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Rapid detection and species identification of *Mycobacterium* spp. using real-time PCR and DNA-Microarray

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Abstract

Infections with mycobacteria are an important issue in public health care. Here we present a 'proof-of-principle' concept for the identification of 37 different *Mycobacterium* species using 5′ exonuclease real-time PCR and DNA microarray based on the region upstream of the 65 kDa heat shock protein. With our two PCR probes, one complementary to all mycobacteria species, the other specific for the M. tbc-complex, 34 species were properly classified by real-time PCR. After reamplification and hybridization to a DNA microarray, all species showed a specific pattern. All 10 blindly tested positive cultures revealed a positive real-time PCR signal with the genus probe. After reamplification and hybridization, six samples could unambiguously be identified. One sample showed a mixture of presumably three species-specific patterns and sequencing the 16S rRNA confirmed the presence of a mixture. The hybridization results of three specimens could not be interpreted because the signal to background ratio was not sufficient. Two samples considered as negative controls (LAL Reagent Water (Cambrex) and DNA of *Candida albicans*) gave neither a genus nor a M. tbc-complex positive PCR signal. Based on these results we consider our method to be a promising tool for the rapid identification of different mycobacteria species, with the advantage of possible identification of mixed infections or contaminations.

Keywords: Diagnostics; Microarray; Mycobacteria; Real-time PCR; Smart Cycler® II; Tuberculosis

1. Introduction

Infections with mycobacteria lead to severe lung disease and remain a serious threat to public health. In addition, infections with nontuberculous mycobacteria (NTM) have increased mainly in patients with a compromised immune system (Wolinsky, 1992; Katoch, 2004; Paolo and Nosanchuk, 2004). Even mixed infections with *Mycobacterium tuberculosis* (*M. tbc*) and NTM have been reported (Libanore et al., 1992). Early effective treatment preventing the development of resistance and further spread of infections requires sensitive and fast detection and identification of the different mycobacterial species.

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Traditional cultures of the pathogen, however, are time-consuming due to the long replication time of many clinically relevant species. Biochemical identification may take even longer. Direct microscopic examination is a fast and useful method but it lacks sensitivity and specificity. Commercially available molecular detection and identification systems usually offer a rapid and specific detection for the *M. tuberculosis*-complex (*M. tbc*-complex) but not for NTM.

Real-time PCR (Wittwer et al., 1997) is a methodology with high sensitivity that permits the simultaneous amplification and detection of a given target sequence directly from clinical specimens. It is highly specific and can even be used for the differentiation of closely related species (O'Mahony and Hill, 2002; Englund, 2003; Rondini et al., 2003) or for the identification of not-cultivable species (Maibach and Altwegg, 2003; Kramme et al., 2004). Techniques to identify bacterial isolates include sequencing (Kirschner et al., 1993; Kox et al., 1995; Roth et al., 1998; Ringuet et al., 1999), restriction fragment length polymorphism analysis (Plikaytis et al., 1992; Telenti et al., 1993) and hybridization with species-specific probes in various formats (Kirschner et al., 1996; Fukushima et al., 2003).

In this 'proof of concept' study we established a two-step procedure for the identification of different *Mycobacterium* species including *M. tbc*-complex and NTM. For this purpose we targeted the region upstream of the 65 kDa heat shock protein (65kDa hsp) gene. We believe that this region has great potential for the identification of mycobacteria due to its specificity for mycobacteria and the presence of a conserved as well as species-specific regions (Martinetti Lucchini et al., 1995). In a first step, we used a duplex 5′ exonuclease real-time PCR to detect the genus *Mycobacterium* and members of the *M. tbc*-complex. In a second step, species were identified by hybridization to an oligonucleotide microarray.

2. Materials and methods

2.1. Strains and DNA extraction

Most of the used 89 strains were stored in the Microbanks™ System (Pro-lab Diagnostics, Neston, UK) at −80 °C. One microbanks bead was resuspended in 500 µl physiological saline and inactivated by boiling at 80 °C for 10 min. Extraction of the genomic DNA was done with InstaGene Matrix (Bio-Rad, Reinach, Switzerland) following the manufacturers instructions. Genomic DNA of *M. leprae*, extracted from a biopsy of a skin lesion, was kindly provided by S. Kramme.

2.2. Real-time PCR, reamplification and sequencing

Based on the available sequence data, a real-time PCR assay using the 5′ exonuclease format was designed that allows amplification of a DNA fragment from all mycobacteria and identification based on two different probes. One of these probes recognizes all mycobacteria while the other one was considered specific for members of the *M. tbc*-complex. Because of the limited length of the conserved sequence and the corresponding low melting temperature of the genus-specific probe, primers 65kDaf and 65kDar (Pao et al., 1990) were shortened and renamed to 65kDaf2 (5′-TAGGTCGGGACGGTGAG-3′) and 65kDar3 (5′-TTGCGAAGTGATTCCTCC-3′) (Microsynth GmbH, Balgach, Switzerland; Fig. 1). All species were amplified except *M. celatum*, *M. heckeshornense* and *M. leprae*. The former two species were amplified using a second reverse primer (65kDar4; 5′-CCAGCTC-GATCTCCTTG-3′; Fig. 1), deduced from NCBI-

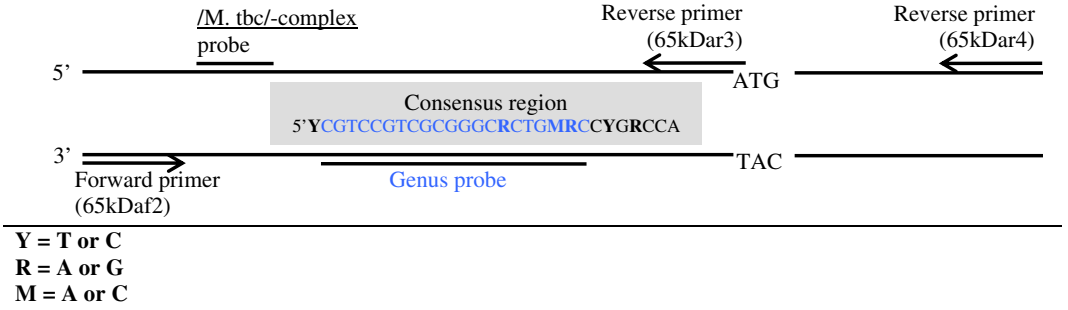

Fig. 1. Schematic illustration of the primer and probe design for the 5′ exonuclease real-time PCR. Location of primers and probes used for real-time PCR to detect the mycobacteria genus and to distinguish between *M. tbc*-complex and NTM are shown. ATG indicates the start codon of the 65kDa hsp.

sequences AF547817 and AF547843 and located at the 5′ end of the 65kDa hsp gene, whereas no further primers were searched for *M. leprae*. The 5′ exonuclease probes detecting all mycobacteria is located within the conserved sequence upstream of the 65 kDa hsp gene (Fig. 1). It contains three degenerated nucleotides, is labelled at the 5′-end with FAM and at the 3′-end with Dabcyl (5′-FAM-CGTCCGTCGCGGGC-RCTGMRC-Dabcyl-3′; Microsynth GmbH). The second probe detecting all members of the *M. tbc*-complex was searched manually within the variable region (Fig. 1), labelled at the 5′-end with Cy3 and at the 3′-end with BHQ2 (5′-Cy3-AGTTGTCCTCGC-TGCCACTCGCT-BHQ2-3′; Proligo, Paris, France). Real-time PCR was conducted on a Smart Cycler® II (Cepheid, Sunnyvale, USA). For the detection of mycobacteria from cultured material, real-time PCR was performed in a total volume of 25 μl. A PCR mix contained 1 μM of each primer 65kDaf2 and 65kDar3, 0.3 μM each of the genus and *M. tbc*-complex specific probe and 1× QuantiTect Probe PCR Kit (Qiagen AG, Basel, Switzerland). Amplification included an initial denaturation step at 95 °C for 15 min, followed by 50 cycles of denaturation at 95 °C for 15 s, annealing at 51 °C for 15 s and elongation at 72 °C for 30 s. For each amplification two positive and one negative control were included (*M. tbc*, *M. fortuitum* and LAL Reagent Water (Cambrex, Ververs, Belgium)). Results were expressed as threshold cycle values (Ct-value). The threshold line was set at 30 fluorescent units. Amplicons were checked on a 2% agarose gel. The reverse strand was then labelled by reamplification using the normal forward primer 65kDaf2 and a 5′Cy3 fluorescence-labelled reverse primer (5′Cy3-AGTTGTCCTCGCTGCCACTCGCT-3′). Reamplification was performed using the same conditions as for the real-time PCR.

Prior to sequencing the double-stranded PCR products were purified with the QIAquick PCR purification kit (Qiagen AG) following the manufacturers instructions. Forward and reverse linear amplification was performed in 10 μl using 2 μl of the purified PCR product (about 20–100 ng), 2 μl BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Rotkreuz, Switzerland), 1 μl BigDye Sequencing Buffer and 1 μM primer 65kDaf2 (5′-TAGGTCGGGACGGTGAG-3′) or 65kDar3 (5′-TTGCGAAGTGATTCCTCC-3′). Linear amplification consisted of 25 cycles of denaturation at 96 °C for 10 s and elongation at 60 °C for 60 s using the Perkin-Elmer GeneAmp 9600 (Applied Biosystems). Fluorescence labeled DNA was purified with Performa® DTR Gel Filtration Cartridges (Edge Biosystems, Geithersburg, USA) according to the manufacturers protocol and analyzed on an ABI Prism 3100 Avant Genetic Analyzer (Applied Biosystems).

2.3. DNA microarray

Oligonuclotide probes complementary to the labelled reverse strand were designed with the 'Probe Search' software (Jud and Altwegg, 2003 (unpublished)) and produced by Microsynth GmbH. A length of 20–27 bases and a melting temperature of 55–65 °C (4*(C+G)+2*(A+T)) were set as searching criteria. All microarray probes were labelled with a 3′ aminogroup and a T12-stem to decrease steric hindrance (Pfunder et al., 2004). Whenever possible a probe was selected with at least two mismatches to all other species (Peplies et al., 2003) (Table 2).

Spotting, hybridization and detection was done as previously described (Pfunder et al., 2004). In addition to the species-specific probes, a hybridization control (genus probe with the same sequence as that used for real-time PCR; Fig. 1) was applied in triplicate. The hybridization mix contained 25 μl of the unpurified reamplified DNA, 60 μl 20× SSC, 4 μl Denhardt's solution (Fluka Chemie GmbH, Buchs, Switzerland) and 2 μl 10% SDS. DNA was hybridized for 1 h. Signal intensities were normalized to the fluorescence of the genus probe. Hybridization experiments were accepted only if the genus probe signals/background ratio * 100% was at least 300%. Hybridizations to heterologous probes with values above 50% of the fluorescence of the homologous probe were considered as cross-reactions.

The performance of the microarray was tested blindly with 10 primary cultures positive for NTM, LAL Reagent Water and a culture of *Candida albicans*.

3. Results

3.1. Sequence analysis

Sequencing of 89 mycobacterial strains confirmed the presence of a consensus region as previously described by Martinetti Lucchini et al. (1995). This constant region is 29 bp long and contains six twofold degenerated positions (Fig. 1). DNA of *M. celatum* and *M. heckeshornense* could not be amplified with the standard primer pair 65kDaf2/65kDar3. However these two species were amplified and sequenced with the primer pair 65kDaf2/65kDar4 under the same conditions as with the primer pair 65kDaf2/65kDar3. DNA of *M. leprae* could not be amplified and sequenced with the standard primer pair 65kDaf2/65kDar3 (data not shown).

Table 1
Sequence information of strains used to establish real-time PCR and probes for DNA-microarray

Mycobacterium spp.	Fragment length*	Number of analyzed strains	Intraspecies variability (%)**	Species with more than 93% sequence homology
M. abscessus	108	2	1.63	94.6–93.5% with *M. chelonae*
M. africanum	161	1		99.4–100% with all species belonging to the *M. tbc*-complex
M. alvei	118	1		
M. avium subsp. *avium*	120	2	0	100% with *M. avium* subsp. *paratuberculosis*
M. avium subsp. *paratuberculosis*	120	3	0	100% with *M. avium* subsp. *avium*
M. bovis	161	1		99.4–100% with all species belonging to the *M. tbc*-complex
M. bovis BCG	161	1		99.4–100% with all species belonging to the *M. tbc*-complex
M. canettii	161	1		99.4–100% with all species belonging to the *M. tbc*-complex
M. celatum	97	2	0	
M. chelonae	108	2	0	94.6–93.5% with *M. abscessus*
M. fortuitum	118	2	3.17	97.6–100% with *M. peregrinum* 96.5% with *M. senegalense*
M. genavense	128	1		
M. gordonae	139	2	3.60	
M. haemophilum	149	1		
M. heckenshornense	101	1		99–100% with *M. xenopi*
M. heidelbergense	126	2	0	
M. intracellulare	107	3	0	
M. kansasii Typ I	135	2	0	
M. kansasii Typ II		1		
M. kansasii Typ III		1		
M. lentiflavum	128[a]	2	0	
M. leprae		1		
M. malmoense	136/137	3	0.74–1.10	
M. marinum	152	7	0	100% with *M. ulcerans*
M. microti	161	1		99.4–100% with all species belonging to the *M. tbc*-complex
M. mucogenicum	115	2	4.17	94.25–98.9% with *M. ratisbornense*
M. parafortuitum	119	1		
M. peregrinum	118	2	0	97.6–100% with *M. fortuitum*, 96.5% with *M. senegalense*
M. ratisbornense	115	2	1.56	94.25–98.9% with *M. mucogenicum*
M. scrofulaceum	133	2	1.14	
M. senegalense	118	1		96.5% with *M. fortuitum* and *M. peregrinum*
M. septicum	118	1		
M. simiae	118	2	1.37	
M. smegmatis	119	2	2.74	
M. szulgai	136	3	0	
M. terrae	118	2	3.89	
M. tbc	161	14	0–0.6	99.4–100% with all species belonging to the *M. tbc*-complex
M. ulcerans	152	2	0	100% with *M. marinum*
M. xenopi	101	2	0.99–1.81	99–100% with *M. heckeshornense*

Representation of the 37 used mycobacteria species. For species represented by two or more strains, the intraspecies-variability was calculated as follows: Number of mismatches / length of the sequence * 100%. Only the closely related species with a sequence homology of more then 93% are displayed.

*Length of the amplicons using the primer pair 65kDaf2/65kDar3, length including primer sequences.

**Intraspecies variability (Number of mismatches / length of the sequence * 100%).

[a] Neither amplification nor sequencing possible, sequence information from NCBI, accession number: AL450380.

3.2. Differentiation between M. tbc-complex and NTM by real-time PCR

Using real-time PCR with two different probes, 34 of the 37 species tested were correctly assigned to the *M. tbc*-complex or to NTM. Analysis on a 2% agarose gel revealed variable amplicon lengths as expected (Martinetti Lucchini et al., 1995; Table 1). The remaining three species *M. celatum*, *M. heckeshornense* and *M. leprae* showed little or no amplification with the

Table 2
Information about probes used for the microarray including a 3′ aminolinker and a T_{12}-stem

Probe number	Name and specificity of the probe	Length[a]	T_m (°C)[b]	GC-content	Binding site[c]	Expected cross-reactions[d]
P1	Genus[e]	21	65.3	71–75%		
P2	Abscessus	22	60.9	59%	23	
P3	Alvei	21	60.9	62%	27	
P4	AviumParatuberculosis[f]	20	63.5	75%	70	
P5	Celatum	22	59.7	55%	63	
P6	Chelonae	21	62.1	67%	27	
P7	Fortuitum	22	59.7	55%	31	*M. peregrinum*, *M. senegalese*, each 0 mismatches
P8	Genavense	22	59.7	55%	29	
P9	Gordonae	22	60.9	59%	7	
P10	Haemophilum	21	62.1	67%	108	
P11	Heckeshornense	21	62.1	67%	65	*M. xenopi,* 0–1 mismatch (15th nucleotide of the probe)
P12	Heidelbergense	21	62.1	67%	31	
P13	Intracellulare	21	62.1	67%	7	*M. avium* subsp. *avium*, MAP,[g] *M. heckeshornense*, *M. xenopi*, each one mismatch (9th nucleotide of the probe)
P14	Kansasii I	20	63.5	75%	39	
P15	Kansasii II	21	62.1	67%		
P16	Kansasii III	21	60.9	62%		
P17	Lentiflavum	22	60.9	59%	29	
P18	Malmoense	23	59.7	52%	91;92[h]	
P19	Marinum[i]	22	60.9	59%	99	
P20	Mucogenicum	20	63.5	75%	38	*M. ratisbornense*, 0 mismatch
P21	Parafortuitum	23	59.7	52%	30	
P22	Peregrinum	22	59.7	55%	26	*M. fortuitum*, *M. senegalese*, each 0 mismatches
P23	Ratisbornense	23	59.7	52%	16	*M. mucogenicum*, 0 mismatch to one of the *M. mucogenicum*-sequences, 1 mismatch to the other *M. mucogenicum*-sequence (3th nucleotide)
P24	Scrofulaceum	21	62.1	67%	40	
P25	Senegalense	20	63.5	75%	89	*M. alvei*, *M. parafortuitum*, each 0 mismatch *M. mucogenicum*, *M. ratisbornense*, *M. septicum*, *M. smegmatis*, each one mismatch (9th nucleotide of the probe)
P26	Septicum	23	59.7	52%	28	
P27	Simiae	20	63.5	75%	31	
P28	Smegmatis	21	60.9	62%	36	
P29	Szulgai	21	60.9	62%	92	
P30	Tbc[j]	20	63.5	75%	119	
P31	Terrae	21	62.1	67%	36	
P32	Xenopi	20	62.2	70%	27	*M. avium* subsp. *avium*, MAP,[g] *M. celatum*, *M. heckeshornense* each 0 mismatch *M. intracellulare*, one mismatch (9th nucleotide of the probe)

standard primers 65kDaf2/65kDar3 and thus could not be assigned to one of the two groups.

3.3. DNA microarray

After reamplification of real-time PCR positive samples, species identification was done by hybridization to a DNA microarray containing specific probes for 24 species. If possible, the species-specific probes contained at least two mismatches to all other species. Cross-reactions were expected for eight probes with less than two mismatches to some other species (Table 2). For closely related (sub-)species with 100% sequence homology in the analyzed region, e.g. *M. avium* subsp. *avium*/*M. avium* subsp. *paratuberculosis*, *M. marinum*/*M. ulcerans* and the species of the *M. tbc*-complex (Table 1), only one probe per pair or group was designed. Upon hybridization to the microarray, 34 species showed a specific pattern and a genus-probe intensity >300% of the local background. Species with 95–100% sequence homology (Table 1) always showed the same hybridization pattern. Only hybridization data of the species *M. celatum*, *M. heckeshornense* and *M. leprae* did not reach the set threshold (data not shown).

Twenty of the 34 species with sufficient signal intensity showed only interactions with the homologous probe and the predicted cross-reactions based on fewer than two mismatches (data not shown). For the remaining 14 species unexpected cross-reactions were seen while some of the expected cross-reactions were hardly visible (Fig. 2). Surprisingly, the probes considered specific for the *M. tbc*-complex and *M. terrae* exhibited interactions with nearly every hybridized reamplification product, rarely though with an intensity high enough to be considered as true cross-reaction, i.e., the signal intensity was below 50% as compared to the homologous probe.

We then tested the specificity of our method by blind testing of ten positive cultures and two negative controls (LAL Reagent Water and *C. albicans* DNA). Using real-time PCR all ten culture positive samples revealed a positive genus signal whereas none of them was positive for the *M. tbc*-complex. The two negative controls remained negative. Subsequently the ten positive samples were subjected to microarray analysis. Six samples could unambiguously be identified (Fig. 3), three samples had to be discarded because fluorescence intensity of the genus signal never reached the set threshold of 300% over local background. One sample showed a mixture of presumably three different patterns, and 16s rRNA sequencing confirmed the presence mixed sequences (data not shown).

4. Discussion

In this 'proof-of-concept' study, we describe a two-step procedure for the identification of 37 different *Mycobacterium* species which is based on the sequence upstream of the 65 kDa heat shock protein gene. In a first step, a real-time PCR with two probes allows discrimination between NTM and *M. tbc*-complex. For a diagnostic laboratory, this would allow assignment of about 75% of all positive cultures to the *M. tbc*-complex. For the remaining 25%, i.e. those positive with the genus probe but negative for the *M. tbc*-complex, reamplification and hybridization would be needed for species identification.

Using a modification of the previously published primers (Pao et al., 1990) all mycobacterial species tested except *M. celatum*, *M. heckeshornense* and *M. leprae* were successfully amplified. *M. celatum* and *M. heckeshornense* could be amplified and sequenced with forward primer 65kDaf2 in combination with a second reverse primer 65kDar4 located in the 65kDa hsp gene. However, sequencing the 65kDar3 primer region did not reveal any mismatches. Due to the good amplification efficiency with the primer-combination 65kDaf2/

Notes to Table 2:
Information about all probes used for DNA-microarray analysis such as probe number, name, length, melting temperature (according to manufacturer's information) and GC-content. The binding site is defined as distance of the 3′ end of the probe to the 5′ end of the reverse strand (in bases). Cross-reactions are expected for species with one or no mismatch between the species-specific probe and the sequence of another species.
[a] Length of the probe without the T_{12}-stem.
[b] Manufacturer's information.
[c] Distance of the 3′ end of the probe to the 5′ end of the reverse strand in bases.
[d] Only one or no mismatch between sequence and probe.
[e] The same sequence as used for the real-time PCR.
[f] One probe was designed for *M. avium* subsp. *avium* and *M. avium* subsp. *paratuberculosis.*
[g] MAP: *M. avium* subsp. *paratuberculosis*.
[h] Two different cultures revealed different sequence length (see Table 1).
[i] One probe designed for the detection of *M. marinum* and *M. ulcerans.*
[j] One probe designed for the detection of species belonging to the *M. tbc*-complex; not the same sequence as used as probe for the real-time PCR.

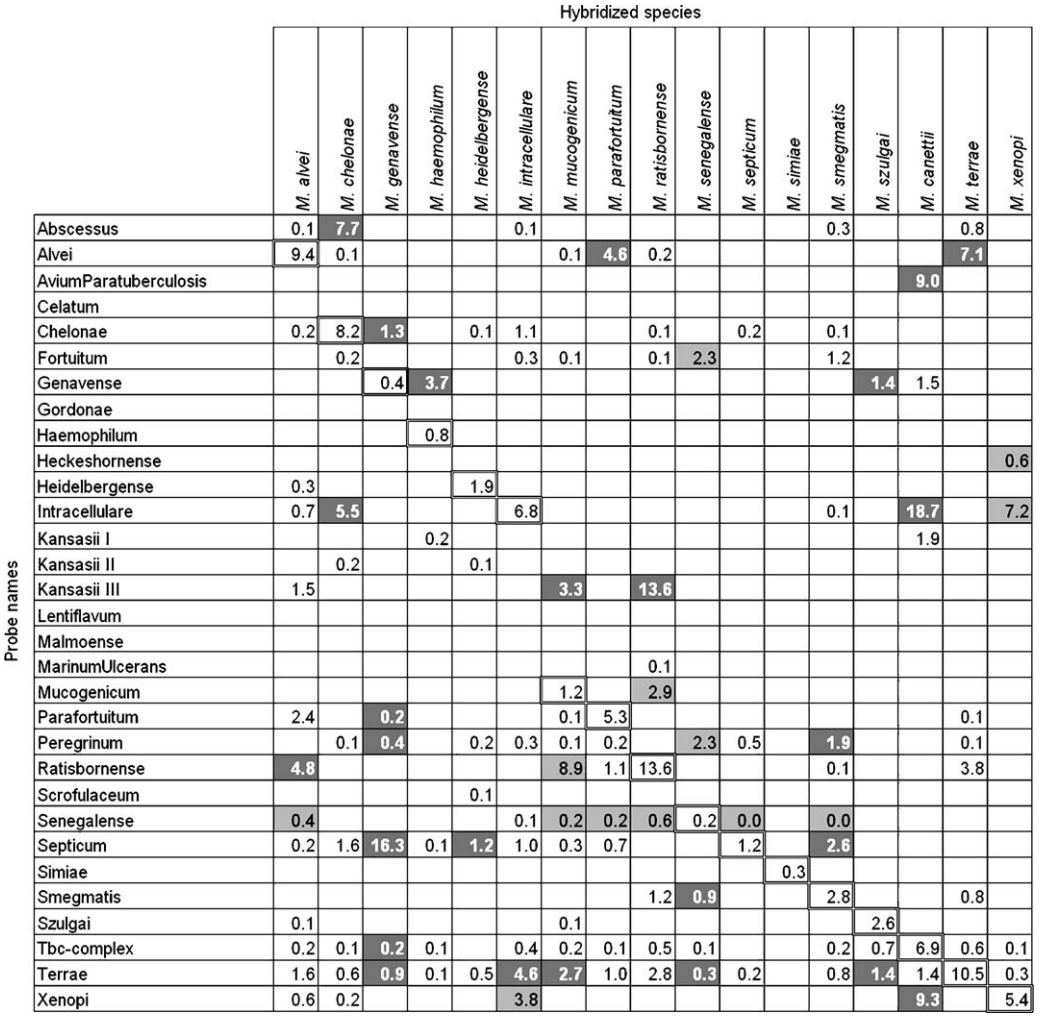

Fig. 2. 14 of 36 hybridized species—unexpected cross-reactions. Out of all 36 hybridized species (*M. leprae* was excluded) 34 species revealed analyzable results with a species-specific pattern. The species *M. alvei*, *M. mucogenicum*, *M. parafortuitum*, *M. ratisbornense*, *M. septicum* and *M. smegmatis* did not show expected cross-reactions with the probe Senegalense. Fourteen species showed unexpected cross-reactions (dark grey boxes). *M. simiae* gave unique results of a weak species-specific binding. Out of all tested species *M. celatum* and *M. heckeshornense* gave no analyzable results. Numbers indicate arbitrary fluorescence values normalized with the fluorescence of the Genus probe. Probes are further characterized in Table 2. Species-specific probe. Expected cross-reactions (two or less mismatches). Not expected cross-reactions with a fluorescence intensity > 50% of the fluorescence intensity of the species-specific probe.

65kDar4 a mismatch regarding the primer 65kDaf2 can be excluded. Thus the reason for the failure to amplify the original fragment remains obscure. *M. leprae* DNA could not be amplified at all although in the complete sequence of *M. leprae* (NCBI-accession number: AL450380) the primer regions of 65kDaf2 and 65kDar3 and the consensus sequence were found. The most likely explanation for this would be degradation of the DNA upon transport, storage or contamination with DNase. Other DNA of *M. leprae* was not available. Therefore this species was excluded from the study.

Microarray-based species identification is already a widely used technology (Stenger et al., 2002; Volokhov et al., 2003; Clewley, 2004). Of the 36 hybridized species (*M. leprae* was excluded) 34 gave analyzable results and showed a specific pattern. As some species have a sequence homology of more than 95% (Table 1), we expected them to show a similar pattern. This was indeed the case. Due to the low amplification efficiency of the species *M. celatum* and *M. heckeshornense* with the primer pair 65kDaf2/65kDar3, we never achieved the set threshold for the genus probe signal, i.e. more than 300% above the local background. Our genus

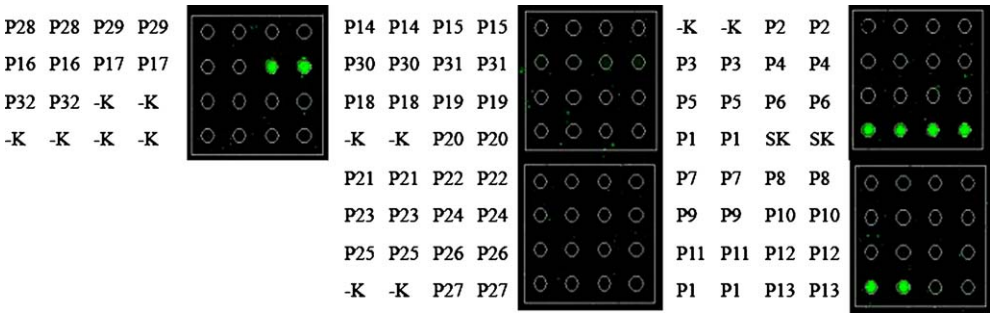

Fig. 3. Microarray pattern analysis after hybridization of a positive culture containing *M. lentiflavum*. Green fluorescence reveals positive hybridization to the probe. P1 indicates the hybridization control (Genus probe), SK the spotting control (fluorescence labelled oligo) and −K the negative control of spotted water samples. P1–P32 represent the species-specific probes outlined in Table 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

probe contained three twofold degenerated positions. Spotting each of the corresponding eight different probes might enhance the genus signal and therefore minimize not analyzable data. Even though every species showed a specific pattern, there was high heterogeneity in hybridization efficiency of different probes targeting the same DNA. This observation is not new (Southern et al., 1999; Peplies et al., 2003) and in our case it might be due to hybridization of non-standardized DNA-amounts (Pfunder et al., 2004) or due to steric hindrance (Peplies et al., 2003; Pfunder et al., 2004). This would explain the weak hybridization efficiency of the probes Haemophilum and Senegalense but not the weak species-specific hybridization of the probes Genavense and Simiae. On the other hand there are several options for improving the hybridization step: hybridizing under more stringent conditions would result in fewer cross-reactions (Peplies et al., 2003; Letowski et al., 2004). However, it was also shown that increasing the hybridization and washing temperatures can have very little effect on specificity (Pfunder and Frey, 2005). Adding urea or formamide to the hybridization-mix may be a good alternative (Peplies et al., 2003). Helper oligos have been described to decrease false-negative pairing but also to decrease specificity (Peplies et al., 2003). To enhance redundancy but also for identification of closely related species each array should include different species-specific probes, or probes from different regions (Fukushima et al., 2003). Choosing another hybridization platform could have the advantage of a label-free and therefore cheaper detection method (McKendry et al., 2002).

Our approach for a diagnostic microarray-chip promises a flexible, simple and fast mycobacterial species identification. Compared to direct sequencing, hybridization on a microarray has the advantage of possible detection of mixed infections or laboratory contaminations with environmental mycobacteria. Using multiplex PCR even more information might be generated, e.g. simultaneous identification and drug resistance testing (Troesch et al., 1999; Mikhailovich et al., 2001).

Our results with 10 blindly tested positive cultures and repeated hybridizations indicate good specificity and reliability of the two-step procedure. However, prior to introducing the procedure into the routine diagnostic laboratory, additional prospective testing with cultures from clinical specimen is necessary. Application of the procedure directly to clinical specimens seems possible but requires further sensibility and specificity evaluations.

Our ‘proof of concept’ study suggests that identification of cultures as well as direct detection and identification from clinical specimens based on real-time PCR and DNA-microarray is a fast, specific and reliable method. Targeting the region upstream of the 65kDa hsp seems to be as powerful as targeting the 16S rRNA, with the advantage of the mycobacteria specificity of the modified primer pair (Pao et al., 1990). The algorithm chosen (PCR followed by microarray hybridization for NTM) has the advantage of an immediate result for about 75% of all positive cultures, since NTM account only for about 25% of all isolates. Further, species identification by hybridization on a microarray may even be possible for mixed cultures.

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