Identification of Mycobacterium species by comparative analysis of the dnaA gene

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Abstract
For the establishment of a diagnostic tool for mycobacterial species, a part of the dnaA gene was amplified and sequenced from clinically relevant 27 mycobacterial species as well as 49 clinical isolates. Sequence variability in the amplified segment of the dnaA gene allowed the differentiation of all species except for Mycobacterium tuberculosis, Mycobacterium africanum and Mycobacterium microti, which had identical sequences. Partial sequences of dnaA from clinical isolates belonging to three frequently isolated species revealed a very high intraspecies similarity, with a range of 96.0–100%. Based on the dnaA sequences, a species-specific primer set for Mycobacterium kansasii and Mycobacterium gastri was successfully designed for a simple loop-mediated isothermal amplification method. These results demonstrate that the variable sequences in the dnaA gene were species specific and were sufficient for the development of an accurate and rapid diagnosis of Mycobacterium species.

Introduction
Increasing reports of opportunistic infection by nontuberculous mycobacteria (NTM) in immunocompromised patients such as AIDS patients and elderly people are a matter of serious concern to public health (Horsburg, 1991; Montessori et al., 1996; Primm et al., 2004). The routine diagnosis of mycobacterioses relies primarily on the detection of acid-fast-stained bacilli in the samples by microscopic observation, and the infecting mycobacterial species can be identified with conventional tests including observation of colony morphology and pigmentation, growth rate, and biochemical characteristics (Cernoch et al., 1994; Metchock et al., 1999). Disadvantages of this approach include the time taken to provide clinically relevant information. The clinician must initiate therapy for Mycobacterium tuberculosis against NTM infection several weeks before species identification (Montessori et al., 1996), which may increase healthcare costs, and may reduce the social activity of the patients. Therefore rapid detection and identification of the species level of mycobacteria is required, both to decide whether measures are needed to prevent the spread of the disease and for adequate therapy (American Thoracic Society, 1997).

The mycobacterium species often implicated in NTM infection are Mycobacterium avium–Mycobacterium intracel- lulare complex (MAC), Mycobacterium kansasii, Mycobacterium chelonae, Mycobacterium abscessus, and Mycobacterium xenopi (Wayne & Sramek, 1992; Metchock et al., 1999; Primm et al., 2004). Mycobacterium gordonae, Mycobacterium gastri, or most of the rapidly growing species are rarely pathogenic, but are often encountered as contaminant in clinical samples. Therefore, the discrimination of these species from pathogenic ones is an important diagnostic issue (Primm et al., 2004).

Several studies have been conducted to develop rapid methods based on molecular technique for identifying mycobacterial species in recent years. The DNA sequences reported for such usage are those of 16S rRNA gene (Kirschner et al., 1993; De Beenhouwer et al., 1995; Cloud et al., 2002), recA (Blackwood et al., 2000), rpoB (Kim et al., 1999), gyrB (Kasai et al., 2000), hsp65 (Plikaytis et al., 1992; Brunello et al., 2001), or 16S–23S internal transcribed spacer (ITS) (De Smet et al., 1995; Roth et al., 1998). The 16S RNA gene and ITS-based methods are currently widely accepted as rapid and accurate for identifying mycobacteria (Plikaytis et al., 1992; De Smet et al., 1995; Park et al., 2000; Turenne et al., 2001). However, some species have the same sequence or a very high similarity (Kim et al., 1999; Kasai et al., 2000). This fact indicates the need to develop more reliable and user-friendly molecule-based diagnostic tools.

Recently, Notomi et al. (2000) have reported a novel nucleic acid amplification method, termed loop-mediated
isothermal amplification (LAMP), that amplifies DNA with high specificity, efficacy, and rapidity under isothermal conditions. The LAMP reaction requires a Bst DNA polymerase with strand displacement activity and a set of four specially designed primers that recognize six distinct sequences on the target DNA, the specificity of which should be extremely high. The amplification products are stem-loop DNA structures with several inverted repeats of the target. The advantage of the LAMP method is that the reaction is performed under isothermal conditions of between 60 and 65 °C. As a result, it requires only simple and cost-effective reaction equipment. The LAMP method has emerged as a powerful tool to facilitate genetic testing for various infectious diseases (Enosawa et al., 2003; Iwamoto et al., 2003; Kuboki et al., 2003; Ihira et al., 2004; Parida et al., 2004; Thai et al., 2004).

The purpose of our work is to identify a species-specific region of *Mycobacterium* sp., and to develop a LAMP assay that can differentiate clinically relevant species.

### Materials and methods

#### Bacterial strains and preparation of genomic DNA

The bacteria used in this study comprised 27 strains and 49 clinical isolates as shown in Table 1. All strains except for *Mycobacterium leprae* were cultured on 1% Ogawa medium (Nissui, Tokyo, Japan) at 37 °C. *Mycobacterium leprae* was prepared from infected nude mouse food pad (Shepard, 1960). Genomic DNA was extracted from mycobacterial strains as follows. Mycobacterial cells were resuspended in 1.8 mL of sterile phosphate-buffered saline (PBS) containing 0.1 mm diameter zirconia/silica beads (BioSpec Products Inc., Bartlesville, OK). The mixture was beaded for 20 s with a Beads Homogenizer Model BC-20 (Central Scientific Commerce, Tokyo, Japan), transferred to a 1.5 mL microcentrifuge tube, and the genomic DNA was purified with proteinase K treatment and phenol/chloroform extraction followed by ethanol precipitation, then suspended in 100 μL distilled water.

| Species                          | Strains                        | Accession number | Primer set | Kan32 | Gas583 |
|----------------------------------|--------------------------------|------------------|------------|-------|--------|
| *Mycobacterium abscessus*        | JATA 63-01 (ATCC 19977)        | A8087684         | –          | –     | –      |
| *Mycobacterium africanum*        | KK 13-02 (ATCC 25420)          | A8087685         | –          | –     | –      |
| *Mycobacterium avium*            | JATA 51-01 (ATCC 25291)        | A8087686         | –          | –     | –      |
| *Mycobacterium bovis*            | JATA 12-01 (ATCC 19210)        | A8087687         | –          | –     | –      |
| *Mycobacterium chelonae*         | JATA 62-01 (ATCC 35752)        | A8087688         | –          | –     | –      |
| *Mycobacterium fortuitum*        | JATA 61-01 (ATCC 6841)         | A8087689         | –          | –     | –      |
| *Mycobacterium gastri*           | KK 44-02 (ATCC 15754)          | A8087690         | –          | +     | –      |
| *Mycobacterium gordonae*         | JATA 33-01 (ATCC 14470)        | A8087691         | –          | –     | –      |
| *Mycobacterium intracellulare*   | JATA 52-01 (ATCC 13950)        | A8087692         | –          | –     | –      |
| *Mycobacterium kansasii*         | KK 21-01 (ATCC 12478)          | A8087693         | +          | –     | –      |
| *Mycobacterium leprae*           | Thai-53                        | A8087694         | –          | –     | –      |
| *Mycobacterium malmoense*        | JATA 47-01 (ATCC 29571)        | A8087695         | –          | –     | –      |
| *Mycobacterium marinum*          | JATA 22-01 (ATCC 927)          | A8087696         | –          | –     | –      |
| *Mycobacterium microti*          | KK 14-01 (ATCC 19422)          | A8087697         | –          | –     | –      |
| *Mycobacterium nonchromogenicum* | JATA 45-01 (ATCC 19530)        | A8087698         | –          | –     | –      |
| *Mycobacterium parafortuitum*    | ATCC 25807                     | A8087699         | –          | –     | –      |
| *Mycobacterium phlei*            | ATCC 19249                     | A8087700         | –          | –     | –      |
| *Mycobacterium scrofulaceum*     | JATA 31-01 (ATCC 19981)        | A8087701         | –          | –     | –      |
| *Mycobacterium simiae*           | KK 23-08 (ATCC 25275)          | A8087702         | –          | –     | –      |
| *Mycobacterium szegmatis*        | JATA 64-01                     | A8087703         | –          | –     | –      |
| *Mycobacterium szulgai*          | JATA 32-01                     | A8087704         | –          | –     | –      |
| *Mycobacterium terrae*           | KK 46-01 (ATCC 15755)          | A8087705         | –          | –     | –      |
| *Mycobacterium triviale*         | KK 50-02 (ATCC 23292)          | A8087706         | –          | –     | –      |
| *Mycobacterium tuberculosis*     | JATA 11-01 (H37Rv)            | A8087707         | –          | –     | –      |
| *Mycobacterium ulcerans*         | KK 43-01                      | A8087708         | –          | –     | –      |
| *Mycobacterium vaccae*           | KK 66-01                      | A8087709         | –          | –     | –      |
| *Mycobacterium xenopi*           | KK 42-01 (ATCC 19250)          | A8087710         | –          | –     | –      |

All strains were kindly donated by Dr Kashiwabara, NIID.
Clinical isolates were identified by Amplicore Mycobacterium kit (Roche Pharma, Basel, Switzerland) or conventional biochemical test (Jamal et al., 2000).

**Amplification of the region within dnaA gene**

Highly polymorphic regions flanked by conserved regions were identified by aligning the Mycobacterium spp. dnaA sequences, which were available in GenBank at the time this study was initiated. These regions were used to design a pair of degenerate primers, U1F 5’-GTS CAR AAC GAR ATC GAR CG-3’ and U1R 5’-CCB GAY TCR CCC CAG ATG AA-3’. A schematic representation of the primer design is shown in Fig. 1a. PCR was performed in a TAKARA Thermal Cycler MP (TAKARA Biomedical, Otsu, Japan) with a reaction mixture consisting of 1 µL of genomic DNA, each deoxynucleoside triphosphate at a concentration of 200 µM, each primer at a concentration of 0.4 µM, 1 × PCR buffer with 1.5 mM MgCl₂ (TAKARA Biomedical), and 1.25 U of ExTaq (TAKARA Biomedical), with 10 µL PCRX Enhancer System solution (Gibco BRL, Rockville, MD) in a total volume of 50 µL. The PCR thermocycles were 3 min at 94 °C, followed by 30 cycles of 94 °C for 10 s, 50 °C for 20 s, and 72 °C for 45 s, with a final extension step at 72 °C for 7 min. PCR products were visualized by UV illumination of an ethidium bromide and were visualized on a UV transilluminator at 302 nm. The specificity of the LAMP assay was further validated by restriction enzyme digestion with NaeI and HaeIII for M. kansasii and M. gastri, respectively. The diluted genomic DNA was used for determining the sensitivity of the species-specific LAMP assay.

**Species-specific LAMP assay for Mycobacterium kansasii and Mycobacterium gastri**

A set of four primers comprising two inner primers and two outer primers that recognized six distinct regions on the target sequence were designed with PrimerExplorer Ver.3 (Fujita, Tokyo, Japan). The detailed sequences of the primers are shown in Fig. 3. The two inner primers are called the forward inner primer (FIP) and the backward inner primer (BIP), and each contains two distinct sequences corresponding to the sense and antisense sequences of the target DNA, one for priming in the first stage and the other for self-priming in late stages. FIP contains the sequence complementary F1 (F1c) and F2. BIP contains the complementary B1 (B1c) and B2. The two outer primers consist of F3 and B3. The LAMP reaction was carried out in 25 µL of reaction mixture by using the Loopamp DNA amplification kit (Eiken Chemical Co. Ltd., Tochigi, Japan) containing 2.4 µM (each) FIP and BIP, 0.2 µM (each) of the outer primers, F3 and B3, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 8 mM MgSO₄, 10 mM (NH₄)₂SO₄, 0.1% Tween 20, 0.8 M betaine, 1.4 mM (each) of dNTP, 8 U of Bst DNA polymerase (New England BioLabs, Beverly, MA), and the template DNA. Amplification was undertaken in 0.5 µL microtubes in a heatblock under isothermal conditions of 63 °C for 60 min, followed by 80 °C for 2 min to terminate the reaction. Positive and negative controls were included in each run, and precautions to prevent cross-contamination were observed. Two microliter aliquots of LAMP products were subjected to electrophoresis on a 4% agarose gel in Tris-borate-EDTA buffer followed by staining with ethidium bromide and were visualized on a UV transilluminator at 302 nm. The specificity of the LAMP-amplified products was further validated by restriction enzyme digestion with NaeI and HaeIII for M. kansasii and M. gastri, respectively. The diluted genomic DNA was used for determining the sensitivity of the species-specific LAMP assay.
Results

Comparison of partial dnaA sequence to identify the Mycobacterium species

For the species identification of mycobacterial species, we analyzed some possible variable regions of mycobacterial sequences deposited in the GenBank, and found the 5′ part of the dnaA gene as a candidate target for PCR amplification. The PCR products with U1F and U1R, from 27 mycobacterial species, showed the ragged pattern around 400 bp in size (data not shown). Therefore, we determined nucleotide sequences, corresponding to position 228–612 bp of Mycobacterium tuberculosis, of all 27 species (Fig. 1a). The alignment of the sequence shows that the region (298–508 bp) in the amplified products had the highest species-specific variability (Fig. 1b). The size of the variable fragment in dnaA ranged from 154 bp in M. triviale to 232 bp in M. kansasii. The variable region exhibits a reasonable number of nucleotide substitution and insertion or deletion sites, which is important for the development of a differential diagnostic tool. The lowest interspecies similarity was 28.2% in M. leprae versus M. vaccae. The similarity between M. avium and M. intracellulare was 78.3% and that between M. marinum and M. ulcerans was 97.7%. Pathogenic M. kansasii were easily differentiated from nonpathogenic M. gastri (83.6%). The sequences of M. tuberculosis, M. microti, M. africanum, and M. bovis were found to be identical, except for one nucleotide substitution that occurred in M. bovis. When clinical isolates from clinically relevant mycobacterial strains were analyzed, the following minor variation was found among each species: 97.7–100% (M. avium) and 96.0–100% (M. intracellulare). We did not find any intraspecies variation in 10 clinical isolates and the standard strain of M. kansasii. Because other reports using different systems revealed the existence of more than one sequence (Yang et al., 1993; Alcaide et al., 1997), we may need to examine a bigger number of clinical isolates.

The unrooted phylogenetic tree showed that the 27 mycobacterial species were resolved by the variable region in the dnaA sequence (Fig. 2). All rapidly growing species, M. abscessus, M. chelonae, M. fortuitum, M. parafortuitum, M. phlei, M. vaccae, and M. smegmatis, made a cluster that was clearly separated from those of the other species so far examined. On the other hand, M. kansasii, M. gastri, M. avium, and M. intracellulare are clinically relevant species; however, the branch of the former two species was obviously segregated from one of the later two species, which was supported by high bootstrap values. The results indicated that the partial dnaA sequence could be useful for the differentiation of NTM (Fig. 2).

Identification of mycobacteria by dnaA sequence-targeted species-specific LAMP assay

Several sets of primers designed from the dnaA sequence were evaluated for their specificity and sensitivity by the LAMP method. One set of primers named Kan-32 for M. kansasii and Gas-583 for M. gastri was selected (Fig. 3), and...
Thus, we concluded that each primer set was species specific. A right arrow indicates the sense sequence which is used as the primer. A left arrow indicates that a complementary sequence is used as the primer.

The sizes of the fragments generated after digestion, and 123 and 98 bp by HaeI digestion, were in good agreement with sizes predicted theoretically from the expected DNA structure: 100 and 93 bp by HaeII digestion, and 123 and 98 bp by HaeII digestion (Fig. 4a). Thus, we concluded that each primer set was species specific.

We next assessed the sensitivity of the assay. Serially diluted M. kansasii or M. gastri genomic DNA was used. The results of a typical experiment are shown in Fig. 4b. Amplified DNA was readily visible when 500 copies of genomic DNA were present in a 60 min incubation assay. The detection limit did not change with a longer incubation period (data not shown).

### Discussion and conclusions

For the identification of species, a target gene must be conserved among strains and species. As the DnaA protein is generally conserved among microbial organisms (Mizrahi et al., 2000), this coding region could be used for the target analysis. Four functional domains of the DnaA protein have been defined (Messer et al., 1998). Domain 1 is involved in oligomerization and interaction with DnaB, Domain 2 constitutes a flexible loop, Domain 3 has ATPase function, and Domain 4 is sufficient for specific binding to DNA. The variable region that we identified in the dnaA sequence was equivalent to the Domain 2 coding nucleotide sequence by using these primer sets, a successful LAMP product appeared as a ladder of multiple bands (Fig. 3a).

The species specificity and intraspecies stability of each primer set were examined with purified DNA from 27 mycobacterial species and 10 clinical isolates of M. kansasii. The amplified products had corresponding DNA structures, after a 60 min incubation at 63°C. To confirm that the amplification products were digested with restriction enzymes and the size of the fragments was analyzed by electrophoresis. The detection limit did not change with a longer incubation period (data not shown).

### Table 1

| Primer Set | Species Targeted |
|------------|------------------|
| Kan 32     | M. kansasii      |
| Gas 583    | M. gastri        |

### Location of Oligonucleotide Primer Sets

A partial sequence of the dnaA gene (GenBank accession number AB087693) and for Mycobacterium gastri partial dnaA gene (GenBank accession number AB087690). A right arrow indicates the sense sequence which is used as the primer. A left arrow indicates that a complementary sequence is used as the primer. The unique restriction enzyme recognition sites in the amplified product are shown with a bold bar.

### Fig. 3

Location of oligonucleotide primer sets Kan 32 and Gas 583, used for the loop-mediated isothermal amplification method. For Mycobacterium kansasii partial dnaA gene (GenBank accession number AB087693) and for Mycobacterium gastri partial dnaA gene (GenBank accession number AB087690). A right arrow indicates the sense sequence which is used as the primer. A left arrow indicates that a complementary sequence is used as the primer. The unique restriction enzyme recognition sites in the amplified product are shown with a bold bar.

### Fig. 4

We subjected each sample to amplification using Kan-32 or Gas 583 primer sets, a successful LAMP product appeared as a ladder of multiple bands (Fig. 3a).

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The percent similarity between these mycobacteria provides useful information for discrimination of these species, at least in complex media. These observations may indicate the usefulness of the dnaA gene with respect to sequence and length among M. smegmatis, M. tuberculosis, and M. leprae (Fshi et al., 1996). However, comparative studies of this region using 27 mycobacteria have not been reported and, as far as we know, this is the first report indicating the usefulness of the dnaA Domain 2 sequence as a differential diagnostic tool.

An accurate and rapid bacterial identification greatly contributes to this field of medication. Several methods based on molecular biological techniques have been reported. The sequences that have been reported include hsp65, 16S rRNA gene, and ITS (Plikaytis et al., 1992; De Smet et al., 1995; Springer et al., 1996; Messer & Weigel, 1997; Roth et al., 1998; Brunello et al., 2001). Each gene has several advantages and disadvantages. An excessive degree of variability is found in the hsp65 gene (Telenti et al., 1993), which may hinder the development of reliable probes. While 16S rRNA gene sequence is identical in M. kansasii and M. gastri and shows narrow divergencicity within species (Taylor et al., 1997), ITS sequence can be used to distinguish between M. kansasii and M. gastri (Roth et al., 1998). While M. kansasii is a representative pathogenic mycobacteria, M. gastri does not induce an apparent disease. The discrimination between these mycobacteria provides useful information to select the appropriate therapy. The percent similarity of ITS between two species was 93% (Roth et al., 1998), and that of the dnaA variable region was found to be 83.6%. These observations may indicate the usefulness of the dnaA gene for discrimination of these species, at least in complement with ITS.

The recent trend in genetic testing is to make systems fully automatic with high-throughput analysis. Although this may be an ideal approach, it requires expensive equipment as well as a well-trained person in diagnostic laboratories. The LAMP method could be conducted under isothermal conditions ranging from 60 to 65 °C by a single enzyme. The only equipment needed for LAMP reaction is a regular laboratory water bath or a heat block that furnishes a constant temperature around 63 °C. LAMP does not require a thermal cycling step, and an isothermal reaction for a short time (60 min) is enough to amplify the target DNA to a detectable level. As PCR and other molecular biological techniques are conducted in well-equipped laboratories, these methodologies are often impracticable under a field diagnosis.

In this paper, we demonstrated that the dnaA region could be an effective new nucleotide region for the diagnosis of NTM infection and that the LAMP method could be applied for a dnaA gene-based differential diagnostic tool.

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**References**

Alcaide F, Richter I, Bernasconi C, Springer B, Hagenau C, Schulze-Robbecke R, Tortoli E, Martin R, Bottger EC & Telenti A (1997) Heterogeneity and clonality among isolates of Mycobacterium kansasii: implications for epidemiological and pathogenicity studies. J Clin Microbiol 35: 1959–1964.
American Thoracic Society (1997) Diagnosis and treatment of diseases caused by nontuberculous mycobacteria. *Am J Respir Crit Care Med* **156**: S1–S25.

De Beenhouwer H, Liang Z, De Rijk 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.

Blackwood KS, He C, Gunton J, Turenne CY, Wolfe J & Kabani AM (2000) Evaluation of recA sequences for identification of *Mycobacterium* species. *J Clin Microbiol* **38**: 2846–2852.

Brunello F, Ligozzi M, Cristelli E, Bnora S, Tortoli E & Fontana R (2001) Identification of 54 mycobacterial species by PCR-restriction fragment length polymorphism analysis of the *hsp65* gene. *J Clin Microbiol* **39**: 2799–2806.

Cernoch PL, Enns RK, Saubolle MA & Wallace RA Jr (1994) Laboratory diagnosis of the Mycobacterioses. Comitech 16A (Weissfield AS, Coordinating ed.), American Society for Microbiology, Washington, DC.

Cloud JL, Neal H, Rosenberry R, Turenne CY, Juma M, Hillyard DR & Carroll KC (2002) Identification of *Mycobacterium* spp. by using a commercial 16S ribosomal DNA sequencing kit and additional sequencing libraries. *J Clin Microbiol* **40**: 400–406.

De Smet KA, Brown IN, Yates M & Ivanyi J (1995) Ribosomal internal transcribed spacer sequences are identical among *Mycobacterium avium–intracellulare* complex isolates from AIDS patients, but vary among isolates from elderly pulmonary disease patients. *Microbiology* **141**: 2739–2747.

Enosawa M, Kageyama T, Sawai K, Watanabe K, Notomi T, Onoe S, Mori Y & Yokomizo Y (2003) Use of loop-mediated isothermal amplification of the IS900 sequence for rapid detection of cultured *Mycobacterium avium* subsp. *paratuberculosis*. *J Clin Microbiol* **41**: 4359–4365.

Fsihi H, De Rossi E, Salazar L, Cantoni R, Labo M, Riccardi G, Takiff HE, Eiglmeier K, Bergh S & Cole ST (1996) Gene arrangement and organization in approximately 76 kb fragment encompassing the oriC region of the chromosome of *Mycobacterium leprae*. *Microbiology* **142**: 3147–3161.

Horsburg CR Jr (1991) *Mycobacterium avium* complex infection in the acquired immunodeficiency syndrome. *N Engl J Med* **324**: 1332–1338.

Ihira M, Yoshikawa T, Enomoto Y, et al. (2004) Rapid diagnosis of human herpesvirus 6 infection by a novel DNA amplification method, loop-mediated isothermal amplification. *J Clin Microbiol* **42**: 140–145.

Iwamoto T, Sonobe T & Hayashi K (2003) Loop-mediated isothermal amplification for direct detection of *Mycobacterium tuberculosis* complex, *M. avium*, and *M. intracellulare* in sputum samples. *J Clin Microbiol* **41**: 2616–2622.

Jamal MA, Maeda S, Nakata N, Kai M, Fukuuchi K & Kashiwabara Y (2000) Molecular basis of clarithromycin-resistance in *Mycobacterium avium intracellulare* complex. *Tuberc Lung Dis* **80**: 1–4.

Kasai H, Ezaki T & Harayama S (2000) Differentiation of phylogenetically related slowly growing mycobacteria by their gyrB sequences. *J Clin Microbiol* **38**: 301–308.

Kim BJ, Lee SH, Lyu MA, Kim SJ, Bai GH, Chae GT, Kim EC, Cha CY & Kook YH (1999) Identification of mycobacterial species by comparative sequence analysis of the RNA polymerase gene (*rpoB*). *J Clin Microbiol* **37**: 1714–1720.

Kirschner P, Springer B, Vogel U, Meier A, Wrede A, Kiekenbeck M, Range FC & Bottger EC (1993) Genotypic identification of mycobacteria by nucleic acid sequence determination: report of a 2-year experience in a clinical laboratory. *J Clin Microbiol* **31**: 2882–2889.

Kuboki N, Inoue N, Sakurai T, Di Cello F, Grab DJ, Suzuki H, Sugimoto C & Igarashi I (2003) Loop-mediated isothermal amplification for detection of African trypanosomes. *J Clin Microbiol* **41**: 5517–5524.

Messer W, Blaesing F, Majka J, et al. (1998) Functional domains of *Dna A* proteins. *Biochimie* **81**: 819–825.

Messer W & Weigel C (1997) *Dna A* initiator – also a transcription factor. *Mol Microbiol* **24**: 1–6.

Metcchock BG, Nolte FS & Wallace RJ Jr (1999) *Mycobacterium*. Manual of Clinical Microbiology, 7th edn (Murray PR, Baron EL, Pfaffer MA, Tenover FC & Yolken RH, eds), pp. 399–437. American Society for Microbiology, Washington, DC.

Mizrahi VS, Dawes S & Rubin H (2000) DNA replication. *Molecular Genetics of Mycobacteria* (Hatful GF & Jacobs WR Jr, eds), pp. 159–172. American Society for Microbiology, Washington, DC.

Montessori V, Phillips P, Montaner J, Haley L, Craib K, Bussuille E & Black W (1996) Species distribution in human immunodeficiency virus-related mycobacterial infections: implications for selection of initial treatment. *Clin Infect Dis* **22**: 989–992.

Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N & Hase T (2000) Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res* **28**: E63.

Parida M, Posadas G, Inoue S, Hasebe F & Morita K (2004) Real-time reverse transcription loop-mediated isothermal amplification for rapid detection of West Nile virus. *J Clin Microbiol* **42**: 257–263.

Park H, Jang H, Kim C, Chung B, Chang CL, Park SK & Song S (2000) Detection and identification of mycobacteria by amplification of the internal transcribed spacer regions with genus- and species-specific PCR primers. *J Clin Microbiol* **38**: 4080–4085.

Plikaýtis BB, Plikaýtis BD, Yakrus MA, Butler WR, Woodley CL, Silcox VA & Shinnick TM (1992) Differentiation of slowly growing *Mycobacterium* species, including *Mycobacterium tuberculosis*, by gene amplification and restriction fragment length polymorphism analysis. *J Clin Microbiol* **30**: 1815–1822.

Primm TP, Lucero CA & Falkinham JO III (2004) Health impacts of environmental mycobacteria. *Clin Microbiol Rev* **17**: 98–106.

Roth A, Fischer M, Hamid ME, Michalke S, Ludwig W & Mauch H (1998) Differentiation of phylogenetically related slowly growing mycobacteria based on 16S–23S rRNA gene internal transcribed spacer sequences. *J Clin Microbiol* **36**: 139–147.
Shepard CC (1960) The experimental diseases that follows the injection of human leprosy bacilli into foot-pads of mice. J Exp Med 112: 445–454.

Springer B, Stockman L, Teschner K, Roberts GD & Bottger EC (1996) Two-laboratory collaborative study on identification of mycobacteria: molecular versus phenotypic methods. J Clin Microbiol 34: 296–303.

Taylor TB, Patterson C, Hale Y & Safranek WW (1997) Routine use of PCR-restriction fragment length polymorphism analysis for identification of mycobacteria growing in liquid media. J Clin Microbiol 35: 79–85.

Telenti A, Marchesi F, Balz M, Bally F, Bottger EC & Bodmer T (1993) Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. J Clin Microbiol 31: 175–178.

Thai HTC, Le MQ, Vuong CD, Parida M, Minekawa H, Notomi T, Hasebe F & Morita K (2004) Development and evaluation of a novel loop-mediated isothermal amplification method for rapid detection of severe acute respiratory syndrome coronavirus. J Clin Microbiol 42: 1956–1961.

Thompson JD, Higgins DG & Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22: 4673–4680.

Turenne CY, 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.

Wayne LG & Sramek HA (1992) Agents of newly recognized or infrequently encountered mycobacterial diseases. Clin Microbiol Rev 5: 1–25.

Yang M, Ross BC & Dwyer B (1993) Isolation of a DNA probe for identification of Mycobacterium kansasii, including the genetic subgroup. J Clin Microbiol 31: 2769–2772.