

A paradigm for the molecular identification of *Mycobacterium* species in a routine diagnostic laboratory

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The aim of this study was to improve the identification of *Mycobacterium* species in the context of a UK teaching hospital. Real-time PCR assays were established to enable the rapid differentiation between *Mycobacterium tuberculosis* (MTB) complex and *Mycobacterium* species other than *tuberculosis* (MOTT), followed by 16S rRNA gene sequencing for the speciation of MOTT. Real-time PCR assays gave comparable results to those from the reference laboratory. The implementation of these PCR assays using an improved bead extraction method has enhanced the mycobacterial diagnostic service at the Royal Free Hospital by providing a rapid means of differentiating between MTB complex and MOTT, and would be simple to implement in similar laboratories. Sequence analysis successfully identified a range of *Mycobacterium* spp. representative of those encountered in the clinical setting of the authors, including *Mycobacterium avium* complex, *Mycobacterium fortuitum* group, *Mycobacterium chelonae*–*Mycobacterium abscessus* group, *Mycobacterium xenopi* and *Mycobacterium goodnae*. It provides a useful tool for the identification of MOTT when clinically indicated.

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INTRODUCTION

The rapid detection and identification of clinically important *Mycobacterium* spp. is essential for patient management and infection control. *Mycobacterium tuberculosis* (MTB) remains one of the leading causes of morbidity and mortality worldwide, but *Mycobacterium* species other than *tuberculosis* (MOTT) are increasingly important pathogens. Speciation is particularly important when choosing antibiotic regimens for immunocompromised patients, in whom the presence of any acid-fast bacilli may be considered significant (Katoch, 2004).

In recent years, the development of molecular techniques has had a major impact on the diagnosis of mycobacterial infections (Davies *et al.*, 1999; Conaty *et al.*, 2005). Methods for the detection and identification of mycobacteria include nucleic acid probes (Arnold *et al.*, 1989), conventional PCR amplification, PCR hybridization with species-specific probes (Fiss *et al.*, 1992; Zolg & Philipp-Schulz, 1994; De Beenhouwer *et al.*, 1995; Portaels *et al.*, 1997; Hong *et al.*, 2004), PCR RFLP analysis (Vanechoutte *et al.*, 1993; Kasai *et al.*, 2000; Lee *et al.*, 2000; Roth *et al.*, 2000; Kim *et al.*, 2005) and nucleic acid sequence analysis. Genes that have been targeted for sequence analysis include

hsp65 (Ringuet *et al.*, 1999), 32 kDa protein gene (Soini *et al.*, 1994), *gyrB* (Kasai *et al.*, 2000), *recA* (Blackwood *et al.*, 2000), *rpoB* (Kim *et al.*, 1999; Adekambi *et al.*, 2003) and the 16S rRNA gene (Han *et al.*, 2002; Hall *et al.*, 2003; Pauls *et al.*, 2003). Duplex, multiplex and real-time PCR assays for the identification of mycobacteria have also been described (Broccolo *et al.*, 2003; Shrestha *et al.*, 2003; Tanaka *et al.*, 2003; Kim *et al.*, 2004; Kurabachew *et al.*, 2004).

Several commercial systems using various technologies for the detection and identification of *Mycobacterium* spp. are in routine use, including the INNO-LiPA Mycobacteria assay (Innogenetics; Tortoli *et al.*, 2001), GenoType Mycobacterium (Hain Diagnostika; Padilla *et al.*, 2004), the Cobas Amplicor PCR system (Roche; Bogard *et al.*, 2001), the LCx *Mycobacterium tuberculosis* ligase chain reaction assay (Abbott Laboratories; Lindbrathen *et al.*, 1997), the BD ProbeTec strand displacement amplification (Becton Dickinson; McHugh *et al.*, 2004) and the amplified *Mycobacterium tuberculosis* direct test (Gen-Probe Inc; O'Sullivan *et al.*, 2002).

The Royal Free Hospital is a UK teaching hospital and tertiary referral centre for transplantation and HIV, receiving 146 mycobacterium isolates in 2005. Due to our large immunocompromised population, a significant proportion (35%) of these were MOTT and it is essential that these are

Abbreviations: MOTT, *Mycobacterium* species other than *tuberculosis*; MTB, *Mycobacterium tuberculosis*.

rapidly identified to enable appropriate patient management. All new isolates are currently sent to a reference laboratory (Mycobacterial Reference Unit, Dulwich, UK, now at Barts & Royal London, Queen Mary School of Medicine and Dentistry, London, UK) for identification. We proposed to improve the mycobacterial diagnostic service at the Royal Free Hospital by the validation and implementation of real-time PCR assays for the differentiation between MTB complex and MOTT, and 16S rRNA gene sequence analysis for the speciation of MOTT isolates.

METHODS

Bacterial strains. A total of 194 isolates from 194 patients were analysed. Group 1 ($n=166$) was retrospectively tested, and included all available isolates from different patients between September 2004 and May 2006, and group 2 ($n=28$) included all isolates prospectively collected between April 2006 and June 2006. The real-time PCR assays were performed for all 194 isolates. Sequencing was performed for 32 isolates from group 1.

DNA extraction

In our routine diagnostic practice, sodium hydroxide decontaminated samples were inoculated into an automated culture system (BacT Alert; bioMérieux) that reads positive when growth is detected. Selective 7H9 broths and two LJ slopes (one with pyruvate and one without) were then inoculated and DNA was extracted.

Crude extraction method. This was performed for all group 1 isolates ($n=166$). Volumes of 500 μ l 7H9 broth culture or a 10 μ l loop full of cell growth in 500 μ l Tris-EDTA (TE) (10 mM Tris, 0.1 mM EDTA, pH 8.0) buffer were centrifuged at 7500 g for 3 min, pellets were resuspended in 500 μ l TE buffer and incubated at 80 °C for 1 h.

Chloroform extraction method. Isolates producing no results using the real-time PCR assays were retested following chloroform extraction ($n=21$). Briefly, heat killed bacteria were incubated with lysozyme (Sigma) for 1 h at 37 °C, followed by digestion with 50 μ g proteinase K (Sigma) in 10% SDS for 10 min at 65 °C. A further incubation with 1% (w/v) cetyltrimethylammonium bromide in ≥ 0.5 M NaCl for 10 min at 65 °C was followed by partition using chloroform/isoamyl alcohol (24 : 1, v/v) (Gillespie *et al.*, 2000).

Bead extraction method. This was performed for all isolates in group 2 ($n=28$). Volumes of 500 μ l 7H9 broth culture or a 10 μ l loop full of cell growth in 500 μ l TE buffer were centrifuged at 14 243 g for 3 min, pellets were resuspended in 200 μ l TE buffer and transferred into microfuge tubes containing 0.1 g 80 mesh glass beads and 0.1 g 425–600 micron glass beads (Sigma). These were vortexed for 5 min, incubated at 80 °C for 20 min, centrifuged at 14 243 g for 5 min and 100 μ l supernatant was retained.

MTB *rpoB* real-time PCR assay. Primer set Tbc1 and TbcR5 (Kim *et al.*, 2004) was used to amplify a 235 bp region of the MTB complex *rpoB* gene in a reaction volume of 25 μ l containing 12.25 μ l Absolute QPCR SYBR green (ABgene), 2.5 μ l extracted DNA, 0.5 mM each primer and PCR quality water.

MOTT *rpoB* real-time PCR assay. Primer set M5 and RM3 (Kim *et al.*, 2004) was used to amplify a 136 bp region of the MOTT *rpoB* gene in a final volume of 25 μ l containing 12.25 μ l Absolute QPCR SYBR green, 2.5 μ l extracted DNA, 1 mM each primer and PCR quality water. PCR cycles for both assays were performed using a

Rotor-Gene (Corbett Research) and consisted of 15 min at 95 °C, followed by 30 cycles of 95 °C for 15 s and 72 °C for 30 s, with a final extension at 72 °C for 10 min. Post-amplification melt curves were generated and analysed using the Rotor-Gene software to determine the melting temperature of the PCR products, which is based on their size and composition. A positive MTB complex identification was defined as amplification in the quantification channel with the normal fluorescence threshold set at 0.5 and a melt curve peak at 90–91 °C. A positive MOTT identification was defined as amplification in the quantification channel with the normal fluorescence threshold set at 0.5 and a melt curve peak at 88–90 °C.

Mycobacterium 16S rRNA gene PCR. The primer set described by Han *et al.* (2002) was used to amplify a 640–665 bp region of the 16S rRNA gene. PCR reactions were 100 μ l in volume, consisting of 1 \times Taq buffer, 2 mM MgCl₂, 0.1 mM dNTPs, 2 units Taq polymerase (Invitrogen), 1 mM each primer, 20 μ l extracted DNA and PCR quality water. PCR cycles used were 95 °C for 5 min, followed by 40 cycles of 94 °C for 20 s, 55 °C for 20 s and 75 °C for 40 s.

DNA sequence analysis. Amplicons purified with a QIAquick purification kit (Qiagen) were quantified by comparison with a Bioline Hyperladder I (Bioline) and sequenced using the Big Dye terminator cycle sequencing ready reaction kit (Applied Biosystems). Briefly, 2 ng cleaned-up DNA was added to 3 μ l buffer, 1 μ l cycle sequencing ready reaction mix, 0.16 mM forward or reverse primer and PCR quality water. The reaction parameters were 96 °C for 1 min, followed by 25 cycles of 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min (Techne). For each isolate, two forward and two reverse cycle sequencing reactions were performed. Cycle sequencing products were precipitated, rehydrated in loading buffer and analysed using an ABI 377 automated sequencer according to manufacturer's instructions (Applied Biosystems). Chromatograms were visualized using Chromas software (<http://www.techelysium.com.au/chromas.html>). Reverse compliments were generated using the Sequence Manipulation Site (<http://www.ualberta.ca/~stothard/javascript/index.html>) and forward sequences were aligned using ClustalW (<http://www.ebi.ac.uk/clustalw>). GenBank BLAST searches (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi>) were performed for species identification.

Identification of mycobacterial species at the Mycobacterium Reference Unit. At the beginning of the study phenotypic methods based on biochemical tests, growth characteristics and drug susceptibility profiles were used (Collins *et al.*, 1997). During the course of the study these were replaced by a commercially available identification assay that uses PCR followed by reverse hybridization of the amplified products to a probe array (GenoType Mycobacterium; Hain Diagnostika; Padilla *et al.*, 2004).

RESULTS AND DISCUSSION

Using real-time PCR assays 172 (89%) isolates gave concordant results with the reference laboratory identifications and 22 (11%) isolates failed to amplify for both real-time assays (Table 1). The real-time PCR assays were able to detect a wide range of different *Mycobacterium* spp. including 4 *Mycobacterium abscessus*, 24 *Mycobacterium avium* complex, 1 *Mycobacterium celatum*, 4 *Mycobacterium chelonae*, 20 *Mycobacterium fortuitum*, 7 *Mycobacterium gordonae*, 5 *Mycobacterium kansasii*, 1 *Mycobacterium lentiflavum*, 2 *Mycobacterium mucogenicum*, 4 *Mycobacterium peregrinum*, 1 *Mycobacterium simiae*, 1 *Mycobacterium szulgai*, 6 *Mycobacterium xenopi* and 2 *Mycobacteria* spp. as identified by the reference laboratory. Of the 22 isolates failing

Table 1. Real-time PCR results compared to reference laboratory reports

		Reference laboratory		
		MTB complex	MOTT	Total
Real-time assays	MTB	98	–	98
	MOTT	–	74	74
	No result	14	8	22
	Totals	112	82	194

Table 2. Real-time PCR results using different extraction methods

	MTB complex	MOTT	No result	Total
Crude	74	64	28	166
Chloroform*	6	1	21	28
Bead	18	9	1	28

*Performed on isolates not giving results following the crude extraction method.

to give results, 21 had been extracted using the crude and chloroform DNA extraction methods. A further seven isolates had failed to give results following the crude extraction method but these were resolved by re-extracting DNA using the chloroform extraction method (Table 2). The majority of isolates (19 of the 21) failing to give an identification following chloroform extraction were from 2005, it is therefore likely that the age of the isolates affected the results. Chloroform extraction methods are time consuming and labour intensive, and therefore not suitable for rapid identification assays. To address this, in 2006 a bead extraction method was adopted and used to test samples prospectively. Using this method all samples

except one (96%) were identified as MTB or MOTT (Table 2). The real-time PCR assays have proved to be a rapid and reliable method of differentiating between MTB complex and MOTT. They demonstrated a specificity value of 100%, and a sensitivity value of 83% using the crude extraction method, which was improved to 96% using the bead extraction method although this was with a smaller sample number (Table 2). Using the bead extraction method and the real-time PCR assays, an identification of MTB complex or MOTT can be achieved in 2.5 h. We have found the combination of bead extraction and real-time PCR to be a rapid method that is user friendly and reagent costs (approx. £3 per patient) are relatively inexpensive compared to commercially available tests (approx. £15 per patient depending on assay selected). It can therefore be easily implemented in any diagnostic laboratory with a real-time amplification platform to differentiate *Mycobacterium* spp. from culture.

During this study 16S rRNA sequence analysis was successfully performed for 30 MOTT isolates giving results comparable to a reference laboratory (Table 3). Two isolates failed to give a 16S rRNA PCR product. Sequence analysis of 16S rRNA did not differentiate between *M. kansasii* and *Mycobacterium gastri*; *M. chelonae*–*M. abscessus* group and ‘*Mycobacterium fuerth*’; *M. mucogenicum* and ‘*Mycobacterium ratisbonense*’; or *M. peregrinum*, *Mycobacterium septicum* and *M. fortuitum*. The inability to differentiate between *M. kansasii* and *M. gastri* using 16S rRNA gene sequence analysis has been noted previously, but these species can be distinguished by culture characteristics (Han *et al.*, 2002). Although treatment is the same for both species, *M. kansasii* isolates are usually regarded as clinically significant, but there have been few reported cases of clinically significant *M. gastri* infections (Subcommittee of the Joint Tuberculosis Committee of the British Thoracic Society, 2000; Velayati *et al.*, 2005). ‘*M. fuerth*’ and ‘*M. ratisbonense*’ are newly described species previously shown to be identical to *M. chelonae* and *M.*

Table 3. Comparison of 16S rRNA gene sequence analysis for group 1 isolates ($n=32$) with reference laboratory identifications

Reference laboratory identification (no.)	Sequencing identification (no.)	Homology (%)	Fragment size (bp)
<i>M. avium</i> complex (11)	<i>M. avium</i> complex (7)	97–100	500–634
	No identification (4)	NA	NA
<i>M. fortuitum</i> (11)	<i>M. fortuitum</i> (8)	99–100	457–621
	<i>M. fortuitum</i> group (3)	92–99	623–627
<i>M. kansasii</i> (1)	<i>M. kansasii</i> , <i>M. gastri</i>	100	532
<i>M. chelonae</i> (1)	<i>M. chelonae</i> – <i>M. abscessus</i> group	100	574
<i>M. abscessus</i> (1)	<i>M. chelonae</i> – <i>M. abscessus</i> group, ‘ <i>M. fuerth</i> ’	99	535
<i>M. gordonae</i> (1)	<i>M. gordonae</i>	100	511
<i>M. xenopi</i> (2)	<i>M. xenopi</i> (2)	96–99	591–635
<i>M. mucogenicum</i> (1)	<i>M. mucogenicum</i> , ‘ <i>M. ratisbonense</i> ’	100	527
<i>M. peregrinum</i> (3)	<i>M. peregrinum</i> , <i>M. septicum</i> , <i>M. fortuitum</i>	100	448
	<i>M. fortuitum</i>	–	–
	<i>Mycobacterium</i> spp.	100	525

mucogenicum, respectively, using 16S rRNA gene sequence analysis (Turenne *et al.*, 2001), and there is no evidence that either cause infection in humans. There are reports of *M. peregrinum* and *M. septicum* causing infection in immunocompromised patients but there are no guidelines for treatment (Schinsky *et al.*, 2000; Sakai *et al.*, 2005). Both species have been proposed as members of the *M. fortuitum* group (Brown-Elliott & Wallace, 2002). In this study the identification was taken as the closest match generated by GenBank BLAST search analysis. Similarities ranged from 96 to 100% homology over fragment sizes of 448 to 635 bp. Currently there are no standards for the interpretation and analysis of sequence data in a diagnostic setting; therefore, all results should be interpreted on an individual basis in conjunction with the clinical information.

As demonstrated in a recent study conducted by Yam *et al.* (2006) 16S rRNA sequencing is an effective tool for the identification of *Mycobacterium* spp. It has been proposed that sequence analysis of several genes such as *hsp65*, *rpoB* and *sod*, in addition to the 16S rRNA gene, increases the robustness and power of discrimination to provide a more accurate identification (Devulder *et al.*, 2005). However, the increased commitment in time and labour must be weighed against the clinical value of increased discrimination in the context of our setting. Sequence analysis of the 16S rRNA gene has proven to be clinically useful in our hospital; however, the cost (£18 per patient) and time commitment (18 h total time, including 4 h hands on time) of sequencing is only justified when the clinical circumstance requires a rapid identification for patient management. For example, in November 2004 *Mycobacteria* spp. were isolated from the blood cultures of three haematology patients on the same ward over a period of 10 days. There were concerns that this may be due to transmission within the ward. Sequencing of the isolates identified two as *M. chelonae* and one as *M. fortuitum*, and following analysis of the *M. chelonae* sequences the isolates were considered to be unrelated. This indicated that the three positive blood cultures were independent episodes with no cross transmission.

From our analysis of all specimens submitted to the Royal Free Hospital TB service over a 21 month period we have adopted a model for the rapid discrimination between MTB and MOTT, appropriate to a clinical practice with low levels of MTB in the community but a substantial immunosuppressed population at risk of MOTT infection. Implementation of a real-time PCR assay has provided us with a rapid means of identifying MTB in-house, and 16S rRNA gene sequence analysis provides early diagnostic input to the management of patients who are often on complex treatment regimens.

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REFERENCES

- Adekambi, T., Colson, P. & Drancourt, M. (2003). *rpoB*-based identification of nonpigmented and late-pigmenting rapidly growing mycobacteria. *J Clin Microbiol* **41**, 5699–5708.
- Arnold, L. J., Hammond, P. W., Wiese, W. A. & Nelson, N. C. (1989). Assay formats involving acridinium-ester-labeled DNA probes. *Clin Chem* **35**, 1588–1594.
- Blackwood, K. S., He, C., Gunton, J., Turenne, C. Y., Wolfe, J. & Kabani, A. M. (2000). Evaluation of *recA* sequences for identification of *Mycobacterium* species. *J Clin Microbiol* **38**, 2846–2852.
- Bogard, M., Vincelette, J., Antinozzi, R., Alonso, R., Fenner, T., Schirm, J., Aubert, D., Gaudreau, C., Sala, E. & other authors (2001). Multicenter study of a commercial, automated polymerase chain reaction system for the rapid detection of *Mycobacterium tuberculosis* in respiratory specimens in routine clinical practice. *Eur J Clin Microbiol Infect Dis* **20**, 724–731.
- Broccolo, F., Scarpellini, P., Locatelli, G., Zingale, A., Brambilla, A. M., Cichero, P., Sechi, L. A., Lazzarin, A., Lusso, P. & Malnati, M. S. (2003). Rapid diagnosis of mycobacterial infections and quantitation of *Mycobacterium tuberculosis* load by two real-time calibrated PCR assays. *J Clin Microbiol* **41**, 4565–4572.
- Brown-Elliott, B. A. & Wallace, R. J. (2002). Clinical and taxonomic status of pathogenic nonpigmented or late-pigmenting rapidly growing mycobacteria. *Clin Microbiol Rev* **15**, 716–746.
- Collins, E. H., Grange, T. M. & Yates, M. D. (1997). *Tuberculosis Bacteriology: Organisation and Practice*, 2nd edn, London: Butterworth Heinemann.
- Conaty, S. J., Claxton, A. P., Enoch, D. A., Hayward, A. C., Lipman, M. C. I. & Gillespie, S. H. (2005). The interpretation of nucleic acid amplification tests for tuberculosis: do rapid tests change treatment decisions?. *J Infect* **50**, 187–192.
- Davies, A. P., Newport, L. E., Billington, O. J. & Gillespie, S. H. (1999). Length of time to laboratory diagnosis of *Mycobacterium tuberculosis* infection: comparison of in-house methods with reference laboratory results. *J Infect* **39**, 205–208.
- De Beenhouwer, H., Liang, Z., de Rizk, P., van Eekeren, C. & Portaels, F. (1995). Detection and identification of mycobacteria by DNA amplification and oligonucleotide specific capture plate hybridization. *J Clin Microbiol* **33**, 2994–2998.
- Devulder, G., Perouse de Montclos, M. & Flandrois, J. P. (2005). A multigene approach to phylogenetic analysis using the genus *Mycobacterium* as a model. *Int J Syst Evol Microbiol* **55**, 293–302.
- Fiss, E. H., Chehab, F. F. & Brooks, G. F. (1992). DNA amplification and reverse dot blot hybridization for detection and identification of mycobacteria to the species level in the clinical laboratory. *J Clin Microbiol* **30**, 1220–1224.
- Gillespie, S. H., Dickens, A. & McHugh, T. D. (2000). False molecular clusters due to non-random association of IS6110 with *Mycobacterium tuberculosis*. *J Clin Microbiol* **38**, 2081–2086.
- Hall, L., Doerr, K. A., Wohlfiel, S. L. & Roberts, G. D. (2003). Evaluation of the MicroSeq system for identification of mycobacteria by 16S ribosomal DNA sequencing and its integration into a routine clinical mycobacteriology laboratory. *J Clin Microbiol* **41**, 1447–1453.
- Han, X. Y., Pham, A. S., Tarrand, J. J., Sood, P. K. & Luthra, R. (2002). Rapid and accurate identification of mycobacteria by sequencing hypervariable regions of the 16S ribosomal RNA gene. *Am J Clin Pathol* **118**, 796–801.

- Hong, S. K., Kim, B. J., Yun, Y. J., Lee, K. H., Kim, E. C., Park, E. M., Park, Y. G., Bai, G. H. & Kook, Y. H. (2004). Identification of *Mycobacterium tuberculosis* by PCR-linked reverse hybridization using specific *rpoB* oligonucleotide probes. *J Microbiol Methods* **59**, 71–79.
- Kasai, H., Ezaki, T. & Harayama, S. (2000). Differentiation of phylogenetically related slowly growing mycobacteria by their *gyrB* sequences. *J Clin Microbiol* **38**, 301–308.
- Katoch, V. M. (2004). Infections due to non-tuberculous mycobacteria (NTM). *Indian J Med Res* **120**, 290–304.
- Kim, B. J., Lee, S. H., Lyu, M. A., Kim, S. J., Bai, G. H., Kim, S. J., Chae, G. T., Kim, E. C., Cha, C. Y. & Kook, Y. H. (1999). Identification of mycobacterial species by comparative sequence analysis of the RNA polymerase gene (*rpoB*). *J Clin Microbiol* **37**, 1714–1720.
- Kim, B. J., Hong, S. K., Lee, K. H., Yun, Y. J., Kim, E. C., Park, Y. G., Bai, G. H. & Kook, Y. H. (2004). Differential identification of *Mycobacterium tuberculosis* complex and nontuberculous mycobacteria by duplex PCR assay using the RNA polymerase gene (*rpoB*). *J Clin Microbiol* **42**, 1308–1312.
- Kim, H., Kim, S.-H., Shim, T.-S., Kim, M.-N., Bai, G.-H., Park, Y.-G., Lee, S.-H., Cha, C.-Y., Kook, Y.-H. & Kim, B.-J. (2005). PCR restriction fragment length polymorphism analysis (PRA)-algorithm targeting 644 bp Heat Shock Protein 65 (*hsp65*) gene for differentiation of *Mycobacterium* spp. *J Microbiol Methods* **62**, 199–209.
- Kurabachew, M., Enger, O., Sandaa, R., Skuce, R. & Bjorvatn, B. (2004). A multiplex chain reaction assay for genus-, group- and species-specific detection of mycobacteria. *Diagn Microbiol Infect Dis* **49**, 99–104.
- Lee, H., Park, H.-J., Cho, S.-N., Bai, G.-H. & Kim, S.-J. (2000). Species identification of mycobacteria by PCR-restriction fragment length polymorphism of the *rpoB* gene. *J Clin Microbiol* **38**, 2966–2971.
- Lindbrathen, A., Gaustad, P., Hovig, B. & Tonjum, T. (1997). Direct detection of *Mycobacterium tuberculosis* from patients in Norway by ligase chain reaction. *J Clin Microbiol* **35**, 3248–3253.
- McHugh, T. D., Pope, C. F., Ling, C. L., Patel, S., Billington, O. J., Gosling, R. D., Lipman, M. C. & Gillespie, S. H. (2004). Prospective evaluation of BD ProbeTec strand displacement amplification (SDA) system for diagnosis of tuberculosis in non-respiratory and respiratory samples. *J Med Microbiol* **53**, 1215–1219.
- O'Sullivan, C. E., Miller, D. R., Schneider, P. S. & Roberts, G. D. (2002). Evaluation of Gen-Probe amplified *Mycobacterium tuberculosis* direct test by using respiratory and non-respiratory specimens in a tertiary care center laboratory. *J Clin Microbiol* **40**, 1723–1727.
- Padilla, E., Gonzalez, V., Manterola, J. M., Perez, A., Quesada, M. D., Gordillo, S., Vilaplana, C., Pallares, M. A., Molinos, S. & other authors (2004). Comparative evaluation of the new version of the INNO-LiPA Mycobacteria and GenoType mycobacterium assays for identification of *Mycobacterium* species from MB/BacT liquid cultures artificially inoculated with mycobacterial strains. *J Clin Microbiol* **42**, 3083–3088.
- Pauls, R. J., Turenne, C. Y., Wolfe, J. N. & Kabani, A. (2003). A high proportion of novel mycobacteria species identified by 16S rDNA analysis among slowly growing AccuProbe-negative strains in a clinical setting. *Am J Clin Pathol* **120**, 560–566.
- Portaels, F., Aguiar, J., Fissette, K., Fonteyne, P. A., de Beenhouwer, H., de Rijk, P., Guedenon, A., Lemans, R., Steunou, C. & other authors (1997). Direct detection and identification of *Mycobacterium ulcerans* in clinical specimens by PCR and oligonucleotide specific capture plate hybridization. *J Clin Microbiol* **35**, 1097–1100.
- Ringuet, H., Akoua-Koffi, C., Honore, S., Varnerot, A., Vincent, V., Berche, P., Gaillard, J. L. & Pierre-Audigier, C. (1999). *hsp65* sequencing for identification of rapidly growing mycobacteria. *J Clin Microbiol* **37**, 852–857.
- Roth, A., Reischl, U., Streubel, A., Naumann, L., Koppenstedt, R. M., Habicht, M., Fischer, M. & Mauch, H. (2000). Novel diagnostic algorithm for identification of mycobacteria using genus-specific amplification of the 16S–23S rRNA gene spacer and restriction endonucleases. *J Clin Microbiol* **38**, 1094–1104.
- Sakai, T., Kobayashi, C. & Shinohara, M. (2005). *Mycobacterium peregrinum* infection in a patient with AIDS. *Intern Med* **44**, 266–269.
- Schinsky, M. F., McNeil, M. N., Whitney, A. M., Steigerwalt, A. G., Lasker, B. A., Floyd, M. M., Hogg, G. G., Brenner, D. J. & Brown, J. M. (2000). *Mycobacterium septicum* sp. nov., a new and rapidly growing species associated with catheter-related bacteraemia. *Int J Syst Evol Microbiol* **50**, 575–581.
- Shrestha, N. K., Tuohy, M. J., Hall, G. S., Reischl, U., Gordon, S. M. & Procop, G. W. (2003). Detection and differentiation of *Mycobacterium tuberculosis* and nontuberculous mycobacterial isolates by real time PCR. *J Clin Microbiol* **41**, 5121–5126.
- Soini, H., Bottger, E. C. & Viljanen, M. K. (1994). Identification of mycobacteria by PCR-based sequence determination of the 32-kilodalton protein gene. *J Clin Microbiol* **32**, 2944–2947.
- Subcommittee of the Joint Tuberculosis Committee of the British Thoracic Society (2000). Management of opportunistic mycobacterial infections: Joint Tuberculosis Committee guidelines 1999. *Thorax* **55**, 210–218.
- Tanaka, I. I., Anno, I. S., Andrade Leite, S. R., Cooksey, R. C. & Leite, C. O. F. (2003). Comparison of a multiplex-PCR assay with mycolic acids analysis and conventional methods for the identification of mycobacteria. *Microbiol Immunol* **47**, 307–312.
- Tortoli, E., Nanetti, A., Piersimoni, C., Chichero Farina, C., Mucignat, G., Scarparo, C., Bartolini, L., Valentini, R., Nista, D. & other authors (2001). Performance assessment of new multiplex probe assay for identification of mycobacteria. *J Clin Microbiol* **39**, 1079–1084.
- Turenne, C. Y., Tschetter, L., Wolfe, J. & Kabani, A. (2001). Necessity of quality controlled 16S rRNA gene sequence databases: identifying nontuberculous *Mycobacterium* species. *J Clin Microbiol* **39**, 3637–3648.
- Vanechoutte, M., Beenhouwer, H. D., Claeys, G., Verschraegen, G., De Rouck, A., Paepe, N., Elaichouni, A. & Portaels, F. (1993). Identification of *Mycobacterium* species by using amplified ribosomal DNA restriction analysis. *J Clin Microbiol* **31**, 2061–2065.
- Velayati, A. A., Boloorsaze, M. R., Farnia, P., Mohammadi, F., Karam, M. B., Soheyla-Zahirifard, M. D. & Masjedi, M. R. (2005). *Mycobacterium gastri* causing disseminated infection in children of same family. *Pediatr Pulmonol* **39**, 284–287.
- Yam, W.-C., Yuen, K.-Y., Kam, S.-Y., Yiu, L.-S., Chan, K.-S., Leung, C.-C., Tam, C.-M., Ho, P.-O., Yew, W.-W. & other authors (2006). Diagnostic application of genotypic identification of mycobacteria. *J Med Microbiol* **55**, 529–536.
- Zolg, J. W. & Philippi-Schulz, S. (1994). The superoxide dismutase gene, a target for detection and identification of *Mycobacterium* by PCR. *J Clin Microbiol* **32**, 2801–2812.