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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 IS6110 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 IS6110 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 IS6110 (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 IS6110 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' C₆ 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 93 probes specific for the *M. tuberculosis* complex, MYC15a and MYC16a designed on IS6110, 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. malmhoense/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. ravenuspurgense*, *C. glutamicum*, and *N. asteroides* also reacted, albeit weakly, with

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Address correspondence to Gianni Pozzi, g.pozzi@unisi.it.

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TABLE 1 Bacterial strains

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

TABLE 2 Microarray hybridization results^a

Strain	MYC10a	MYC15a ^b	MYC16a ^b	MYC3a	MYC4a	MYC5a	MYC6a	MYC8a	MYC17a	MYC19a	MYC31a
<i>Mycobacterium tuberculosis</i> H37 Rv	+++	+++	+++	+++							
<i>Mycobacterium africanum</i> Myc83	+++	+++	+++	+++							
<i>Mycobacterium bovis</i> BCG	+++	+++	+++	+++							
<i>Mycobacterium avium</i> 15445	+++				+						
<i>Mycobacterium intracellulare</i> 551	+++					+++					
<i>Mycobacterium kansasii</i> 97758	+++						+++				
<i>Mycobacterium xenopi</i> 44382	+++								+++		
<i>Mycobacterium haemophilum</i> 44634	+++									+++	
<i>Mycobacterium fortuitum/peregrinum</i> complex C6	+++										+++
<i>Mycobacterium marinum</i> Myc42	+++										
<i>Mycobacterium malmoense</i> Myc39	+++										
<i>Mycobacterium ulcerans</i> 19423	+++										
<i>Mycobacterium gordonae</i> 14832	+++										
<i>Mycobacterium szulgai</i> A4	+++										
<i>Mycobacterium chelonae</i> A2	+++							+++			
<i>Mycobacterium fortuitum</i> Myc56	+++										+++
<i>Escherichia coli</i> 25922											
<i>Brevibacterium ravenspurgenense</i> 21258	+										
<i>Nocardia asteroides</i> 43255	+										
<i>Corynebacterium glutamicum</i> 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 IS6110.

TABLE 3 Results with clinical samples

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

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,

(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 IS6110 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.

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TABLE S1. Oligonucleotide primers and probes^a

Gene	Name ^b	Target	Oligonucleotide sequence (5'-3')
16S rDNA	MYC1p	<i>Mycobacterium</i>	AGTGGCGAACGGGTGAGTAA
16S rDNA	MYC2p	<i>Mycobacterium</i>	CGTATCTCAGTCCCAGTGTG
IS6110	MYC13p	<i>M. tuberculosis</i> complex	GACCACCAGCACCTAACC
IS6110	MYC14p	<i>M. tuberculosis</i> complex	GACCCGCCAGCCCAGGAT
16S rDNA	MYC3a	<i>M. tuberculosis</i> complex	GATAGGACCACGGGATGCATGTCT
16S rDNA	MYC4a	<i>M. avium</i>	GGATAGGACCTCAAGACGCATGTCTT
16S rDNA	MYC5a	<i>M. intracellulare</i>	GGATAGGACCTTTAGGCGCATGTCT
16S rDNA	MYC6a	<i>M. simiae</i> , <i>M. kansasii</i> , <i>M. scrofulaceum</i>	GGACCACTTGGCGCATGCC
16S rDNA	MYC8a	<i>M. abscessus</i> <i>M. chelonae</i>	GGATAGGACCACACTTCATGGTGAG
16S rDNA	MYC10a	<i>Mycobacterium</i>	GGGATAAGCCTGGGAAACTGGGTCTA
16S rDNA	MYC17a	<i>M. xenopi</i>	ATAGGACCATTCTGCGCATGTGG
16S rDNA	MYC19a	<i>M. haemophilum</i>	GGACCTCAAGGCGCATGCCTTTGT
16S rDNA	MYC31a	<i>M. fortuitum</i> ^c	GAATATGACCACGCGCTTCATGGTGT
IS6110	MYC15a	<i>M. tuberculosis</i> complex	GTGGGTAGCAGACCTCACCTATGTGTC
IS6110	MYC16a	<i>M. tuberculosis</i> complex	TCGCCTACGTGGCCTTTGTCA

^a The genes used for primers and probes design were: (i) IS6110 of *M. tuberculosis H37Rv* (GenBank acc. No. X17348) and (ii) the 16S rRNA genes of *M. tuberculosis H37Rv* (GeneID 2700429), *Mycobacterium avium* (GeneID 4530020), *Mycobacterium intracellulare* (GenBank acc. No. AJ536036), *Mycobacterium simiae* (GenBank acc. No. X52931.1), *Mycobacterium kansasii* (GenBank acc. No. AJ536035.1), *Mycobacterium scrofulaceum* (GenBank acc. No. X52924.1), *Mycobacterium abscessus* (GenBank acc. No. AJ536038.1), *Mycobacterium chelonae* (GenBank acc. No. AM884324.1), *Mycobacterium xenopi* (GenBank acc. No. AJ536033.1), *Mycobacterium haemophilum* (GenBank acc. No. X88923.1), *Mycobacterium fortuitum* (GenBank acc. No. X52933)

^b Final letters “p” and “a” in the name indicate primers and probes respectively

^c *M. fortuitum* 16S rDNA sequence shows some variability among isolates. Probe is designed to target the most common 16S rDNA sequence present in GenBank for *M. fortuitum* (27/49)