JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 2010, p. 247–250 Vol. 48, No. 1
0095-1137/10/$12.00 doi:10.1128/JCM.01084-09

Rapid Method for Identification of Six Common Species of Mycobacteria Based on Multiplex SNP Analysis▽

Hongxiu Wang,[1] Jun Yue,[1]* Min Han,[1] Jinghui Yang,[1] and Yanlin Zhao[2]

Key Laboratory of Mycobacteria Tuberculosis, Shanghai Pulmonary Hospital, Tongji University, Shanghai 200433, People's Republic of China,[1] and Tuberculosis Reference Laboratory, Beijing Thoracic Tumor and Tuberculosis Research Institute, Beijing 101149, People's Republic of China[2]

Received 3 June 2009/Returned for modification 23 July 2009/Accepted 26 October 2009

A multiplex method using the SNaPshot technique was developed to screen for six common mycobacterial species: *Mycobacteria tuberculosis*, *M. avium*, *M. intracellulare*, *M. chelonae*, *M. kansasii*, and *M. gordonae*. A total of 468 mycobacterial clinical isolates were subjected to analysis for the presence of the six mycobacterial species by the multiplex SNaPshot method. Of the 468 mycobacterial isolates, 464 (99.15%) could be correctly identified by this assay. The multiplex SNaPshot technique is a promising discriminatory tool for rapid and accurate identification of frequently encountered clinical mycobacterial species.

Even though *Mycobacterium tuberculosis* continues to be a serious health concern worldwide, it has been increasingly recognized that nontuberculous mycobacteria (NTM) are important human pathogens (4, 16, 23). NTM are ubiquitous organisms, with nearly 100 different species found in soil and water that can act as opportunistic pathogens in humans, causing a wide variety of skin and soft tissue infections, lymphadenitis, and lung disease (6, 15). The early differentiation of *M. tuberculosis* from NTM and the identification of species among NTM are crucial for immediate implementation of the appropriate therapy because susceptible drugs vary widely among different species (9).

Conventionally, identification of mycobacteria is carried out by time-consuming biochemical tests that are not always accurate (5, 17, 25). Chromatographic techniques such as high-performance liquid chromatography (HPLC), gas-liquid chromatography (GLC), and thin-layer chromatography (TLC) are labor-intensive, difficult, or expensive (21, 26). DNA sequence analysis of the 16S rRNA gene region is now regarded as the gold standard for the identification of mycobacteria (13, 22, 25, 27). However, equipment and running costs are high. Simple genotypic assays for the identification of mycobacteria, such as Accuprobe (Gen-Probe Inc., San Diego, CA) (1), INNO-LiPA (27), and Genotype Mycobacterium (Hain Diagnostika) (19) are available commercially. Even though these tests are simple, they are often suited for small test volumes and are too expensive for high-throughput laboratories to use in a routine clinical diagnostic setting.

In this study, we developed a novel multiplex SNaPshot method using fluorescently labeled terminators and capillary electrophoresis to screen for six common clinically encountered mycobacterial species (*M. tuberculosis*, *M. avium*, *M. intracellulare*, *M. chelonae*, *M. kansasii*, and *M. gordonae*) based on eight single nucleotide polymorphisms (SNPs) located in conserved regions of the 16S rRNA and Hsp65 genes.

MATERIALS AND METHODS

Reference strains and clinical isolates. Thirteen reference strains of mycobacteria and 468 clinical isolates (Table 1) were subjected to the multiplex SNaPshot analysis. The reference strains and 20 clinical isolates were kindly provided by Beijing Thoracic Tumor and Tuberculosis Research Institute. A total of 448 clinical isolates were obtained in our microbiology laboratory, which is the reference laboratory for tuberculosis diagnosis in southeastern China. All isolates were identified by their colony morphology, pigmentation, growth characteristics, biochemical reactions (such as nitrate reduction and 3-day arylsulphatase test) (28), and restriction fragment analysis of the heat shock protein gene (30). When necessary, Accuprobe assays (Gen Probe, Inc.) and 16S rRNA gene sequencing were also used to differentiate the isolates to species level.

DNA preparation. DNA template was prepared by boiling a 1-μl loopful of actively growing mycobacteria grown on Lowenstein-Jensen medium in 200 μl of Milli-Q water for 10 min, followed by rapid chilling in an ice bath for 5 min. The aliquot of supernatant after centrifugation was used as the DNA template.

Primer design. The PCR and SNaPshot primers designed for the study are provided in Table 2 and Table 3. The primers for SNaPshot extension reactions were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

PCR amplification. PCR amplification was performed in 20 μl of a reaction mixture containing 1× GC buffer I (Takara), 3.0 mM Mg^{2+}, a 0.3 mM concentration of each deoxynucleoside triphosphate (dNTP), a 0.1 μM concentration of each primer, 1 U of Hotstar *Taq* polymerase (Qiagen Inc.), and 1 μl of template DNA. The PCR cycling conditions were 95°C for 15 min; 12 cycles of 94°C for 20 s, 65°C for 40 s (decreasing 0.5°C/cycle), and 72°C for 100 s; 23 cycles of 94°C for 20 s, 59°C for 30 s, and 72°C for 90 s; and a final step at 72°C for 2 min. Following PCR, 15 μl of the PCR product was incubated with 5 units of shrimp alkaline phosphatase (SAP; Applied Biosystems) and 2 units of ExoI (Applied Biosystems) for 60 min at 37°C, followed by 15 min at 75°C for enzyme inactivation.

SNaPshot multiplex single base extension reaction. The SNaPshot extension reactions were carried out in a final volume of 10 μl containing 2 μl of purified PCR product, 5 μl of SNaPshot Ready Mix (ABI Prism SNaPshot Multiplex Kit), 1 μl of a mixture of each of the nine SNaPshot primers (each at 0.8 μM), and 2 μl of ultrapure water. The cycling conditions were as follows: hot start at 96°C for 1 min, followed by 28 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 s, and extension at 60°C for 30 s, with a final step at 60°C for 1 min. After primer extension, the unincorporated fluorescently labeled dideoxynucleoside triphosphates (ddNTPs) were removed by the addition of 1 unit of SAP and incubation for 1 h at 37°C for deactivation. The SNaPshot reaction products (0.5 μl) were mixed with 0.5 μl of Liz120 size standard (Applied Biosystems) and 9 μl of Hi-Di formamide and denatured at 95°C for 5 min. The fluorescently labeled fragments were resolved by capillary electrophoresis on an ABI Prism

* Corresponding author. Mailing address: Key Laboratory of Mycobacteria Tuberculosis, Shanghai Pulmonary Hospital affiliated to Tongji University, 507 Zhengming Rd., Shanghai 200433, People's Republic of China. Phone: 86 21 65115006 3027. Fax: 86 21 5566 3327. E-mail: yuejunnan@yahoo.com.cn.

▽ Published ahead of print on 4 November 2009.

TABLE 1. Mycobacterial strains tested in this study

Mycobacterium species	Strain(s) and/or sample type (no. of isolates)
M. tuberculosis	ATCC 27294, ATCC 35820, ATCC 35822, ATCC 35826, ATCC 35827, clinical isolates (169)
M. kansasii	ATCC 12478, clinical isolates (38)
M. gordonae	ATCC 14470, clinical isolates (48)
M. avium	ATCC 25291, ATCC 35718, clinical isolates (41)
M. intracellulare	ATCC 13950, ATCC 35772, clinical isolates (68)
M. chelonae	ATCC 35749, ATCC 35752, clinical isolates (87)
M. kumamotonense	Clinical isolate (1)
M. fortuitum	Clinical isolates (8)
M. parascrofulaceum	Clinical isolates (6)
M. monacense	Clinical isolates (2)

3130×1 genetic Analyzer (Applied Biosystems) and electrophoresed using Pop-7 Polymer. The resulting data were analyzed with GeneMapper, version 4.0, software (Applied Biosystems).

RESULTS

Designed primers. Two sets of genus-specific primers for mycobacteria (Table 2) were designed to amplify a 354-bp fragment of Hsp65 and a 436-bp fragment of 16S rRNA, respectively. Table 3 shows the sequences of all forward and reverse extension primers, which were designed to anneal immediately adjacent to the nucleotide at eight SNPs (Table 4) at positions 125, 141, 231, 264, and 471 in the alignment of 16S rRNA gene and at positions 163, 235, and 265 in the alignment of Hsp65.

Genotype by the multiplex SNaPshot method. A method for simultaneous identification of the six most common mycobacterial species has been developed in this study. The SNaPshot technique (Applied Biosystems) is a method used specifically to genotype single nucleotide polymorphisms (SNPs). It involves PCR amplifications of a region of interest, purification of the product, and annealing of a SNaPshot primer that ends one nucleotide 5′ of a known SNP. A single base extension reaction is then performed in the presence of the four fluorescently labeled ddNTPs. Upon excitation with a laser, the different fluorescent dyes emit a color that is specific for each ddNTP, i.e., green for A, blue for G, black for C, and red for T. By SNaPshot analysis, each SNP's genotype is determined by both the position (size) of the peak and the color of the emitted fluorescence. In the case of our study, the electropherogram (Fig. 1) generated from each species demonstrated that the mycobacterial species examined could be clearly identified and differentiated from one another by this genotyping technique.

TABLE 2. The sequences of primers used in this research

Primer target	Orientation	Sequence (5′→3′)
16S rRNA	Forward	GAGTGGCGAACGGGTGAGTAAC
16S rRNA	Forward	CCTACGTATTACCGCGGCTGCT
Hsp65	Forward	GAGGACCCGTACGAGAAGATCG
Hsp65	Reverse	GTTGGACTCCTCGASGGTGATG

TABLE 3. Extension primer sequences for the SNaPshot reaction

Primer target	Primer name	Sequence (5′→3′)
16S rRNA	125SF	TTTTTTTTTTAACGGGTGAGTAACA CGTGGG
	141SR	TTTTTTTCAGTTTCCCAGGCTTAT CCCR
	231SR	TTTTTAAGCTGATAGGCCGCGGGC
	264SR	CGTCGTCGCCTTGGTRGGCC
	471SR1	TTTTTTTTTTTTTTTTTTTTTTTTTTTG CTTCTTCTCCACCTACCGTCA
	471SR2	TTTTTTTTTTTTTTTTTTTTTTTTTC CTTCTTCTGTAGGTACCGTCA
Hsp65	163SR	TTTTTTTTTTTTTTTTTTTTTTTTTTT TTTTTCCTTGGTCTCGACSTCCT TGGC
	235SR	TTTTTTTTTTGTCCATSGCCTCGGC GATCAGGTC
	265SF	TTTTTTTTTTTTTTTTTTATCGCCGA GGCSATGGACAAGGT

Sensitivity and specificity. To validate the novel multiplex SNaPshot method, we analyzed 13 reference strains and 468 clinical isolates. All standard strains tested produced results as expected. For 468 DNA samples, the sensitivity of the assay was evaluated as 100% for *M. tuberculosis*, *M. gordonae*, *M. avium*, and *M. intracellulare*; 97.7% for *M. chelonae*; and 94.7% for *M. kansasii*; the specificity was 100% for all six of the most common mycobacterial species. A total of 99.15% (464/468) of mycobacterial isolates could be correctly identified by this assay. Only four mycobacterial isolates produced ambiguous identification results by multiplex SNaPshot assay.

DISCUSSION

In recent years, with an increase in diseases caused by nontuberculous mycobacteria (NTM) (8, 10) and advancement in mycobacterial culture methods (11), a substantial workload consisting of mycobacterium species identification has been imposed on routine clinical laboratories. This is especially remarkable in high-throughput laboratories where many mycobacterial isolates have to be rapidly identified in order for results to be clinically relevant.

In our study, we sought to establish a new molecular method for identification of mycobacteria that, on the one hand, would be capable of simultaneously identifying several of the most common mycobacteria to the species level with high accuracy

TABLE 4. Single nucleotide polymorphisms in 16S rRNA and Hsp65 gene sequences in six mycobacterium species

Organism	Sequence at the indicated position of:							
	16s rRNA					Hsp65		
	125	141	231	264	471	163	235	265
M. tuberculosis	T	T	A	C	T	C	T	G
M. avium	C	T	G	C	T	G	C	C
M. chelonae	T	C	A	T	G	C	C	T
M. gordonae	T	T	G	T	C	G	T	C
M. intracellulare	C	T	G	T	T	G	T	C
M. kansasii	C	C	G	C	T	C	C	C

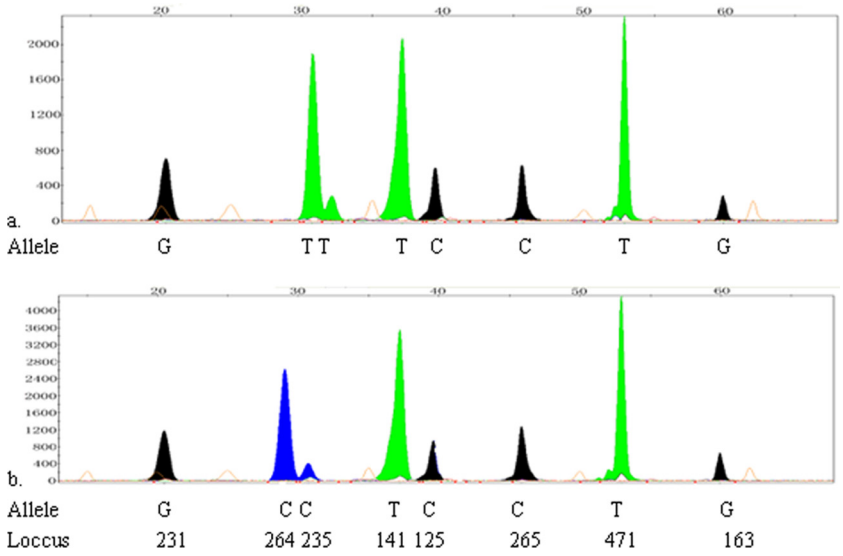

FIG. 1. Electropherograms for *M. intracellulare* (a) and *M. avium* (b) by multiplex SNaPshot analysis. The *x* axis represents the size (bp) of the primer pair with the incorporated nucleotides, while the *y* axis corresponds to the relative fluorescent units of the peak. Each fluorescent dye corresponds to a different nucleotide: blue represents G, green represents A, red represents T, and black represents C. The orange peak represents the size standard. The allele labeled below the peak is the same as the base incorporated when the forward extension primer is used; it is complementary to the base incorporated when the reverse extension primer is used.

and reliability and, on the other hand, would be simple enough for application even in routine laboratories. Hence, we developed the multiplex SNaPshot assay to simultaneously identify pathogenic tuberculosis mycobacteria and the five most common NTM species, namely, *M. avium*, *M. intracellulare*, *M. chelonae*, *M. gordonae*, and *M. kansasii*, in a single assay. Although the multiplex SNaPshot method for single-nucleotide polymorphism analysis is one of the most stimulating SNP genotyping techniques in genetic research (3, 2, 7, 24, 29), this is the first attempt to employ it in identification mycobacterial species.

In our study, 468 mycobacterial isolates from clinical specimens were analyzed by multiplex SNaPshot assay. The assay showed high sensitivities (94.7 to 100%) and 100% specificities. A total of 99.15% (464/468) of the mycobacterial isolates could be correctly identified by this assay. These results revealed the suitability of applying this technique in a clinical laboratory for the screening of commonly encountered mycobacterial species, especially in a high-throughput laboratory; this test can help the laboratory handle more than 80% of NTM identifications in a highly effective way, and thus more resources could be saved for other laboratory activities. Of two *M. kansasii* isolates, one gave mixed signals to both *M. kansasii* and *Rhodococcus* bacteria, which are nonmycobacterium organisms; one possible and reasonable explanation was that the sample might have been contaminated. Another sample gave mixed signals to both *M. kansasii* and other NTM species. Two *M. chelonae* isolates gave mixed signals to both *M. chelonae* and other NTM species. Ambiguous identification results obtained by the multiplex SNaPshot assay were mostly due to incomplete purification of the samples, causing extraneous peaks, which would be unlikely to affect the overall efficiency of the assay.

We believe the assay offers a rapid, robust, and highly reliable alternative to a classical biochemical method. First, this assay is rapid. In our clinical laboratory testing setting, the multiplex SNaPshot assay could be completed within 2 days. This assay can shorten the turnaround time compared with our conventional biochemical identification procedures, which normally take 4 to 10 days (depending on species) to complete. Second, this technique can facilitate identification of the cause of infection as *M. avium*, *M. intracellulare*, or the *M. avium* complex (MAC) group, which will lead to a better understanding of the epidemiology and clinical importance of the clusters that make up the MAC (14, 17). Third, this assay is flexible. In addition to the six common mycobacterial species in our study, the assay can also identify other mycobacterial species in a single assay from pure and mixed cultures according to different identification requirements since nontuberculous mycobacterial infections vary geographically by species (12, 18, 20). In contrast, the AccuProbe system was limited to the identification of *M. tuberculosis* complex, *M. avium-M. intracellulare* complex, *M. kansasii*, and *M. gordonae*. Lastly, this strategy has numerous technical advantages: high-throughput genotyping is possible as the process can be automated, and the interpretation of the peak patterns is very simple.

In conclusion, our results confirm the usefulness of the multiplex SNaPshot for rapid and accurate identification of the six most commonly encountered clinical mycobacterial species, which facilitates the diagnosis, thereby providing agile clinical interventions. We believe the multiplex SNaPshot assay is a promising new tool for species identification for clinical laboratories.

ACKNOWLEDGMENTS

This work was supported by National Great Research Program of China (2008zx10003-009).

We thank all study participants for their cooperation, for technical help, and for sample collection. We are grateful to all members of the Mycobacteriology Laboratory at Shanghai Pulmonary Hospital, Tongji University, for their knowledge, helpfulness, and willingness to share.

REFERENCES

1. **Arnold, L. J., P. W. Hammond, W. A. Wiese, and N. C. Nelson.** 1989. Assay formats involving acridinium-ester-labeled DNA probes. Clin. Chem. **35:** 1588–1594.
2. **Bardien, S., H. Human, T. Harris, G. Hefke, R. Veikondis, H. S. Schaaf, L. van der Merwe, J. H. Greinwald, J. Fagan, and G. de Jong.** 2009. A rapid method for detection of five known mutations associated with aminoglycoside-induced deafness. BMC Med. Genet. **10:**2.
3. **Ben-Avi, L., R. Durst, S. Shpitzen, E. Leitersdorf, and V. Meiner.** 2004. Apolipoprotein E genotyping: accurate, simple, high throughput method using ABI Prism SNaPshot Multiplex System. J. Alzheimers Dis. **6:**497–501.
4. **Bodle, E. E., J. A. Cunningham, P. Della-Latta, N. W. Schluger, and L. Saiman.** 2008. Epidemiology of nontuberculous mycobacteria in patients without HIV infection, New York City. Emerg. Infect. Dis. **14:**390–396.
5. **Chadwick, M. V.** 1982. Mycobacteria. Institute of Medical Laboratory Sciences Monographs. John Wright, Inc., Bristol, United Kingdom.
6. **Chetchotisakd, P., S. Kiertiburanakul, P. Mootsikapun, S. Assanasen, R. Chaiwarith, and S. Anunnatsiri.** 2007. Disseminated nontuberculous mycobacterial infection in patients who are not infected with HIV in Thailand. Clin. Infect. Dis. **45:**421–427.
7. **Filippini, S., A. Blanco, A. Fernández-Marmiesse, V. Álvarez-Iglesias, C. Ruíz-Ponte, Á. Carracedo, and A. Vega.** 2007. Multiplex SNaPshot for detection of *BRCA1/2* common mutations in Spanish and Spanish related breast/ovarian cancer families. BMC Med. Genet. **8:**40.
8. **Glassroth, J.** 2008. Pulmonary disease due to nontuberculous mycobacteria. Chest **133:**243–251.
9. **Griffith, D. E., T. Aksamit, B. A. Brown-Elliott, A. Catanzaro, C. Daley, F. Gordin, S. M. Holland, R. Horsburgh, G. Huitt, M. F. Iademarco, M. Iseman, K. Olivier, S. Ruoss, C. F. von Reyn, R. J. Wallace, Jr., and K. Winthrop on behalf of the ATS Mycobacterial Diseases Subcommittee.** 2007. An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. Am. J. Respir. Crit. Care Med. **175:** 367–416.
10. **Griffith, D. E.** 2007. Therapy of nontuberculous mycobacterial disease. Curr. Opin. Infect. Dis. **20:**198–203.
11. **Hanna, B. A., A. Ebrahimzadeh, L. B. Elliott, M. A. Morgan, S. M. Novak, S. Rusch-Gerdes, M. Acio, D. F. Dunbar, T. M. Holmes, C. H. Rexer, C. Savthyakumar, and A. M. Vannier.** 1999. Multicenter evaluation of the BACTEC MGIT 960 system for recovery of mycobacteria. J. Clin. Microbiol. **37:**748–752.
12. **Jarzembowski, J. A., and M. B. Young.** 2008. Nontuberculous mycobacterial infections. Arch. Pathol. Lab. Med. **132:**1333–1341.
13. **Johansen, I. S., B. H. Lundgren, J. P. Thyssen, and V. O. Thomsen.** 2002. Rapid differentiation between clinically relevant mycobacteria in microscopy positive clinical specimens and mycobacterial isolates by line probe assay. Diagn. Microbiol. Infect. Dis. **43:**297–302.
14. **Kahlon, S. S., J. W. East, and J. C. Sarria.** 2008. *Mycobacterium avium-intracellulare* complex immune reconstitution inflammatory syndrome in HIV/AIDS presenting as osteomyelitis. AIDS Read. **18:**515–518.
15. **Katalinić-Janković, V., S. P. Grle, M. Obrovac, E. Cvetnić, and T. Alfirević.** 2007. Infections due to nontuberculous mycobacteria. Lijec. Vjesn. **129:**146–151.
16. **Khan, K., J. Wang, and T. K. Marras.** 2007. Nontuberculous mycobacterial sensitization in the United States: national trends over three decades. Am. J. Respir. Crit. Care Med. **176:**306–313.
17. **Kobashi, Y., and M. Oka.** 2008. Long-term observation of pulmonary *Mycobacterium avium* complex disease treated with chemotherapy—following the guidelines for treatment. Kekkaku **83:**779–784. (In Japanese.)
18. **Koh, W. J., O. J. Kwon, K. Jeon, T. S. Kim, K. S. Lee, Y. K. Park, and G. H. Bai.** 2006. Clinical significance of nontuberculous mycobacteria isolated from respiratory specimens in Korea. Chest **129:**341–348.
19. **Padilla, E., V. González, J. M. Manterola, A. Pérez, M. D. Quesada, S. Gordillo, C. Vilaplana, M. A. Pallarés, S. Molinos, M. D. Sánchez, and V. Ausina.** 2004. Comparative evaluation of the new version of the INNO-LiPA Mycobacteria and GenoType Mycobacterium assays for identification of *Mycobacterium* species from MB/BacT liquid cultures artificially inoculated with mycobacterial strains. J. Clin. Microbiol. **42:**3083–3088.
20. **Polanecky, V., P. Kalina, M. Kubín, B. Kozáková, and M. Müllerová.** 2006. Nontuberculous mycobacteria and incidence of mycobacterioses in Prague in 1999–2004. Epidemiol. Mikrobiol. Imunol. **55:**151–157.
21. **Roberts, G. D., E. C. Bottger, and L. Stockman.** 1996. Methods for the rapid identification of mycobacterial species. Clin. Lab. Med. **16:**603–615.
22. **Rogall, T., T. Flohr, and E. C. Bottger.** 1990. Differentiation of *Mycobacterium* species by direct sequencing of amplified DNA. J. Gen. Microbiol. **136:**1915–1920.
23. **Ryoo, S. W., S. Shin, M. S. Shim, Y. S. Park, W. J. Lew, S. N. Park, Y. K. Park, and S. Kang.** 2008. Spread of nontuberculous mycobacteria from 1993 to 2006 in Koreans. J. Clin. Lab. Anal. **22:**415–420.
24. **Sanchez, J. J., C. Børsting, K. Balogh, B. Berger, M. Bogus, J. M. Butler, A. Carracedo, D. S. Court, L. A. Dixon, B. Filipović, M. Fondevila, P. Gill, C. D. Harrison, C. Hohoff, R. Huel, B. Ludes, W. Parson, T. J. Parsons, E. Petkovski, C. Phillips, H. Schmitter, P. M. Schneider, P. M. Vallone, and N. Morling.** 2008. Forensic typing of autosomal SNPs with a 29 SNP-multiplex—results of a collaborative EDNAP exercise. Forensic Sci. Int. Genet. **2:**176–183.
25. **Springer, B., L. Stockman, K. Teschner, G. D. Roberts, and E. C. Bottger.** 1996. Two-laboratory collaborative study on identification of mycobacteria: molecular versus phenotypic methods. J. Clin. Microbiol. **34:**296–303.
26. **Tortoli, E., A. Bartoloni, C. Burrini, A. Mantella, and M. T. Simonetti.** 1995. Utility of high-performance liquid chromatography for identification of mycobacterial species rarely encountered in clinical laboratories. Eur. J. Clin. Microbiol. Infect. Dis. **14:**240–243.
27. **Tortoli, E., A. Nanetti, C. Piersimoni, P. Cichero, C. Farina, G. Mucignat, C. Scarparo, L. Bartolini, R. Valentini, D. Nista, G. Gesu, C. P. Tosi, M. Crovatto, and G. Brusarosco.** 2001. Performance assessment of new multiplex probe assay for identification of mycobacteria. J. Clin. Microbiol. **39:** 1079–1084.
28. **Vestal, A. L.** 1977. Procedure for isolation and identification of mycobacteria. U.S. Department of Health, Education, and Welfare publication no. (CDC) 77-8230. Centers for Disease Control and Prevention, Atlanta, GA.
29. **Wang, R. H., L. M. Liu, and J. L. Zhao.** 2009. A comparative study on genetic polymorphism and genetic relationship of 13 SNPs in three Chinese populations. Yi Chuan **31:**273–279.
30. **Wong, D. A., P. C. Yip, D. T. Cheung, and K. M. Kam.** 2001. Simple and rational approach to the identification of *Mycobacterium tuberculosis*, *Mycobacterium avium* complex species, and other commonly isolated mycobacteria. J. Clin. Microbiol. **39:**3768–3771.