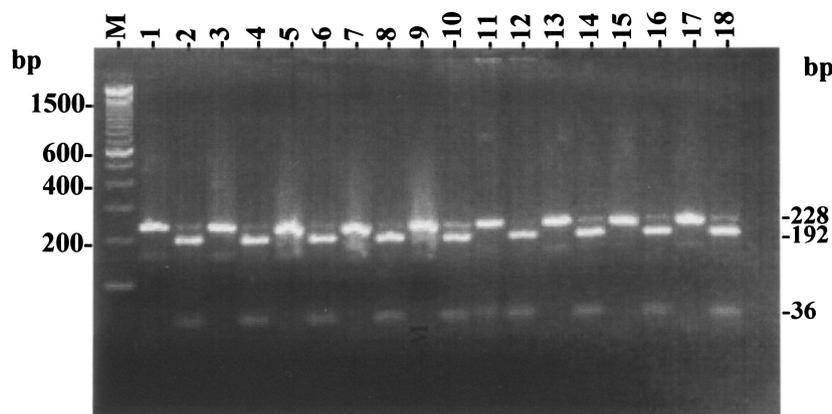


FIG. 3. Confirmation of the mycobacterial origin of PCR amplicons (228 bp) based on *NarI* restriction patterns (192 and 36 bp). Shown are gel patterns for *M. chelonae* reference strain (lane 1 and 2) and for NTM field isolates M-JY1 (lanes 3 and 4), M-JY2 (lanes 5 and 6), M-JY3 (lanes 7 and 8), M-JY4 (lanes 9 and 10), M-JY5 (lanes 11 and 12), M-JY6 (lanes 13 and 14), M-JY7 (lanes 15 and 16), and M-JY8 (lanes 17 and 18); lane M, 100-bp DNA size marker (Invitrogen). For each isolate, the two lanes represent the original amplicon (8  $\mu$ l) and its *NarI* restriction digestion product, respectively.

Due to the relatively resistant cell walls of mycobacteria, various combinations of chemicals (such as SDS and Triton X-100), mechanical devices, heat, and solvents are often applied for the conventional extraction and purification of DNA from mycobacteria (1, 2, 15). However, these protocols are cumbersome and often time-consuming, and they fail to yield PCR-quality DNA; they also increase the chances of contamination or exposure during analysis. Our developed lysis protocol involves direct single-tube cell lysis in the PCR tube using a one-step heating cycle and has the potential for high-throughput applications as well as offering a safer alternative to conventional protocols. Single-tube cell lysis also makes the protocol ideal for use in quantitative PCR applications. The direct cell lysis procedure was also found to be equally effective against other bacteria, including both gram-negative and gram-positive bacteria (data not shown).

A 439-bp region of the *hsp* gene has been used as a PCR target for the identification and species differentiation in mycobacteria by restriction enzyme analysis or nucleotide sequencing (16, 18, 19, 21). In the preceding amplifications using this existing *hsp*-based PCR method, one of the four reference strains and two of the eight isolates yielded weak amplification signals (Fig. 2A [I and II]). Quantification of the amplified PCR products using the Kodak gel documentation system showed a range of DNA concentrations (Fig. 2A [I and II]). Amplicon signal variability among the reference strains (Fig. 1 and 2A) was traced to nucleotide sequence variation in the primer binding regions based on multiple alignment of their *hsp* sequences using MegAlign 5.0 software (DNASTAR, Inc., Madison, Wis.). Hence, in order to achieve unambiguous PCR amplification for all NTM species, including the three strains showing weak amplification, a new genus-specific primer pair based on the sequence alignment of the available *hsp* gene sequences was designed. We selected modified PCR amplification conditions compatible with the melting point values for the new primers as determined by using Gene Runner software. When the optimized lysis protocol was used, the modified protocol yielded a 228-bp PCR product of comparable

intensity and concentration for all tested isolates and strains (Fig. 2B). All control strains of various nonmycobacterial genera were negative, thus confirming the genus-specific nature of the developed protocol. Moreover, the modified PCR conditions optimized for the new primers resulted in a relatively rapid amplification compared to that of the existing method (Table 3).

The *NarI* digestion of the amplicons, performed using recommended digestion conditions (New England Biolabs, Beverly, Mass.), yielded two fragments (192 and 36 bp) for the reference strains and the eight isolates of mycobacteria (Fig. 3). The developed genus-specific protocol for restriction pattern analysis of the amplicons enabled rapid confirmation of the mycobacterial identity of the environmental isolates.

In conclusion, the developed direct cell lysis-based PCR protocol requires less than 2 h for identification of a putative mycobacterial isolate, involving a 6-min lysis step followed by a 50-min amplification and a 30- to 60-min gel analysis. The single-tube protocol is potentially adaptable as a diagnostic tool in routine analytical and clinical laboratories for rapid or high-throughput screening with minimized risks of contamination or exposure. Confirmation of the mycobacterial origin of the PCR product may require an additional hour for restriction analysis. The detection time (2.5 to 3.0 h) could be further shortened in situations in which the real-time format for PCR is available, as the use of such a format would allow early detection of the amplicon as well as confirmation of the mycobacterial origin of the amplicon based on melting-curve analysis.

**Nucleotide sequence accession number.** The sequence described herein has been submitted to GenBank (accession number AY322157).

The study was supported by a Centers for Disease Control and Prevention National Institute of Occupational Safety and Health grant (number 1R01OH007364-01A1 to J.S.Y.).

We thank Milacron management for providing the used MWF samples for isolation of mycobacteria.

## REFERENCES

1. Armand, M. O., M. Mestdagh, and F. Porteaux. 2001. DNA isolation from chloroform/methanol-treated mycobacterial cells without lysozyme and proteinase K. *BioTechniques* **30**:272–274.
2. Bollet, C., M. J. Gevaudan, X. de Lamballerie, C. Zandotti, and P. D. Mico. 1991. A simple method for the isolation of chromosomal DNA from gram positive or acid-fast bacteria. *Nucleic Acids Res.* **19**:1955.
3. Covert, T. C., M. R. Rodgers, A. L. Reyes, and G. N. Stelma, Jr. 1999. Occurrence of nontuberculous mycobacteria in environmental samples. *Appl. Environ. Microbiol.* **65**:2492–2496.
4. Dailoux, M., C. Laurain, M. Weber, and P. Hartemann. 1999. Water and nontuberculosis mycobacteria. *Water Res.* **33**:2219–2228.
5. De Baere, T., R. de Mendonca, G. Claeys, G. Verschraegen, W. Mijs, R. Verhelst, S. Rottiers, L. Van Simaey, C. De Ganck, and M. Vanechoutte. 2002. Evaluation of amplified rDNA restriction analysis (ARDRA) for the identification of cultured mycobacteria in a diagnostic laboratory. *BMC Microbiol.* **2**:4–15.
6. Falkinham, J. O., III. 2002. Nontuberculous mycobacteria in the environment. *Clin. Chest Med.* **23**:529–551.
7. Fraser, V., and R. J. Wallace, Jr. 1996. Nontuberculous mycobacteria, p. 1224–1338. *In* C. Glen. Mayhall (ed.), *Hospital epidemiology and infection controls*. Williams & Wilkins, Baltimore, Md.
8. Freeman, A., J. Lockey, P. Hawley, P. Biddinger, and D. Trout. 1998. Hypersensitivity pneumonitis in a machinist. *Am. J. Ind. Med.* **34**:387–392.
9. Kirschner, P., B. Springer, U. Vogel, A. Meier, A. Wrede, M. Kiekenbeck, F. C. Bange, and E. C. Bottger. 1993. Genotypic identification of mycobacteria by nucleic acid sequence determination: report of a 2-year experience in a clinical laboratory. *J. Clin. Microbiol.* **31**:2882–2889.
10. Lummus, Z. L., J. E. Lockey, and I. L. Bernstein. 1998. Microbial flora of metalworking fluids associated with occupational respiratory disorders. *J. Allergy Clin. Immunol.* **1**:166.
11. Miyazaki, Y., H. Koga, S. Kohno, and M. Kaku. 1993. Predictive value of PCR applied to clinical samples for *Mycobacterium tuberculosis* detection. *J. Clin. Microbiol.* **31**:2228–2232.
12. Muilenberg, M. L., H. A. Berge, and T. Sweet. 1993. Hypersensitivity pneumonitis and exposure to acid-fast bacilli in coolant aerosols. *J. Allergy Clin. Immunol.* **91**:311.
13. Reischl, U., M. Pulz, W. Ehret, and H. Wolf. 1994. PCR-based detection of *Mycobacterium* in sputum samples using a simple and reliable DNA extraction protocol. *BioTechniques* **17**:844–845.
14. Shelton, G. B., W. D. Flanders, and K. G. Morris. 1999. *Mycobacterium* sp. as a possible cause of hypersensitivity pneumonitis in machine workers. *Emerg. Infect. Dis.* **5**:270–273.
15. Talaat, A. M., R. Reimschuessel, and M. Trucksis. 1997. Identification of mycobacteria infecting fish to the species level using polymerase chain reaction and restriction enzyme analysis. *Vet. Microbiol.* **58**:229–237.
16. Telenti, A., F. Marchesi, M. Balz, F. Bally, E. C. Bottger, and T. Bodmer. 1993. Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. *J. Clin. Microbiol.* **31**:175–178.
17. Wallace, R. J., Jr., V. A. Silcox, M. Tsukamura, B. A. Brown, J. O. Kilburn, W. R. Butler, and G. Onyi. 1993. Clinical significance, biochemical features, and susceptibility patterns of sporadic isolates of the *Mycobacterium chelonae*-like organism. *J. Clin. Microbiol.* **31**:3231–3239.
18. Wallace, R. J., Jr., Y. Zhang, R. W. Wilson, L. Mann, and H. Rossmore. 2002. Presence of a single genotype of the newly described species *Mycobacterium immunogenum* in industrial metalworking fluids associated with hypersensitivity pneumonitis. *Appl. Environ. Microbiol.* **68**:5580–5584.
19. Wilson, R. W., V. A. Steingrube, E. C. Bottger, B. Springer, B. A. Brown-Elliott, V. Vincent, K. C. Jost, Jr., Y. Zhang, M. J. Garcia, S. H. Chiu, G. O. Onyi, H. Rossmore, D. R. Nash, and R. J. Wallace, Jr. 2001. *Mycobacterium immunogenum* sp. nov., a novel species related to *Mycobacterium abscessus* and associated with clinical disease, pseudo-outbreaks and contaminated metalworking fluids: an international cooperative study on mycobacterial taxonomy. *Int. J. Syst. Evol. Microbiol.* **51**:1751–1764.
20. Yadav, J. S., I. U. H. Khan, F. Fakhari, and M. B. Soellner. 2003. DNA-based methodologies for rapid detection, quantification, and species- or strain-level identification of respiratory pathogens (*Mycobacteria* and *Pseudomonads*) in metalworking fluids. *Appl. Occup. Environ. Hyg.* **18**:966–975.
21. Yakrus, M. A., S. M. Hernandez, M. M. Floyd, D. Sikes, W. R. Butler, and B. Metchock. 2001. Comparison of methods for identification of *Mycobacterium abscessus* and *M. chelonae* isolates. *J. Clin. Microbiol.* **39**:4103–4110.
22. Yoder, S., C. Argueta, A. Holtzman, T. Aronson, O. G. Berlin, P. Tomasek, N. Glover, S. Froman, and G. Stelma, Jr. 1999. PCR comparison of *Mycobacterium avium* isolates obtained from patients and foods. *Appl. Environ. Microbiol.* **65**:2650–2653.