JCM
Journals.ASM.org

Novel Primer-Probe Sets for Detection and Identification of Mycobacteria by PCR-Microarray Assay

Elisa Lazzeri,[a] Francesco Santoro,[a] Marco R. Oggioni,[a,b] Francesco Iannelli,[a] and Gianni Pozzi[a,b]

Laboratorio di Microbiologia Molecolare e Biotecnologia, Dipartimento di Biotecnologie, Università di Siena,[a] and UOC Batteriologia, Azienda Ospedaliera Universitaria Senese,[b] Siena, Italy

A PCR-microarray assay was developed in which PCR primers and hybridization probes were designed for the 16S rRNA genes of 16 clinically relevant mycobacteria and for IS*6110* of *Mycobacterium tuberculosis*. The assay, based on a multiplex PCR followed by hybridization with oligonucleotide probes, was tested against 16 *Mycobacterium* species and 70 clinical samples.

Tuberculosis, which is caused by members of the *Mycobacterium tuberculosis* complex, is one of the most common human infectious diseases (13, 18). Infections caused by nontuberculous mycobacteria (NTM) are of growing clinical interest, especially in individuals with reduced or compromised immune function. NTM infect many sites within the body but primarily cause pulmonary diseases, cervical lymphadenopathies, and localized skin and soft tissue lesions (4, 7, 17). Prompt and efficient identification of mycobacteria at the species level is extremely important not only for correct management of the diseases caused by NTM but also because differentiation of *M. tuberculosis* from NTM is important for infection control and choice of antitubercular therapy (12). Here we describe novel sets of PCR primers and hybridization probes to be used for development of PCR-microarray assays for detection and identification of clinically relevant mycobacteria.

PCR primers and hybridization probes. Primers and probes were designed for IS*6110* of *M. tuberculosis* and the 16S rRNA genes of 11 mycobacterial species (see Table S1 in the supplemental material), which were aligned using ClustalW (16). PCR primers MYC1p and MYC2p were designed for conserved regions of the 16S rRNA gene to amplify a 235-bp fragment containing hypervariable region A. PCR primers MYC13p and MYC14p were designed for *orfB* of insertion sequence IS*6110* (GenBank accession number X17348), yielding an amplicon 122 bp in size. Species-specific probes were designed on the segment of hypervariable region A of the 16S rRNA genes spanning nucleotides (nt) 140 to 178, whereas genus-specific probe MYC10a matched the adjacent segment spanning nt 100 to 137. Probes MYC15a and MYC16a, specific for the *M. tuberculosis* complex, were also designed for the segment of *orfB* of insertion sequence IS*6110* spanning nt 795 to 869. For the prediction of thermodynamic stability of all probes, the nearest-neighbor method (15) was used. Probes were checked using BLAST (1) to avoid unwanted cross-homology to nontargeted sequences.

Mycobacterial DNA microarray. Epoxy-modified glass slides (Genetix) and a manual microarray system (V&P Scientific) were used for printing. Oligonucleotide probes with a 5′ C_6 amino linker (MWG Biotech) were spotted in three replicates. Procedures for slide printing, labeling of PCR products with Cy5, hybridization, and data analysis were essentially as described previously (3). Slides were read using an Agilent GMS 428 scanner (Genetics MicroSystems), evaluating the absolute signal from each spot. Probes were between 19 and 27 nucleotides, with a melting temperature (T_m) of 56°C to 65°C. The array contained 3 probes specific for the *M. tuberculosis* complex, MYC15a and MYC16a designed on IS*6110*, and MYC3a designed on 16S rRNA genes. Four different species-specific probes were designed on 16S rRNA genes to identify *M. avium* (MYC4a), *M. intracellulare* (MYC5a), *M. xenopi* (MYC17a), and *M. haemophilum* (MYC19a), whereas probe MYC6a was used for identification of *M. kansasii*, as well as *M. simiae* and *M. scrofulaceum*, since in these 3 species the hypervariable region A of the 16S rRNA genes is identical. Also, the rapidly growing *M. abscessus* and *M. chelonae* species have an identical hypervariable region A and could be identified by a single probe (MYC8a). Probe MYC31a was specific for *M. fortuitum*, by far the most clinically relevant species of the *M. fortuitum* group (2). Multiple attempts to design probes for *M. malmoense*/*szulgai*, *M. marinum*/*genavense*, and *Mycobacterium ulcerans* were unsuccessful due to generation of unspecific positive signals (data not shown). For this reason, detection of these mycobacterial species relied on the genus-specific MYC10a probe, followed by identification by DNA sequencing.

Specificity of the multiplex PCR. *In silico* analysis showed that the MYC1p/MYC2p primer pair had the potential to amplify (i) all the 16S rRNA genes of the genus *Mycobacterium* present in GenBank and (ii) the 16S rRNA genes of some species belonging to phylogenetically related genera, such as *Brevibacterium*, *Corynebacterium*, and *Nocardia*, whereas MYC13p/MYC14p were specific for the *M. tuberculosis* complex. Primer pairs MYC1p/MYC2p and MYC13p/MYC14p combined in a multiplex PCR were tested for their capacity to amplify the genomic DNA from the 21 strains described in Table 1. Multiplex PCR results (data not shown) confirmed the *in silico* predictions.

Validation of the primer-probe sets. Primer-probe combinations were tested using genomic DNA of control strains (Table 2). All mycobacterial species were detected by probe MYC10a. As predicted by *in silico* analysis, the DNA of *B. ravenspurgense*, *C. glutamicum*, and *N. asteroides* also reacted, albeit weakly, with

Received 25 August 2012 **Accepted** 2 September 2012
Published ahead of print 12 September 2012
Address correspondence to Gianni Pozzi, gianni.pozzi@unisi.it.
Supplemental material for this article may be found at http://jcm.asm.org/.

doi:10.1128/JCM.02300-12

TABLE 1 Bacterial strains

Complex and/or strain[a]	Source[b]
Mycobacterium tuberculosis complex	
Mycobacterium tuberculosis H37 Rv	ISS (Rome)
Mycobacterium bovis BCG	Institut Pasteur (Paris)
Mycobacterium africanum Myc83	Clinical isolate (Florence)
Mycobacterium avium complex	
Mycobacterium intracellulare Myc48	Clinical isolate (Florence)
Mycobacterium intracellulare 551	Clinical isolate (Naples)
Mycobacterium avium 15445	Clinical isolate (Siena)
Mycobacterium kansasii 97758	Clinical isolate (Siena)
Mycobacterium xenopi 44382	Clinical isolate (Siena)
Mycobacterium chelonae A2	Clinical isolate (Naples)
Mycobacterium fortuitum Myc56	Clinical isolate (Florence)
Mycobacterium haemophilum 44634	DSMZ
Mycobacterium fortuitum/peregrinum complex C6	Clinical isolate (Naples)
Mycobacterium marinum Myc42	Clinical isolate (Naples)
Mycobacterium malmoense Myc39	Clinical isolate (Florence)
Mycobacterium ulcerans 19423	ATCC
Mycobacterium gordonae 14832	Clinical isolate (Siena)
Mycobacterium szulgai A4	Clinical isolate (Naples)
Corynebacterium glutamicum 44475	DSMZ
Nocardia asteroides 43255	DSMZ
Brevibacterium ravenspurgense 21258	DSMZ
Escherichia coli 25922	ATCC

[a] Identifications of all strains used in this study were confirmed by sequencing of the 16S rRNA gene.
[b] Clinical strains were kindly provided by Lanfranco Fattorini (ISS, Rome, Italy), Brigitte Gicquel (Institut Pasteur, Paris, France), Giulia Santoro (Ospedale Monaldi, Naples, Italy), and Enrico Tortoli (Laboratorio di Microbiologia e Virologia, AOU Careggi, Florence, Italy). Abbreviations: ATCC, American Type Culture Collection (Manassas, VA); DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany).

MYC10a. Each of the 3 probes specific for the *M. tuberculosis* complex reacted with *M. tuberculosis*, *M. bovis*, and *M. africanum*, whereas each of the 7 species-specific probes reacted only with one *Mycobacterium* species. Primer-probe combinations were also tested on 70 nonconsecutive clinical samples from various body sites, including 41 from the respiratory tract (Table 3). Clinical samples were processed according to standard procedures (8, 9, 11) at the Bacteriology Unit of the Siena University Hospital (UOC Batteriologia, AOUS). Each sample was analyzed for the presence of mycobacteria by (i) smear microscopy with the Ziehl-Neelsen method, (ii) culture in liquid medium (Bactec MIGIT 960), and (iii) real-time PCR (home-brew assay). Identification of all mycobacterial isolates was confirmed by sequencing of the 16S rRNA genes. Results of the PCR-microarray (i) were in good agreement with results obtained by standard laboratory diagnosis,

TABLE 3 Results with clinical samples

Clinical sample source	Positive samples: *n*	Species detected	No. of smear-positive samples	No. of negative samples	Total no. of samples
Sputum	6	*M. tuberculosis* complex	3	15	25
	2	*M. intracellulare*	1		
	1	*M. avium*	0		
	1	*M. xenopi*	1		
Bronchoalveolar lavage	1	*M. tuberculosis* complex	1	8	9
Bronchial aspirate	2	*M. tuberculosis* complex	2	5	7
Bone biopsy	1	*M. tuberculosis* complex	1	0	1
		M. avium	0		
Fistulas	1	*M. tuberculosis* complex	1	6	8
	1	*M. fortuitum*	0		
Urine				11	11
Blood				2	2
Total	18			52	70

TABLE 2 Microarray hybridization results[a]

Strain	MYC10a	MYC15a[b]	MYC16a[b]	MYC3a	MYC4a	MYC5a	MYC6a	MYC8a	MYC17a	MYC19a	MYC31a
Mycobacterium tuberculosis H37 Rv	+++	+++	+++	+++							
Mycobacterium africanum Myc83	+++	+++	+++	+++							
Mycobacterium bovis BCG	+++	+++	+++	+++							
Mycobacterium avium 15445	+++				+						
Mycobacterium intracellulare 551	+++					+++					
Mycobacterium kansasii 97758	+++						+++				
Mycobacterium xenopi 44382	+++								+++		
Mycobacterium haemophilum 44634	+++									+++	
Mycobacterium fortuitum/peregrinum complex C6	+++										+++
Mycobacterium marinum Myc42	+++										
Mycobacterium malmoense Myc39	+++										
Mycobacterium ulcerans 19423	+++										
Mycobacterium gordonae 14832	+++										
Mycobacterium szulgai A4	+++										
Mycobacterium chelonae A2	+++							+++			
Mycobacterium fortuitum Myc56	+++										+++
Escherichia coli 25922											
Brevibacterium ravenspurgense 21258	+										
Nocardia asteroides 43255	+										
Corynebacterium glutamicum 44475	+										

[a] All strains were tested with all probes. For clarity, only positive results are reported here, whereas negative strain-probe combination results are not shown. +++, very strong signal; ++, strong signal; +, positive signal.
[b] Probes MYC15a and MYC16a target IS*6110*.

(ii) were more reliable than microscopy alone, and (iii) confirmed the presence of *M. tuberculosis*, *M. avium*, *M. intracellulare*, *M. xenopi*, and *M. fortuitum* in positive-control samples.

Many different assays have been developed for the molecular diagnosis of mycobacterial infections (5, 6, 10, 14). This work provides proof of concept that oligonucleotide probes on a microarray can discriminate amplicons of the 16S rRNA genes of different mycobacterial species, and it validates IS*6110* and mycobacterial 16S rRNA genes as targets for DNA-based detection and identification of *M. tuberculosis* complex and NTM by the PCR-microarray approach. The primer-probe combinations produced in this study could be the basis for development of new automated PCR-microarray assays for laboratory diagnosis of mycobacterial infections.

ACKNOWLEDGMENT

This study was supported by FP7 EU grant TM-REST (HEALTH-F3-2008-202145).

REFERENCES

1. **Altschul SF, et al.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25**:3389–3402.
2. **Brown-Elliott BA, Wallace RJJ.** 2002. Clinical and taxonomic status of pathogenic nonpigmented or late-pigmenting rapidly growing *Mycobacteria.* Clin. Microbiol. Rev. **15**:716–746.
3. **Cassone M, et al.** 2006. DNA microarray for detection of macrolide resistance genes. Antimicrob. Agents Chemother. **50**:2038–2041.
4. **Falkinham JO.** 1996. Epidemiology of infection by nontuberculous mycobacteria. Clin. Microbiol. Rev. **9**:178–215.
5. **Fukushima M, et al.** 2003. Detection and identification of *Mycobacterium* species isolates by DNA microarray. J. Clin. Microbiol. **41**:2605–2615.
6. **Helb D, et al.** 2010. Rapid detection of *Mycobacterium tuberculosis* and rifampin resistance by use of on-demand, near-patient technology. J. Clin. Microbiol. **48**:229–237.
7. **Howard ST, Byrd TF.** 2000. The rapidly growing mycobacteria: saprophytes and parasites. Microbes Infect. **2**:1845–1853.
8. **Isenberg HD.** 2004. Clinical microbiology procedures handbook. ASM Press, Washington, DC.
9. **Kent PT, Kubica GP.** 1985. Public health mycobacteriology: a guide for the level III laboratory. Centers for Disease Control and Prevention, Atlanta, GA.
10. **Oggioni MR, et al.** 1995. Identification of *Mycobacterium tuberculosis* complex, *Mycobacterium avium* and *Mycobacterium intracellulare* by selective nested polymerase chain reaction. Mol. Cell. Probes. **9**:321–326.
11. **Pfyffer GE.** 2007. *Mycobacterium* general characteristics, laboratory detection, and staining procedures, p 543–572. *In* Murray PR, Baron EJ, Jorgensen JH, Landry N, Pfaller MA (ed), Manual of clinical microbiology, 9th ed. ASM Press, Washington, DC.
12. **Piersimoni C, Scarparo C.** 2008. Pulmonary infections associated with non-tuberculous mycobacteria in immunocompetent patients. Lancet Infect. Dis. **8**:323–334.
13. **Raviglione MC, Snider DE, Jr, Kochi A.** 1995. Global epidemiology of tuberculosis: morbidity and mortality of a worldwide epidemic. JAMA **273**:220–226.
14. **Richardson ET, Samson D, Banaei N.** 2009. Rapid identification of *Mycobacterium tuberculosis* and nontuberculous mycobacteria by multiplex, real-time PCR. J. Clin. Microbiol. **47**:1497–1502.
15. **SantaLucia J, Jr.** 1998. A unified view of polymer, dumbbell, and oligonucleotide DNA nearest-neighbor thermodynamics. Proc. Natl. Acad. Sci. U. S. A. **95**:1460–1465.
16. **Thompson JD, Higgins DG, Gibson TJ.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22**:4673–4680.
17. **Wallace RJJ, Brown BA, Onyi G.** 1992. Skin, soft tissue, and bone infections due to *Mycobacterium chelonae chelonae*: importance of prior corticosteroid therapy, frequency of disseminated infections, and resistance to oral antimicrobials other than clarithromycin. J. Infect. Dis. **166**:405–412.
18. **WHO.** 2009. Treatment of tuberculosis: guidelines, 4th ed. World Health Organization, Geneva, Switzerland.