

Methodology article

Evaluation of amplified rDNA restriction analysis (ARDRA) for the identification of cultured mycobacteria in a diagnostic laboratory

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Abstract

Background: The development of DNA amplification for the direct detection of *M. tuberculosis* from clinical samples has been a major goal of clinical microbiology during the last ten years. However, the limited sensitivity of most DNA amplification techniques restricts their use to smear positive samples. On the other hand, the development of automated liquid culture has increased the speed and sensitivity of cultivation of mycobacteria. We have opted to combine automated culture with rapid genotypic identification (ARDRA: amplified rDNA restriction analysis) for the detection resp. identification of all mycobacterial species at once, instead of attempting direct PCR based detection from clinical samples of *M. tuberculosis* only.

Results: During 1998–2000 a total of approx. 3500 clinical samples was screened for the presence of *M. tuberculosis*. Of the 151 culture positive samples, 61 were *M. tuberculosis* culture positive. Of the 30 smear positive samples, 26 were *M. tuberculosis* positive. All but three of these 151 mycobacterial isolates could be identified with ARDRA within on average 36 hours. The three isolates that could not be identified belonged to rare species not yet included in our ARDRA fingerprint library or were isolates with an aberrant pattern.

Conclusions: In our hands, automated culture in combination with ARDRA provides with accurate, practically applicable, wide range identification of mycobacterial species. The existing identification library covers most species, and can be easily updated when new species are studied or described. The drawback is that ARDRA is culture-dependent, since automated culture of *M. tuberculosis* takes on average 16.7 days (range 6 to 29 days). However, culture is needed after all to assess the antibiotic susceptibility of the strains.

Background

Rapid and accurate detection, identification and susceptibility testing of mycobacteria remains important i) be-

cause the overall incidence of tuberculosis is increasing, also due to the HIV pandemic [1], ii) because of the increasing resistance to antituberculous agents [2] and iii)

because an increasing number of mycobacterial species are being recognized as potentially pathogenic [3]. The importance of *M. avium* infection has increased in HIV patients [4], and clinical infections have been described with species like *M. heidelbergense*[5], *M. conspicuum*[6], *M. branderi* [7] and *M. interjectum*[8,9], which have been recognized only recently.

Current DNA amplification based diagnostic tests are expensive, have limited sensitivity, are usually restricted to the detection of *M. tuberculosis* only and provide no or limited information on susceptibility (e.g. rifampicin only: RifTB LiPA, Innogenetics, Zwijnaarde, Belgium). Therefore, the need for culture has not been circumvented. The CDC decided to restrict the use of genotypic tests to confirmation of smear positive samples, so that they cannot be used to test the large number of specimens processed for mycobacterial detection every year in an average laboratory [10]. Only recently, the enhanced AMTD (GenProbe, San Diego, CA) gained FDA approval for direct detection of *M. tuberculosis* from smear-negative samples, but several problems are reported [e.g. [11], and the high costs keep restricting its use.

Here, we present our findings with the use of ARDRA [12] for the identification of *Mycobacterium* species. The method consists of amplification of the 16S rRNA gene (rDNA) and subsequent restriction digestion of the amplicon. The restriction patterns obtained with different restriction enzymes and combination of these patterns into a restriction profile was shown to enable identification of most clinically important mycobacteria by comparison of the obtained profiles with a library of ARDRA profiles obtained for reference strains of different species [12]. This PCR-RFLP analysis of the 16S rRNA gene, was published almost simultaneously with the more widely used technique (known as PRA), which is based on the amplification of the *hsp65* gene [13–15].

Results

The initial study describing the applicability of ARDRA for the identification of mycobacteria [12] used universal bacterial primers. However, this sometimes resulted in the false positive amplification from decontaminated samples of organisms other than mycobacteria. Therefore primers were developed, aimed at more specific amplification of mycobacteria. During the three year evaluation period, of which the results are reported here, amplification of nonmycobacterial organisms occurred in two cases. These organisms, namely *Corynebacterium glutamicum* and *Actinomyces odontolyticum*, stained acid fast on direct smear and are relatives of the mycobacteria.

The restriction patterns obtained with the enzymes *HhaI* (isoschizomer of *CfoI*), *MboI* and *RsaI* for the different spe-

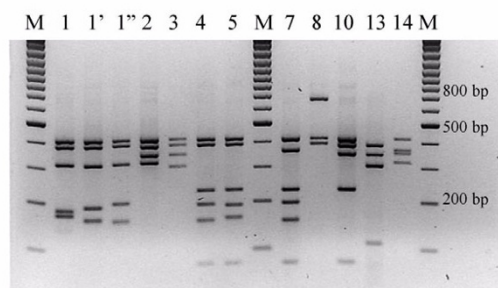


Figure 1

HhaI (*CfoI*) restriction patterns of amplified mycobacterial 16S rRNA genes. Legend: M: marker (100 base pair ladder, Fermentas, Vilnius, Lithuania)

cies are numbered arbitrarily and are presented in Figures 1, 2 and 3 respectively. Figures 4,5,6 represent the restriction patterns obtained when digital restriction is carried out with the same enzymes on published GenBank sequences. The combination of these patterns is designed as ARDRA profiles and these are listed in Table 1. For example, strains of the *M. tuberculosis* complex can be recognized by an ARDRA profile 1-1-1, while *M. goodii* strains have ARDRA profile 8-4-2. For some species the ARDRA pattern obtained with enzyme is already characteristic, e.g. *HhaI* 1 is observed only for species of the *M. tuberculosis* complex.

Most mycobacterial species could be readily identified by comparison of the obtained ARDRA profile with the profiles from Table 1. The species of the *M. tuberculosis* complex can not be differentiated on the basis of the 16S rDNA sequence, and therefore restriction digestion of this gene could not either. Most of the clinically relevant and the most abundant species were readily differentiated from each other. The following species could not be differentiated from each other after digestion with *HhaI*, *MboI*, *RsaI* and *BstUI*: *M. gastri* and *M. kansasii* (1'-4-1-1), *M. bohemicum*, *M. haemophilum* and *M. malmoense* (1'-4-1-3), MCRO6 and *M. nonchromogenicum* (2-6-4-7), *M. chelonae* group I, *M. abscessus* and *M. immunogenicum* (3-3-6-2'), *M. farcinogenes*, *M. fortuitum*, *M. senegalense* and *M. septicum* (4-1-6-7), *M. simiae* and *M. lentiflavum* (5-7-6-2'), *M. goodii* and *M. smegmatis* (10-1-2-2'), *M. tusciae* and *M. flavescens* (10-1-6-2'), and finally *M. genavense* and *M. triplex* (10-7-6-2').

During 1998–2000, approximately 3500 samples were sent to the laboratory for direct smear examination and mycobacterial culture. Of these, 151 specimens, from 149 patients, were culture positive, and 20% of these were also smear positive. Table 2 summarizes the obtained identifi-

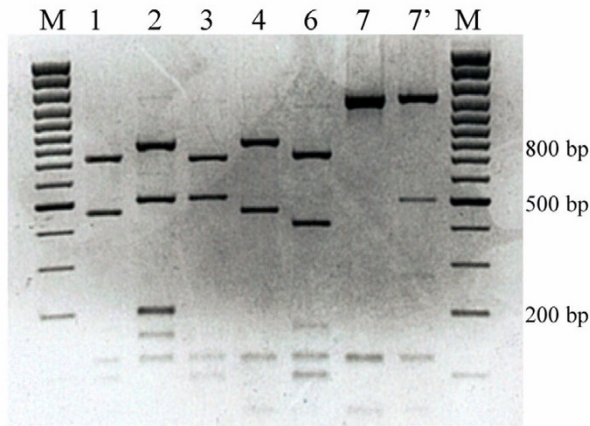


Figure 2
Mbol restriction patterns of amplified mycobacterial 16S rRNA genes. Legend: M: marker (100 base pair ladder, Fermentas, Vilnius, Lithuania)

cations. For 148 of the 151 isolates, identification by ARDRA was straightforward and was obtained after an average of 36 hours after receipt of the cultured strain. Only three difficulties were encountered. One isolate presented with the ARDRA profile 5-4-6, not present in the ARDRA library at that time. Sequencing lead to an identification as *M. interjectum* [8], a species that was not yet covered by the library. A second isolate was first misidentified as *M. xenopi* (profile 1'-4-3), but later on it was observed that the *HhaI* fingerprint differed clearly from the *M. xenopi HhaI* 1' pattern by its low molecular size fragments (Figure 1). This *HhaI* pattern was designated *HhaI* 1'', resulting in the unique ARDRA profile 1''-4-3. The isolate was identified as *M. heckeshornense* by sequencing of the 16S rRNA gene, and can now also be identified by ARDRA. The third problematic isolate had ARDRA profile 1-1-3, again a profile that had never been observed for any mycobacterial strain studied thus far. Sequencing of the 16S rRNA gave a 99.8 % similarity to the 16S rRNA sequence of the type strain of *M. tuberculosis*. The sequence revealed a mutation at *E. coli* position 646 from A to G, abolishing the *RsaI* restriction site GTAC at that place. This mutation shifts the *RsaI* pattern 1 to *RsaI* pattern 3 because the two fragments of resp. 620 and 180 bp are replaced by a single fragment of 800 bp. Further morphological and biochemical tests revealed an identification as probably *M. africanum*, one of the species of the *M. tuberculosis* complex. It should be mentioned that *M. africanum* reference strains used in a previous study [12], were found to have the regular *M. tuberculosis* complex ARDRA profile 1-1-1.

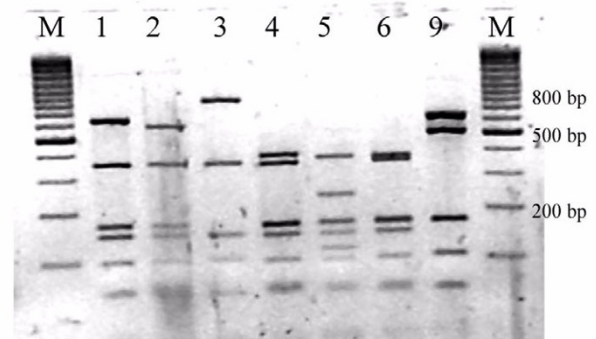
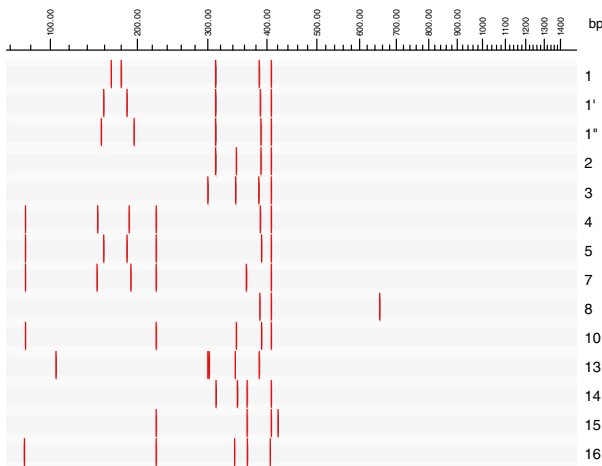


Figure 3
RsaI restriction patterns of amplified mycobacterial 16S rRNA genes. Legend: M: marker (100 base pair ladder, Fermentas, Vilnius, Lithuania)

To construct artificial ARDRA patterns for the recently described species, we applied the programme RFLP on the published Genbank sequences. The resulting, theoretically to be expected, ARDRA profiles are presented in Table 1.

M. tuberculosis was found to be the most prevalent species, with 40% of the isolates, followed by *M. gordonae* and *M. xenopi*, species mostly isolated from non-pulmonary samples with low clinical relevance.

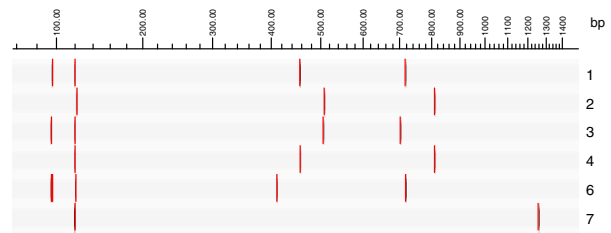
Similarity calculation of published sequences and of sequences obtained in this study (Figure 8) indicates that the *M. abscessus/M. chelonae* complex (*M. chehnae*, *M. abscessus* and *M. immunogenum*) clusters separately from all other mycobacteria. Portaels *et al.* [16] described the presence of four groups within the *M. abscessus/M. chelonae* complex, based on the 16S-23S spacer sequences, as used in the INNO-LiPA Mycobacteria kit (Innogenetics, Zwijnaarde, Belgium). Groups II and IV consisted of genuine *M. chelonae* strains, which were usually from environmental sources. These two groups have characteristic ARDRA profiles, namely 13-3-6 and 3-3-5. Group III, which was found to represent genuine *M. abscessus* and *M. chelonae* group I, which most probably corresponds with *M. immunogenum*, a *M. abscessus*-like species that was recently described as being frequently involved in bronchoscope related pseudo-outbreaks [17], can not be distinguished from each other by ARDRA (profile 3-3-6). Strains of groups I and III are usually isolated from clinical samples, with only group III strains (genuine *M. abscessus*) being pathogenic [16].



Pattern	CfoI restriction fragment lengths (bp)
1	408, 385, 312, 181, 168
1'	408, 388, 312, 187, 160
1''	408, 388, 312, 196, 157
2	408, 387, 347, 312
3	408, 385, 346, 300
4	408, 387, 225, 190, 155, 75
5	408, 387, 225, 187, 160, 75
7	408, 362, 225, 192, 152, 75
8	654, 408, 386
10	408, 389, 347, 225, 75
13	385, 346, 302, 300, 106
14	408, 360, 346, 312, 25
15	422, 408, 363, 225, 25
16	406, 364, 344, 225, 74, 25

Figure 4
HhaI (CfoI) restriction patterns of mycobacterial 16S rRNA genes, theoretically calculated using RFLP (Applied Maths) and published GenBank sequences. Graphical representation and table of restriction fragment lengths for each of the possible patterns.

M. tuberculosis was the most frequently cultured species (40% of all culture positives), followed by *M. goodii* (27%) and *M. xenopi* (15%) The latter two species were mostly isolated from non-pulmonary samples. 46% of the samples culture positive for *M. tuberculosis*, were auramine staining positive, and the average time of incubation until a positive liquid culture of *M. tuberculosis* was 12.9 days for the auramine positive samples and 20 days for auramine negative samples. The range of time to positivity for all *M. tuberculosis* positive samples was between 6 and 29 days. The average culture times for species other than *M. tuberculosis*, as far as data were available, are presented in Table 2.



Pattern	MboI restriction fragment lengths (bp)
1	710, 456, 121, 94, 51, 17
2	808, 508, 121, 17
3	702, 507, 120, 93, 17
4	808, 457, 121, 51, 17
6	718, 411, 121, 96, 94
7	1253, 120, 51, 17

Figure 5
MboI restriction patterns of mycobacterial 16S rRNA genes, theoretically calculated using RFLP (Applied Maths) and published GenBank sequences. Graphical representation and table of restriction fragment lengths for each of the possible patterns.

Discussion

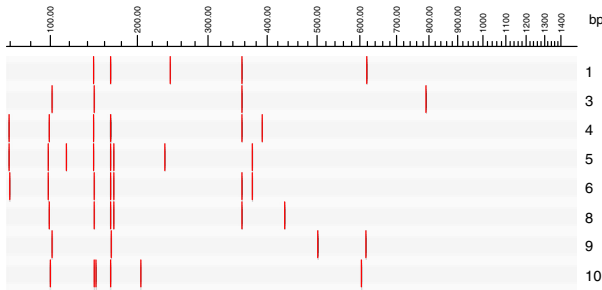
Restriction analysis of the amplified 16S rRNA gene, or amplified rDNA restriction analysis (ARDRA) was introduced into the Laboratory for Bacteriology of the Ghent University Hospital for the identification of cultured mycobacteria in 1993 [12]. Since then, several comparable approaches, based on restriction digestion of the amplified rRNA genes and spacer regions have been described [18–21]. During that period, this approach has been updated and refined. This was possible due to some technical changes, like increased quality control of gel electrophoresis and pattern interpretation and the use of primers specific for species of the order of the Actinomycetales instead of universal bacterial primers. Refinement was also possible because of the improvement of mycobacterial taxonomy and the possibility offered by PCR-RFLP techniques, like ARDRA, to easily adapt to this new information. Indeed, when new species are described, there is no need to develop new probes or primers. Instead, new ARDRA profiles can be easily added to the existing library. Also, ARDRA profiles for newly described species can be predicted by applying computer aided digestion of the available GenBank sequences, given the availability of sequences of sufficient quality [22].

ARDRA was found to be a useful tool for identification of mycobacterial isolates in a clinical routine laboratory, because of its speed – compared to phenotypic identification, its reliability, practical applicability, flexibility and the possibility to identify most nontuberculous mycobacteria together with and at the same cost as *M. tuberculosis*,

Table 1: Library of ARDRA profiles (combination of restriction patterns) obtained for mycobacterial species.

Species	Genbank Number	HhaI	MboI	RsaI	BstUI	Reference strains used ^a
<i>M. tuberculosis</i> complex	X52917	1	1	1	1	ITG 8021, IPB 92/0805
<i>M. conspicuum</i>	X88922	1'	1	2	2	
<i>M. intracellulare</i>	X52927	1'	2	2	3	ITG 5913, ITG 5917
<i>M. gastri</i>	X52919	1'	4	1	1	
<i>M. kansasii</i>	M29575	1'	4	1	1	ITG 8201
<i>M. bohemicum</i>	AJ277283	1'	4	1	3	
<i>M. haemophilum</i>	U06638	1'	4	1	3	ITG 3065
<i>M. malmoense</i>	AF152560	1'	4	1	3	ITG 940611
<i>M. szulgai</i>	X52926	1'	4	2	1	ITG 4981
<i>M. scrofulaceum</i>	X52924	1'	4	2	3	ITG 4988
<i>M. xenopi</i>	X52929	1'	4	3	2	ITG 4986
<i>M. heckeshornense</i>	AF174290	1'	4	3	4	
<i>M. marinum</i>	AF251565	2	1	1	1	ITG 1728
<i>M. asiaticum</i>	M29556	2	1	2	5	ITG 8182
<i>M. terrae</i>	X52925	2	1	4	2	ITG 4922
<i>M. ulcerans</i>	Z13990	2	1	9	1	ITG 724, ITG 1837
<i>M. avium</i>	AF306455	2	2	2	3	ITG 4991, ITG 2666
<i>M. botniense</i>	AJ012756	2	4	3	2	
<i>M. terrae</i> -like MCRO6 ^b	X93032	2	6	4	7	
<i>M. nonchromogenicum</i>	X52928	2	6	4	7	ITG 4980
<i>M. phlei</i>	M29566	3	1	6	2'	
<i>M. elephantis</i>	AJ010747	3	1	8	3'	
<i>M. chelonae</i> group IV ^c	AJ416940	3	3	5	2'	ITG 7701
<i>M. abscessus</i> group III ^c	AJ419970	3	3	6	2'	ITG 98-1296
<i>M. chelonae</i> group I ^c	AJ419969	3	3	6	2'	ITG 95-0026
<i>M. immunogenum</i>	AJ011771	3	3	6	2'	ITG 98-1289
<i>M. farcinogenes</i>	AF055333	4	1	6	7	
<i>M. fortuitum</i>	X52933	4	1	6	7	SLZ A046
<i>M. senegalense</i>	M29567	4	1	6	7	
<i>M. septicum</i>	AF111809	4	1	6	7	ITG 4166
<i>M. intermedium</i>	X67847	5	4	6	1'	
<i>M. interjectum</i>	AJ272088	5	4	6	2'	ITG 96-116
<i>M. heidelbergense</i>	X70960	5	4	8	2'	
<i>M. simiae</i>	X52931	5	7	6	2'	ITG 4485
<i>M. lentiflavum</i>	X80769	5	7	6	2'	
<i>M. peregrinum</i> ^d	X52921	7	1	6	9	ITG 99-2069, ATCC 14467
<i>M. gordonae</i>	M29563	8	4	2	5	ITG 7838
<i>M. wolinskyi</i>	Y12871	10	1	1	2'	
<i>M. species</i> ^a	AF028712	10	1	1	2'	
<i>M. goodii</i>	Y12872	10	1	2	2'	
<i>M. smegmatis</i>	AJ131761	10	1	2	2'	ITG 4995
<i>M. kubicae</i>	AF133902	10	1	6	1'	
<i>M. tusciae</i>	AF058299	10	1	6	2'	
<i>M. flavescens</i>	X52932	10	1	6	2'	VUB A016
<i>M. genavense</i>	X60070	10	7	6	2'	ITG 97-102
<i>M. triplex</i>	U57632	10	7	6	2'	
<i>M. chelonae</i> group II ^c	AJ419968	13	3	6	6	ITG 96-0295
<i>M. branderi</i>	X82234	14	1	1	9	
<i>M. celatum</i> ^e	L08169	14	1	1	10	
<i>M. triviale</i>	X88924	15	1	10	10'	
<i>M. aurum</i>	X55595	16	1	6	8	

a. ITG: Institute for Tropical Medicine, Antwerp, Belgium; IPB: Institute Pasteur du Brabant, Brussels, Belgium; SLZ: Streeklaboratorium Zeeland, Goes, the Netherlands; VUB: Free University of Brussels, Brussels, Belgium. b. Strains designated MCRO6 have been studied by Turenne *et al.*[22] and Torkko *et al.*[33]. c. Sequences determined in this study. Roman numbering according to Portaels *et al.*[16], who distinguished four groups within the *M. abscessus/M. chelonae* complex, based on the 16S–23S rRNA spacer region. d. The GenBank sequence (AF058712) of strain ATCC 14467, submitted to GenBank as *M. peregrinum*, did not cluster within the *M. fortuitum* complex (Figure 8) and was highly similar to sequence Y12871 (*M. wolinskyi*). Moreover, the ARDRA profile calculated from sequence AF058712 (10-1-1-2') did not correspond with the profile we obtained for strain ATCC 14467 (4-1-6-7). The sequence obtained in this study from strain ATCC 14467 (submitted as AJ422046) was identical to the *M. fortuitum* sequence X52933. Sequence AF058712 is indicated as *M. species* in the table. e. Received as *M. xenopi*.



Pattern	RsaI restriction fragment lengths (bp)
1	616, 355, 167, 148, 99, 53, 16
2	556, 355, 167, 148, 99, 60, 53, 16
3	788, 355, 148, 101, 53, 16
4	391, 355, 167, 167, 148, 99, 59, 55, 16
5	373, 237, 171, 167, 147, 117, 98, 59, 53, 16
6	373, 355, 171, 167, 148, 98, 60, 53, 16
8	433, 355, 171, 167, 148, 99, 53, 16
9	616, 502, 167, 102, 53, 16
10	604, 205, 167, 150, 148, 100, 53, 16

Figure 6
RsaI restriction patterns of mycobacterial 16S rRNA genes, theoretically calculated using RFLP (Applied Maths) and published GenBank sequences. Graphical representation and table of restriction fragment lengths for each of the possible patterns.

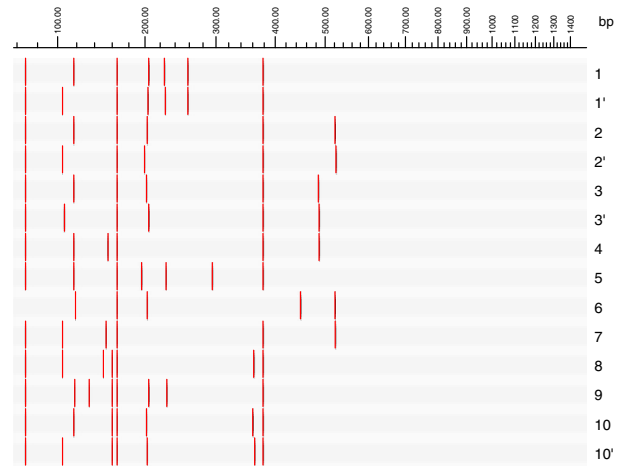
at an affordable price. Of the 151 isolates during the last three years, 148 could be identified without problems. The other three isolates, respectively *M. interjectum*, *M. heckeshornense* and an *M. africanum*-like strain, should be identifiable when met again in the future, since they presented with specific ARDRA profiles.

Practical applicability of ARDRA

The theoretical turnaround time of ARDRA is 6 hours, and the average identification time in practice during this study was 36 hours. It should be emphasized that the technique was not fully implemented in the routine laboratory, but was carried out by the research laboratory technicians, which means that the practical turnaround time should be far less than 36 hours in a routine diagnostic laboratory. Technically, ARDRA is nondemanding, comprising only basic molecular biology techniques like simple DNA extraction, PCR, restriction digestion and submarine agarose gel electrophoresis.

General considerations

We have addressed previously the several limitations of molecular biology based detection in diagnostic bacteriology [23]. Others agree that the expectations that DNA amplification technologies would supplant microscopy, accurately predict culture results and provide an immedi-



Pattern	BstUI restriction fragment lengths (bp)
1	378, 258, 227, 205, 166, 117, 68, 36
1'	378, 258, 227, 204, 166, 105, 68, 36
2	521, 378, 203, 166, 117, 68
2'	521, 378, 200, 166, 105, 68
3	485, 378, 202, 166, 117, 68, 35
3'	485, 378, 205, 166, 117, 68, 36
4	487, 378, 166, 155, 117, 68, 54, 36
5	378, 294, 228, 196, 166, 117, 68
6	521, 448, 203, 166, 119
7	522, 378, 166, 153, 105, 68, 48
8	378, 362, 166, 160, 150, 105, 68, 50
9	378, 229, 205, 166, 160, 134, 117, 68
10	378, 360, 202, 166, 160, 117, 68
10'	378, 363, 203, 166, 160, 105, 68

Figure 7
BstUI restriction patterns of mycobacterial 16S rRNA genes, theoretically calculated using RFLP (Applied Maths) and published GenBank sequences. Graphical representation and table of restriction fragment lengths for each of the possible patterns.

ate definitive diagnosis were premature and that these claims have to be replaced with a more realistic view of the limitations and of the practical value of molecular diagnostics of tuberculosis [10,24,25]. Also the expectation that susceptibility would be carried out solely by means of DNA technology, had to be moderated [26]. Despite the fact that during the last ten years a tremendous effort, both in academic and commercial research, has been put into the applicability of nucleotide amplification techniques for the detection of mycobacteria directly from clinical samples, the CDC approved application of these techniques only for smear positive samples. This implicates that for 52% of the culture positive samples with *M. tuberculosis* encountered in this study, DNA technology would not have accelerated detection, since microscopy

Table 2: Distribution of the *Mycobacterium* species during 1998–2000 in the Ghent University Hospital, number of smear positives and time to positivity of culture.

<i>Mycobacterium</i> species	Number of isolates (%)	Number of samples positive on direct smear ^a (% of culture positive)	Average time until positive culture in days (smear positives; negatives)
<i>M. tuberculosis</i> complex	61 (40)	26 (46 ^b)	16.7 (12.9; 20)
<i>M. avium</i>	12	2	6.5
<i>M. heckeshornense</i>	1	1	32.5
<i>M. chelonae</i>	4	0	18
<i>M. fortuitum</i>	2	0	35.5
<i>M. gordonae</i>	41 (27)	1	22.3
<i>M. interjectum</i>	1	0	30.5
<i>M. intracellulare</i>	1	0	11.6
<i>M. kansasii</i> / <i>M. gastri</i>	2	0	No data
<i>M. malmoense</i>	1	0	No data
<i>M. szulgai</i>	3	0	20.3
<i>M. xenopi</i>	22 (15)	0	38.1
Total	151	40 (26)	

^a: No data for 6 of the 61 samples. All six of these samples were *M. tuberculosis* culture positive. ^b: Calculated as 26 positives on a total of those 56 culture positives for which data on smear positivity were available.

was negative. Recently, AMTD2 (GenProbe) gained FDA approval for testing smear negative sputa, but the cost of the technique keeps limiting its use to only those smear negative samples with strong clinical suspicion of tuberculosis. Moreover, since this kind of direct detection amplification technology is technically demanding or requires specialized equipment and kits, many laboratories carry out these tests only at well-set time intervals [e.g. [11]], delaying diagnosis with several days on average, and as such losing some of the time gain offered by these direct detection methods. As a final remark, one should keep in mind that direct detection without direct antimicrobial susceptibility testing does not obviate the need for culture after all.

ARDRA compared to other culture based genotypic identification techniques

ARDRA and other gene restriction techniques [14,15,21,27,28] have been developed as a practical shortcut to full sequence determination. From this study and others it is clear that the discriminatory power of these RFLP approaches for identification of mycobacteria is almost as high as that of sequencing. The discriminatory power and reliability of a commercially available rRNA-spacer based hybridisation assay (INNO-LiPA Mycobacteria) is high, but again this approach is somewhat more laborious and more expensive than in house PCR-RFLP-based techniques. Restriction digestion of a 439 bp stretch of the *hsp65* gene for identification of mycobacteria was described almost simultaneously with ARDRA and designated PCR-RFLP Analysis (PRA) [15]. Several laboratories have published their experience using this technique [e.g.

[14,27,28]]. Possible drawbacks of *hsp65* gene restriction analysis are the smaller sized restriction fragments and the higher intraspecific variability, which may make interpretation more difficult. The small size differences have led to the use of polyacrylamide gel electrophoresis [27], which is less practical than agarose gel electrophoresis and the interpretation difficulties in general have led to reconsideration of the *hsp65* gene restriction profiles used thus far [28]. Comparable remarks can be made for PCR-RFLP analysis of the rRNA spacer region [21].

Future developments

At present, in an effort to have the best of both worlds, we are performing a double PCR, directly on smear positive, decontaminated samples, extracting DNA with the commercially available QiaAmp Tissue kit (Qiagen, Hilden, Germany). One PCR attempts to amplify the full length 16S rRNA gene (1500 bp), which can be used for ARDRA, at an annealing at 55°C, and one PCR amplifies a 123 bp region of the IS6110 at an optimal annealing of 68.6°C [29]. Both PCRs are carried out simultaneously in a T-gradient thermocycler (Biometra, Göttingen, Germany), programmed to have both annealing temperatures in the 96 well block. In case of the presence of the 123 bp fragment on an agarose gel, the identification of *M. tuberculosis* is completed, and can optionally be confirmed with ARDRA. Due to the higher sensitivity of the IS6110 PCR compared to the rDNA PCR, we could amplify the IS6110 fragment of *M. tuberculosis* directly from all smear positive, *M. tuberculosis* culture positive samples thus far. In case only the 16S rDNA fragment of 1500 bp is present, the absence of *M. tuberculosis* can be confirmed by ARDRA which

also immediately provides with the identification of the *Mycobacterium* species other than tuberculosis. Thus far, this yielded in all cases an identification as a nontuberculous strain, confirming the absence of *M. tuberculosis*. Also in case both amplifications of the smear positive sample remain negative, this can be interpreted as the absence of *M. tuberculosis*. A positive culture is then awaited to identify the nontuberculous organism with ARDRA.

Taxonomical considerations

This study also confirmed the robustness of ARDRA based identifications. For example, for the first study [12], we received strains identified by reference laboratories as *M. avium* and *M. xenopi*, using phenotypic methods. During the first study already, some strains that had been sent as *M. avium* could be shown by ARDRA (and subsequent confirmation by another laboratory) to be *M. scrofulaceum*. For *M. xenopi*, ARDRA indicated the presence of several groups. In the meantime mycobacterial taxonomy has been refined and the groups we indicated as *M. xenopi* A and *M. xenopi* B [12] appear to correspond to genuine *M. xenopi*, resp. *M. celatum* (ITG 6147)[30], as becomes apparent when applying the programme RFLP on the 16S rRNA sequence data we obtained. Similarly, strains that we classified as *M. fortuitum* A and B [12], appear to correspond to *M. fortuitum* subsp. *fortuitum*, resp. *M. peregrinum*.

Materials and Methods

Strains

A collection of well characterized reference strains belonging to different mycobacterial species was used to create an ARDRA profile library. The strains used are listed in Table 1. The 151 clinical strains used in this evaluation were collected during the period between January 1998 and December 2000 in the routine clinical laboratory of the Ghent University Hospital.

Processing and culturing of the samples

Decontamination of the samples was done by mixing 1 ml of sample with 1 ml of decontamination buffer (3% NaOH/N-acetyl L-cysteine (NALC)). After 15 min of incubation at room temperature the mixture was neutralized by adding 40 ml of 0.067 M phosphate buffer (pH 6.8), followed by centrifugation at 11600 g during 15 min. The supernatant was removed and part of the pellet was used for auramine staining and microscopy. The remaining pellet was suspended in 1 ml of phosphate buffer and used for inoculation of culture media. 100 µl was used for inoculation of a solid medium (Ogawa, Sanofi-Pasteur, Marnes la Coquette, France) and 500 µl for the inoculation of a liquid medium. During this study the automated liquid culture system was changed from the Bactec system (Becton Dickinson, Cockeysville, Md.) to the 3D BacT/Alert system (Organon Teknika, Boxtel, The Netherlands).

DNA extraction

Starting from liquid culture, 500 µl of a positive culture was transferred to a 1.5 ml screw cap Eppendorf tube. After centrifugation at 13.000 rpm for 15 minutes, the supernatant was removed and the resulting pellet was resuspended in 50 µl TE buffer (100 mM Tris-HCl – 10 mM EDTA, pH 8.0). The mixture was heated for 30 minutes at 95°C, followed by a freezing step at -20°C for at least 30 minutes. Starting from solid culture, a loopful of a bacterial colony was suspended in 500 µl of TE buffer. The mixture was heated at 95°C for 30 minutes, followed by a freezing step at -20°C for at least 30 minutes. Prior to PCR, DNA extracts were thawed at 4°C and centrifuged shortly to pellet the debris.

ARDRA for mycobacteria consists of the amplification of the 16S rRNA gene, followed by separate restriction digestion with *HhaI*, *MboI* and *RsaI*. The combination of the three obtained fingerprints is designated an ARDRA profile which can be compared with a library of ARDRA profiles, obtained from well-identified mycobacterial strains. In some cases, more discriminatory identification is possible by additional restriction with *BstUI*.

Amplification of the 16S rRNA gene

The primers used to amplify the full length 16S rRNA gene (approximately 1500 bp) were MBUZ1 (GAC GAA CGC TGG CCG CGT GCT TAA C) and MBUZ2 (CGT CCC AAT CGC CGA TC). These primers are designated to amplify only the 16S rRNA gene for species of the order Actinomycetales. The PCR mixture consisted of 25 µl Qiagen Mastermix (Qiagen, Hilden, Germany), 0.2 µM of each primer, 5 µl of DNA extract, and was adjusted to 50 µl with distilled water. Thermal cycling consisted of an initial denaturation of 5 min at 94°C, followed by three cycles of 1 min at 94°C, 2 min at 55°C and 1 min at 72°C, followed by 30 cycles of 20 sec at 94°C, 1 min at 55°C and 1 min 72°C, with a final extension of 7 min at 72°C, and cooling at 10°C.

Amplification of 123 bp of the IS6110 region was carried out as described [29] after DNA extraction from decontaminated sputum samples using the QiaAmp Tissue kit (QiaGen, Hilden, Germany).

Restriction digestion

The restriction enzymes used were *HhaI* (isoschizomer of *CfoI*) (Amersham Pharmacia Biotech Benelux, Roosendaal, the Netherlands), *MboI* (Fermentas, Vilnius, Lithuania), *RsaI* (Amersham Pharmacia). When necessary for further discrimination, digestion with *BstUI* (New England Biolabs, Beverly, Ma.) was carried out. Each 16S rDNA amplicon was divided in three separate tubes in aliquots of 10 µl, to which 10 U of the respective restriction enzymes were added, with 2 µl of the corresponding en-

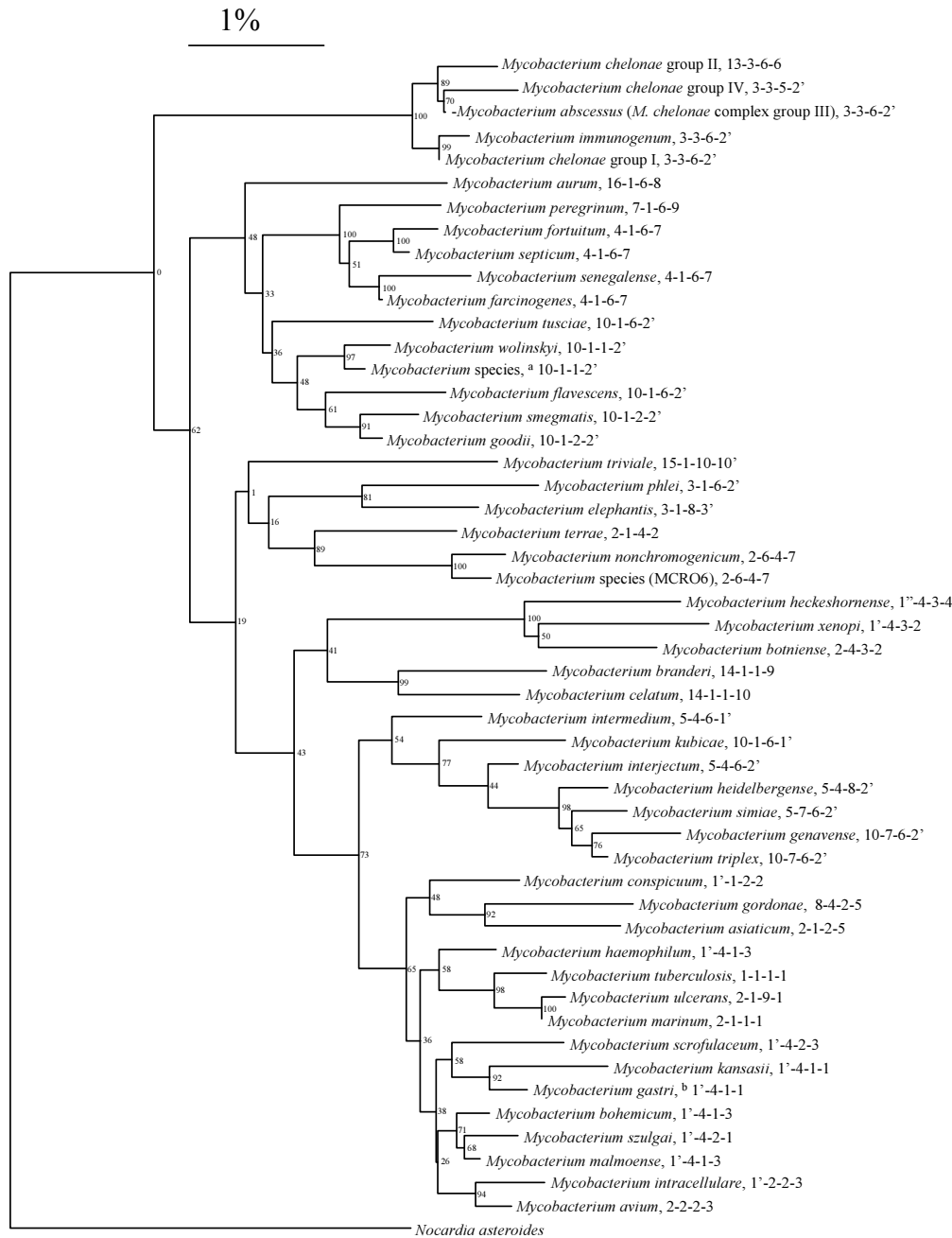


Figure 8

Neighbour-joining similarity tree for 16S rRNA gene sequences of most mycobacterial species. Legend: *N. asteroides* ATCC 49872 (Genbank Z82229) was used as the outgroup. Table I lists the GenBank accession numbers of the sequences used to construct this tree. ARDRA patterns for *HhaI*, *MboI*, *RsaI* and *BstUI* are listed after the species name. a: GenBank AF028712. Erroneously listed in GenBank as *M. peregrinum* (see also legend of Table I). b: *M. gastri* clusters below 100% with *M. kansasii*, although it is generally agreed that the 16S rRNA gene sequences for *M. kansasii* and *M. gastri* are identical. This can be explained by the fact that the only available GenBank *M. gastri* sequence (X52919) contained several ambiguities. c. *M. lentiflavum*, initially not included in the manuscript is not presented in this tree. It clusters close to the branch including *M. heidelbergense*, *M. simiae*, *M. triplex* and *M. genavense*.

Table 3: Overview of the theoretical ARDRA profiles of the species that were newly described since the start of the evaluation period (1998), as obtained after computer aided digestion of the published sequences (see also Figures 4,5,6,7).

Species	Source of isolation	Reference	GenBank Accession Numbers	HhaI	MboI	RsaI	BstUI
<i>M. bohemicum</i>		[34]	AJ277284, U84502	1'	4	1	3
<i>M. botniense</i>	Water	[35]	AF012756	2	4	3	2
<i>M. elephantis</i>	Elephant	[36]	AF010747	3	1	8	3'
<i>M. goodii</i>	Human wounds	[37]	Y12872	10	1	(1)	2'
			Y12872 ^a	10	1	2	2'
<i>M. heckeshornense</i>	Lung disease	[38]	AF1174290	1	4	3	4
<i>M. kubicae</i>		[39]	AF133902	10	1	6	1'
<i>M. murale</i>	Walls of childrens daycare centre	[40]	Unpublished				
<i>M. septicum</i>	Catheter related bacteraemia	[41]	AF111809	4	1	6	7
<i>M. tuberculosis</i> subsp. <i>caprae</i>	Goats in Spain	[42]	AJ131120	1	1	1	1
<i>M. tusciae</i>		[43]	AF058299	10	1	6	2'
<i>M. wolinskyi</i>	Human wounds	[37]	Y12871, Y12873	10	1	1	2'

a. After resolving the ambiguity (N) present at *E. coli* position 95 in the Genbank sequence Y12872. An identical base (G) is found at this position in all mycobacteria.

zyme buffer (10× concentrated, final concentration 2×) and each restriction digestion mixture was adjusted to 20 µl with distilled water and incubated during 2 hours at 37°C in a heater.

Electrophoresis

The DNA restriction fragments were electrophoresed in a 2.5% agarose electrophoresis gel, containing 2% Methaphor (FMC Bioproducts, Rockland, Me.) and 0.5% MP agarose (Roche) in the presence of ethidium bromide (50 ng/ml). The gels were photographed and the fingerprints were compared visually with the overview gels (Figures 1, 2 and 3).

16S rDNA sequencing and comparative analysis

A fragment of the 16S rRNA gene (corresponding to positions 10-1507 in the *Escherichia coli* numbering system) was sequenced as described previously [31]. Sequencing primers were MB UZ1 (GACGAACGCTGGCGGCGTGCTTAAC, *E. coli* position 27-50), MB UZ2 (CGTCCAAATCGCCGATC, 1493 - 1476), MBP1 (CCG-GCCAACTACGTGCCAGC, 502 - 522), MBP2 (CTGGAATTCCTGGTGTAGCGG, 673 - 693), MBP3R (GCATGTCAAACCCAGGTAAGG, 1006 - 986) and MBP4R (CCACCTTCCTCCGAGTTGACC, 1185 - 1165)

The 16S rDNA sequences obtained in this study are indicated in Table 1. All steps of the comparative sequence analysis were performed by using the GeneBase software package (Applied Maths, St. Martens Latem, Belgium), as described [32]. First, pairwise alignment using UPGMA was carried out with a gap penalty of 100 %, a unit gap cost of 20 % and an ambiguity cost of 50 % of the mismatch cost. Subsequently, global alignment - with *N. as-*

teroides ATCC 49872 (Genbank Z82229) used as the outgroup - was carried out on the region corresponding to positions 67 through 1444 of the 16S rRNA gene of *E. coli*, with costs as above. Finally, a similarity matrix of the aligned sequences was constructed by global alignment homology calculation and a gap penalty of 20 %. The neighbour-joining method was used to construct the dendrogram based on this similarity matrix. Bootstrap values were calculated.

Theoretical calculation of restriction patterns was done by means of RFLP (Applied Maths), which makes it possible to obtain restriction patterns using sequences in EMBL format, for every restriction enzyme. The programme Gel-Compar (Applied Maths) was then used to display the obtained fingerprints.

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