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## Notes

## Report of 2 indigenous cases of leprosy from a European country: use of polymerase chain reaction–restriction fragment length polymorphism analysis of *hsp65* gene for identification of *Mycobacterium leprae* directly from a clinical sample

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Received 20 November 2008; accepted 1 March 2009

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**Abstract**

In this article, we report on 2 indigenous cases of leprosy detected in a European country. We also report on the use of polymerase chain reaction–restriction fragment length polymorphism analysis of *hsp65* gene for rapid identification of *Mycobacterium leprae* directly from the clinical sample.

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**Keywords:** Indigenous leprosy; *Mycobacterium leprae*

There are many countries in Asia, Africa, and Latin America with a significant number of leprosy cases (No author listed, 2007). However, this disease is rarely diagnosed in developed countries. In Europe, only sporadic cases have been reported over the last decades, mostly among immigrants or natives that had traveled to an endemic area (Flageul, 2001; Gill et al., 2005; Koch et al., 2006; Stafford and Wilson, 2006). The index of suspicion for leprosy among physicians in community and hospital medicine in developed countries is very low.

Because *Mycobacterium leprae* cannot be cultured in vitro (Vissa and Brennan, 2001), diagnosis is achieved by histopathology and acid-fast staining (AFS). Application of

molecular techniques such as restriction fragment length polymorphism (RFLP) in combination with polymerase chain reaction (PCR) (PCR-RFLP) could be a useful method for identifying and confirming the existence of *M. leprae* bacilli in clinical specimens.

Herein, we report on 2 indigenous cases of leprosy detected on the island of Crete, Greece, and the use of sequencing and PCR-RFLP of the *hsp65* gene for diagnosis.

### 1. Index case

Three years ago (2005), a 39-year-old woman presented in our hospital with a variety of dermal lesions including erythematous macules, papules, nodules, and plaques to infiltration. Lesions tended to become confluent especially on the extremities. The patient had lost her eyebrows and eyelashes and exhibited the so-called lion face. Five years before admission, she underwent surgery for ulcers on her left talus, which were misdiagnosed as atonic ulcers. Upon

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admission to our hospital, samples from nasal mucosa and slit-skin smears from open sites were submitted for AFS. They were positive, confirming the diagnosis of Hansen's disease. The bacterial index (BI) of all samples was 4+ (>9 mycobacteria/1 optical frame, m./o.f.). The numerous acid-fast bacilli in clumps were indicative of lepromatous leprosy. The patient received a multidrug therapy including rifampin (600 mg), ofloxacin (400 mg), and minocycline (100 mg) (ROM). The patient remained in the hospital for 1 month, where the therapeutic scheme was administered daily. Liver and renal function was also monitored on a daily basis. After the initial month, therapy was continued with 23 monthly doses of ROM. Two months after the onset of therapy, the infiltrates resolved, the plaques flattened, and the AFS became negative. Nasal swabs obtained from the patient's family (husband and 2 children) were AFS negative. A thorough questionnaire revealed that 25 years before, a cousin, who was later diagnosed as suffering from leprosy, had lived for months with the patient's family.

## 2. Second case

The 51-year-old husband of the index case was admitted to our hospital in 2008 for skin lesions including papules, plaques with ulcers, and infiltrations, extending especially on the 3rd left metatarsal finger and plantar area. His eyebrows had fallen out 1 year before admission. The patient complained of pain and restriction of movement in his ankles, which had lasted for 2 years. Despite his wife's clinical history and the similarities of the clinical manifestations exhibited, the patient refused to associate the symptoms with leprosy, attributing them to arthritis. Consequently, he did not seek advice. Moreover, he did not comply with the regular checkup for leprosy that had been proposed to him after his wife's diagnosis.

Only 2 months before admission, the patient visited a private dermatologist for the atonic ulcers on his legs. Even then, the lesions were misdiagnosed as mycoses. This was because of the fact that the patient did not mention his wife's medical history, nor was the dermatologist aware of leprosy's clinical manifestations. The therapy given was for yeast and worsened the lesions. When the patient finally visited our hospital, nasal swabs and swabs from the skin lesions were taken for AFS, and all were positive with a BI of 3+ (1–9 m./1 o.f.). The patient received the same therapeutic scheme as the index case and soon improved clinically (resolved infiltrates and flattened plaques, no pain and swelling of the legs). Furthermore, after 3 weeks of treatment, the AFS of the nasal and skin lesion smears revealed a decrease of the BI to 1+ (1–9 m./100 o.f.).

Over the course of the 2nd case, we applied 2 molecular methods for quick identification, namely, partial sequencing and PCR-RFLP of *hsp65*. The PCR-RFLP of *hsp65* had been proposed by Rastogi et al. (1999), and to our knowledge, it has been used only once for the identification of *M. leprae* (Martiniuk et al., 2007).

A modified protocol for direct identification of *M. leprae* from the human clinical sample was used. A sample, taken with a swab from open lesions with exudate, was suspended in distilled sterile water. The mycobacterial cells were heat inactivated at 80 °C for 1 h, and the DNA of *M. leprae* was extracted using the guanidinium thiocyanate lysis buffer (Casas et al., 1995). In brief, the suspension was centrifuged (16 600 × g, 10 min, 4 °C). The pellet was dissolved in 600 µL of lysis buffer (4 mol/L guanidinium thiocyanate, 0.5% *N*-lauryl sarcosine, 1 mmol/L dithiothreitol, 25 mmol/L sodium citrate, and 50 µg of glycogen) and incubated overnight at room temperature. A total of 600 µL of ice-cold (–20 °C) isopropyl alcohol was then added. After 1.5 h, the sample was centrifuged for 10 min (16 600 × g, 4 °C). Isopropyl alcohol was removed, and the pellet was washed with 70% ethanol. The dried pellet was dissolved in 50 µL of DNase and RNase-free double-distilled sterile water. No quantitation of the yield of DNA was performed.

Five microliters of the extracted DNA was used as a target for the PCR amplification of a 439-bp fragment of *hsp65* gene using the protocol and primers Tb11 (5'-ACCAAC-GATGGTGTGTCCAT) and Tb12 (5'-CTTGTCGAACCG-

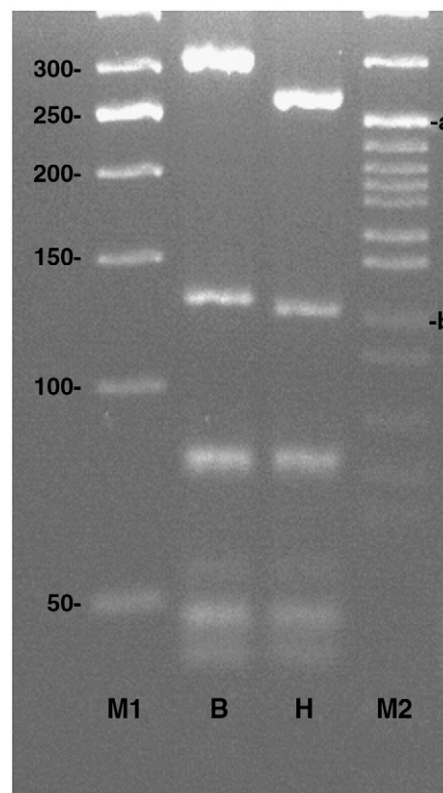


Fig. 1. Agarose gel electrophoresis of fragments produced by the digestion of PCR amplification products of the *hsp65* gene with *BstEII* (lane B) and *HaeIII* (lane H). The set of bands 315/135 in lane B and 265/130 in lane H were the products of digestion. The lower fragments shown in the figure were probably PCR byproducts. Lane M1, GeneRuler 50-bp DNA ladder (Fermentas, Vilnius, Lithuania). Lane M2, Ladder pBR322 DNA-*MspI* Digest (New England Biolabs). The size of the bands is indicated in base pair. Size of bands: a, 242 bp; and b, 123 bp.

CATACCCT) as previously described (Telenti et al., 1993). The PCR product was further analyzed by sequencing and RFLP. Sequencing was performed with an automated DNA sequencer (3730 DNA analyzer, Applied Biosystems, Carlsbad, CA, USA) using the Big Dye Terminator Sequencing Kit (Applied Biosystems), and sequences were compared with the GenBank sequences. The sequence was deposited in GenBank with the accession number FJ497239. It was identical to the previously published sequence of the *M. leprae* 65-kDa antigen (ML0317), confirming the identification of *M. leprae* (Mehra et al., 1986).

RFLP analysis was performed using the *HaeIII* (New England Biolabs, Ipswich, MA, USA) and *BstEII* (New England Biolabs, Ipswich, MA, USA) restriction enzymes as previously described (Telenti et al., 1993). The *BstEII* digestion produced 2 fragments of 315 and 135 bp, and the *HaeIII* digestion produced 2 fragments of 265 and 130 bp (Fig. 1). This profile matched the one previously reported for *M. leprae* (Rastogi et al., 1999).

The 2 cases of leprosy reported in this article demonstrate that a) leprosy is a forgotten but still existent disease in Europe, even among indigenous populations, and b) the index of clinical suspicion for leprosy needs to be raised and physicians should include this disease entity in the differential diagnosis of chronic dermal lesions.

Early diagnosis and proper therapy are of paramount relevance so disabilities and further transmission of the disease can be prevented. The PCR-RFLP method applied in this study performed well, proving that *hsp65* is a useful target for *M. leprae* identification. PCR-RFLP could be a

useful molecular tool as an adjunct to careful clinical and pathologic assessment of patients suspected of suffering from Hansen's disease.

## References

- Casas I, Klapper PE, Cleator GM, Echevarria JE, Tenorio A, Echevarria JM (1995) Two different PCR assays to detect enteroviral RNA in CSF samples from patients with acute aseptic meningitis. *J Med Virol* 47: 378–385.
- Flageul B (2001) Epidemiology study of Hansen's disease in metropolitan France between 1995 and 1998. *Ann Dermatol Venereol* 128:17–20.
- Gill AL, Bell DR, Gill GV, Wyatt GB, Beeching NJ (2005) Leprosy in Britain: 50 years experience in Liverpool. *Q J Med* 98:505–511.
- Koch A, Köstler E, Zieger B, Schönlebe J, Haroske G, Wollina U (2006) Leprosy: two case reports from Dresden, Germany. *Int J Dermatol* 45:1321–1325.
- Martiniuk F, Tambini M, Rahimian J, Moreira A, Yee H, Tchou-Wong KM, Hanna BA, Rom WN, Levis WR (2007) Identification of novel *hsp65* RFLPs for *Mycobacterium leprae*. *J Drugs Dermatol* 6:268–274.
- Mehra V, Sweetser D, Young RA (1986) Efficient mapping of protein antigenic determinants. *Proc Natl Acad Sci U S A* 83:7013–7017.
- No author listed (2007) Global leprosy situation. *Wkly Epidemiol Rec* 82:225–232.
- Rastogi N, Goh KS, Berchel M (1999) Species-specific identification of *Mycobacterium leprae* by PCR-restriction fragment length polymorphism analysis of the *hsp65* gene. *J Clin Microbiol* 37:2016–2019.
- Stafford SJ, Wilson RR (2006) An isolated case of leprosy presenting in a migrant worker in Northern Ireland. *Emerg Med J* 23:699–700.
- 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.
- Vissa VD, Brennan PJ (2001) The genome of *Mycobacterium leprae*: a minimal mycobacterial gene set. *Genome Biol* 2:1023.1–1023.8.