DIAGNOSIS AND LABORATORY TESTS

Leprosy exerts systemic effects in addition to skin lesions, which is evident in the infiltration of bacilli into the nasal mucosa, bones and other organs of multibacillary patients.[53] Severe skeletal lesions, the hallmark of lepromatous leprosy, have been observed in excavated skeletal remains,[54–58] and *M. leprae* DNA has been isolated from such lesions (Fig. 3).[59] Eye damage is frequently seen in multibacillary patients resulting from both nerve damage and direct bacillary invasion.[60] Typically, lagophthalmos is caused by involvement of the zygomatic and temporal branches of the facial nerve. Other facial nerve damage, such as involvement of the ophthalmic branch of the trigeminal nerve, causes anesthesia of the cornea and conjunctiva, resulting in dryness and the risk of ulceration.

A diagnosis of leprosy is made based on cardinal signs such as hypopigmented or reddish patches with definite loss of sensation, thickened peripheral nerves and acid-fast bacilli in slit-skin smears or biopsy materials.[61,62] Smear and biopsy samples are subjected to acid-fast staining in addition to conventional histopathological diagnosis in order to demonstrate the presence of mycobacterium; however, bacilli are not usually detected in paucibacillary cases. The presence of neural inflammation is a histological characteristic of leprosy that can differentiate it from other granulomatous disorders. The polymerase chain reaction (PCR) is a sensitive method for the detection of *M. leprae* DNA that is widely used for differential diagnosis in advanced countries, although it cannot determine if viable organisms are present because DNA can persist long after microorganisms are dead.[15,30,59,63] Serum antibodies against *M. leprae* phenolic glycolipid-I (PGL-I) are found in multibacillary patients and some household contacts, although its specificity is relatively low.[30,64–66] Non-endemic countries do not usually consider leprosy during the differential diagnosis of skin lesions; however, it should be considered in a case of peripheral neuropathy or persistent skin lesions if patients are from endemic countries. Late diagnosis leads to continued transmission and increased risk of disability.[67,68]

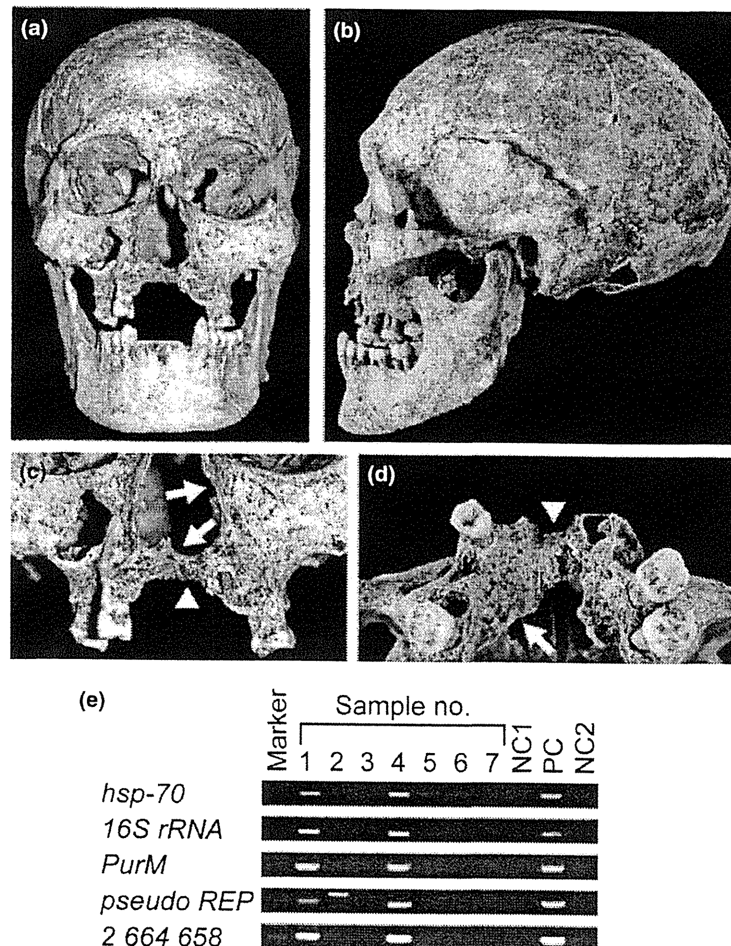

Figure 3. Skeletal lesions of leprosy and isolation of lesion-associated *Mycobacterium leprae* DNA.[59] Frontal view (a) and left side view (b) of archaeological skeletal remains showing erosive deformity of the nasal aperture and disappearance of the anterior nasal spine (arrows) and severe atrophy of the alveolar bone in the maxilla/palatal process with loss of anterior teeth (arrowheads) in panels (c) and (d). Polymerase chain reaction detection of *M. leprae* DNA from skeletal samples (samples 1–4). Samples 5–7 were taken from other skeletons found in the same cemetery, which had no leprous changes as a negative control. *M. leprae* DNA was detected in sample 1 (maxillary palate) and 4 (fibula) (e).

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TREATMENTS

The implementation of MDT for leprosy treatment has been successful over the past three decades. The WHO has designed two easy-to-use blister pack medication kits for paucibacillary and multibacillary patients. The kits contain enough medication for 28 days and are supplied at no cost to registered patients. The treatment for paucibacillary patients include daily doses of 100 mg DDS and a monthly dose of 600 mg rifampicin (RFP) over a 6-month period. Multibacillary patients are administrated 100 mg DDS and 50 mg clofazimine (CLF) once a day in addition to monthly administration of 600 mg RFP and 300 mg CLF for 12 months. Treatment is usually automatically terminated at the end of the proscribed regimen because, in public health terms, it is reasonable to conclude that infectiousness is unlikely after starting MDT (Fig. 4).[69] Many countries, however, prefer longer treatments, especially for

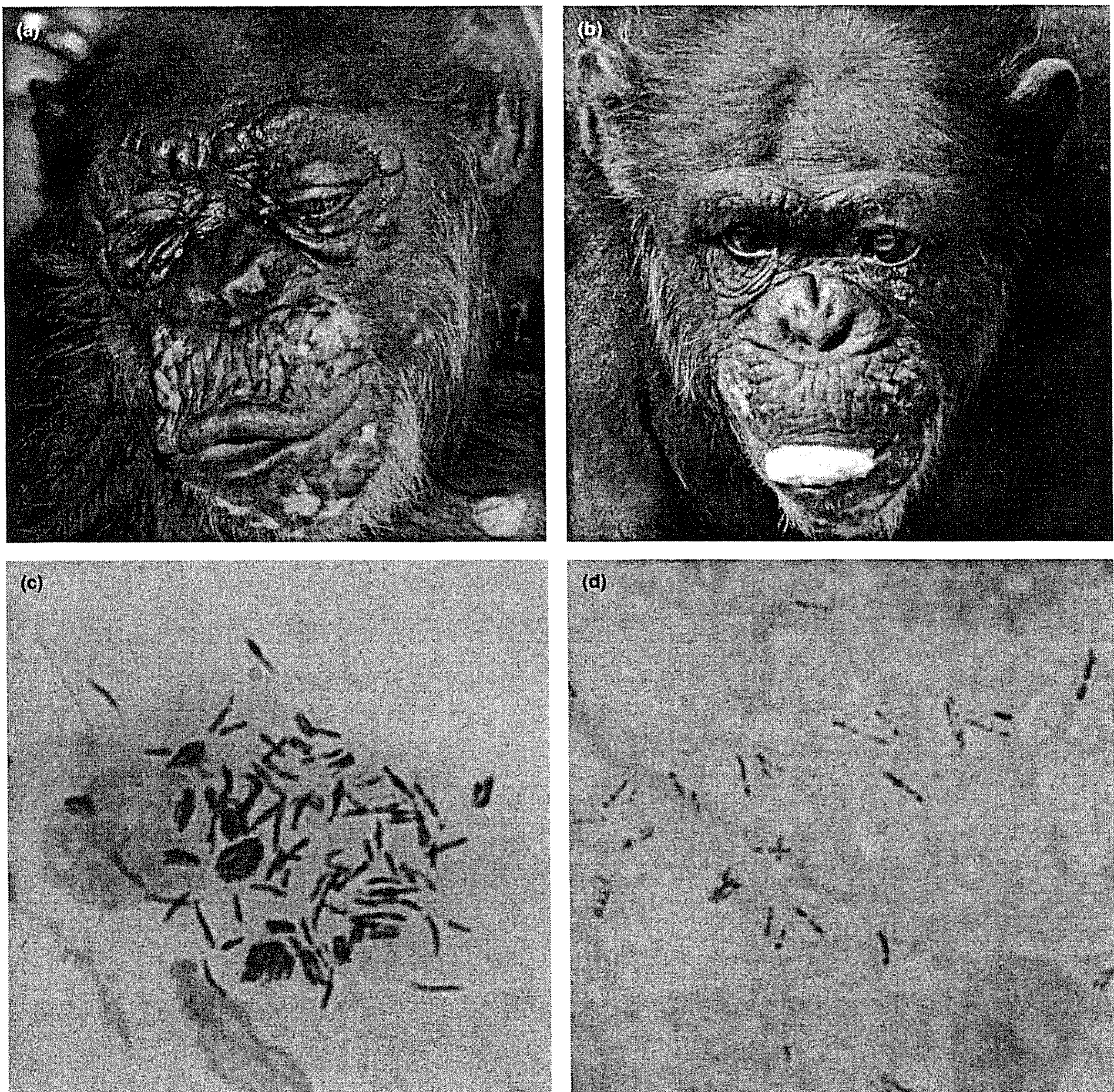

Figure 4. Female chimpanzee at leprosy diagnosis (a) and 3 months after the initiation of multidrug therapy (MDT), showing significant improvement of facial lesions (b).[30] Intact *Mycobacterium leprae* bacilli before treatment (c) fragmented and showed a granular staining pattern 6 months after MDT (d).

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multibacillary cases. Although there has been little standard monitoring of clinical outcomes and relapse rates, accurate diagnosis of relapse requires clinical, bacteriological and histopathological evidence.[70]

Rifampicin is an effective bactericidal agent against *M. leprae*. Within a few days of administrating a single 600-mg dose to multibacillary patients, the bacilli are no longer viable when inoculated into mouse footpads.[71] DDS is bacteriostatic or weakly bactericidal against *M. leprae* and was the mainstay leprosy treatment for many years until widespread resistant strains appeared. CLF binds preferentially to mycobacterial DNA and exerts a slow bactericidal effect on *M. leprae* by inhibiting mycobacterial growth. Skin discoloration ranging from red to black, is one of the most troublesome side-effects of CLF, although the pigmentation fades slowly in most cases after withdrawal. A characteristic ichthyosis is also some times evident. Other effective chemotherapeutic agents against *M. leprae* include ofloxacin (OFLX), minocycline (MINO), levofloxacin (LVFX), sparfloxacin (SPFX), moxifloxacin (MFLX) and clarithromycin (CAM).[72]

As with most chemotherapies, drug-resistant strains are becoming a problem in leprosy, which is a potential threat to the success of current leprosy control efforts. Dapsone resistance is associated with missense mutations in the *folP1* gene encoding dihydropteroate synthase.[73,74] Resistance to RFP is induced by a mutation in *rpoB*, which encodes DNA-dependent RNA polymerase subunit-b.[75] PCR analysis can provide a simple assessment for possible susceptibility to these drugs.[73,74]

LEPRA REACTIONS

Lepra reactions (or reactional states) are acute inflammatory complications that occur in treated or untreated leprosy and often present as medical emergencies. There are two major clinical types of lepra reactions that affect 30–50% of all leprosy patients.[76–78] Severe inflammation associated with these reactions results in nerve injury accompanied by subsequent loss of sensation, paralysis and deformity. The different types of reactions appear to have different underlying immunological mechanisms; however, the factors that initiate them are unknown.

Reversal reactions (type 1 reactions) manifest as erythema and edema of dermal lesions and tender peripheral nerves with rapid loss of nerve function. It generally occurs during the first several months of treatment, and occasionally after MDT is completed.[79,80] Treatment is aimed at controlling acute inflammation, easing pain,

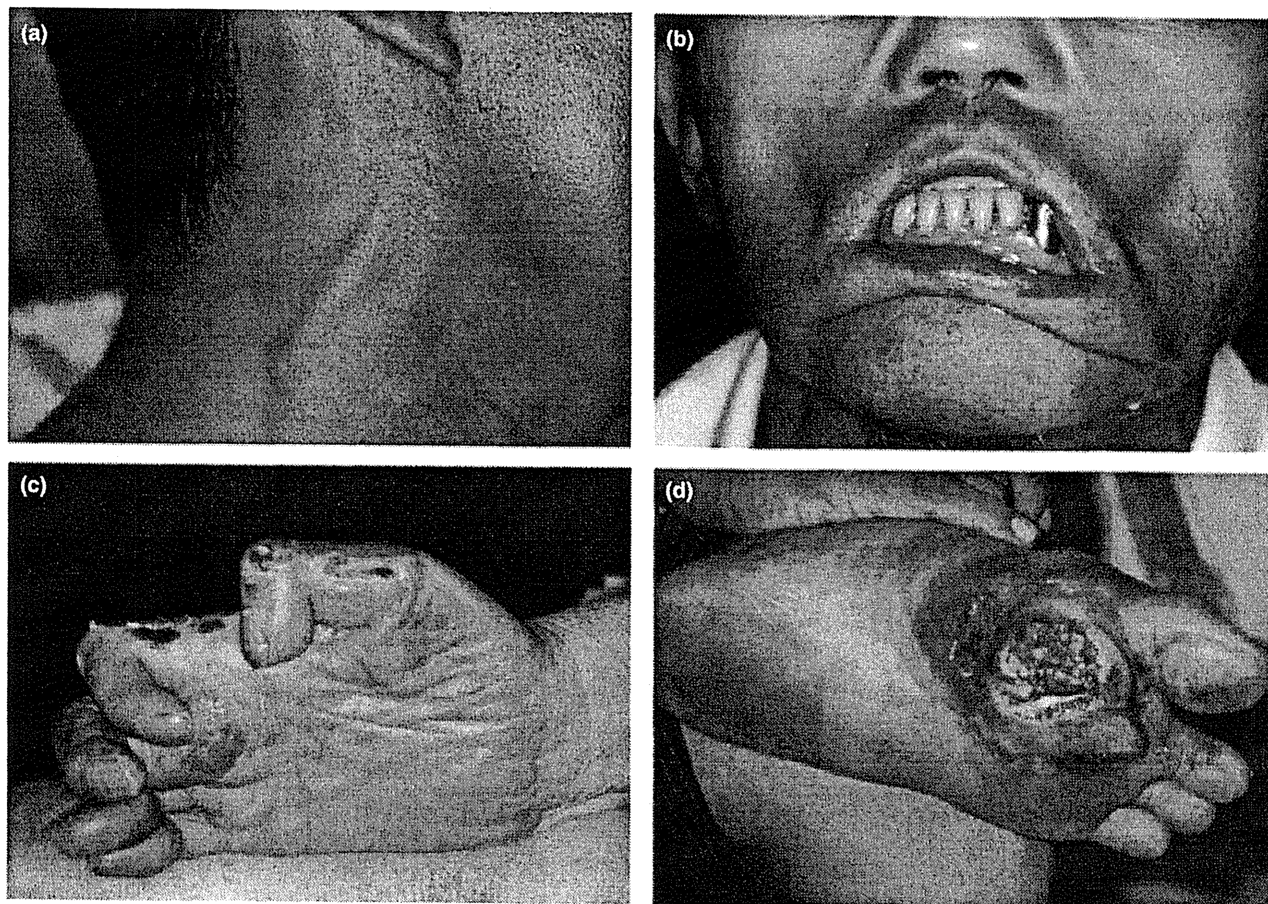

Figure 5. Leprosy with peripheral nerve damage. Swelling of the great auricular nerve (a), facial nerve paralysis (b), dropped wrist, clawed fingers with stiff joints due to ulnar and median nerve damage (c), and foot ulceration due to loss of sensation (d).

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reversing nerve and eye damage, and reassuring the patient. Standard courses of corticosteroids have been used to treat patients for several weeks to months. Erythema nodosum leprosum (ENL or type 2 reactions) occurs in lepromatous and borderline lepromatous patients with higher bacterial loads in their lesions.[81] ENL can begin during the first or second year of treatment. Patients are febrile with skin nodules accompanied by iritis, neuritis, lymphadenitis, orchiitis, bone pain, dactylitis, arthritis, and proteinuria that is difficult to treat.[82] CLF has an anti-inflammatory effect on ENL, and thalidomide is better than steroids in controlling ENL, although thalidomide is not available in many countries because of its teratogenic effects.[83] The use of monoclonal antibodies or inhibitors of TNF-α, as used in rheumatoid arthritis, Crohn's disease and psoriasis, seems to be a logical choice for treatment, but more evidence is needed.[84]

DISABILITY AND STIGMA

Leprosy is a leading cause of permanent physical disability among communicable diseases. The disease and its associated deformities have been responsible for social stigmatization and discrimination against patients and their families in many societies. If unchecked, the disease gradually spreads over the entire body, attacks the soft tissue of the nose and throat, impairs vision and damages the nervous system. The morbidity and disability associated with leprosy are secondary to nerve damage (Fig. 5). Ultimately, the extremities become deformed and paralyzed, and may fall off after repeated but unperceived injuries. Therefore, timely diagnosis and treatment of the patient, before nerve damage has occurred, is extremely important in preventing disabilities. Management of lepra reactions and neuritis is also effective in preventing or minimizing the development of further disabilities.

The occurrence of leprosy in families has led to the misinterpretation that the disease is hereditary. The progressive symptoms and sometimes lethal secondary infections probably led to the assumption that patients are beyond medical support and that death is inevitable. In many societies, public stigmatization and exclusion coexist, and in some countries, the stigma is promoted by legislation against leprosy patients.[85] The accumulation of misnomers and misunderstandings have triggered unreasonable reactions in people, which have been difficult to overcome.

Self-awareness is crucial if the patient is to minimize damage. Treatment and/or surgical management, including reconstructions, should be provided for ulcers, and it is important that the patient understand the need for daily self-care and inspection for trauma.[86,87] Protective footwear and other tools are available to help patients improve their abilities and quality of life.[88] Community-based rehabilitation programs and other socioeconomic rehabilitation are required to support patients and families.[89]

CONCLUSIONS AND FUTURE PERSPECTIVES

Leprosy has affected humans for millennia. However, the MDT regimen recommended by the WHO has had a significant impact in reducing the global burden of leprosy, and research activities have led to increased knowledge of *M. leprae* genomic structure and host responses. Health-care workers and researchers should continue to support the intensive implementation of the elimination strategy and address issues related to the detection of *M. leprae*-infected individuals as a matter of urgency. Sustained quality patient care that is equitably distributed, affordable and easily accessible is still needed. A goal of the WHO is to bring institutional and management changes that strengthen the operational capacity of leprosy control programs. Improvement is needed in efforts to provide appropriate information to societies, dermatologists and patients. *M. leprae* is a very unique microorganism. It is expected that basic research for leprosy can be sustained.

REFERENCES

1 WHO. Global leprosy situation, 2010. *Wkly Epidemiol Rec* 2010; **85**: 337–348.
2 Saikawa K. The effect of rapid socio-economic development on the frequency of leprosy in a population. *Lepr Rev* 1981; **52** (Suppl 1): 167–175.
3 Cole ST, Eiglmeier K, Parkhill J *et al.* Massive gene decay in the leprosy bacillus. *Nature* 2001; **409**: 1007–1011.
4 Brosch R, Gordon SV, Eiglmeier K, Garnier T, Cole ST. Comparative genomics of the leprosy and tubercle bacilli. *Res Microbiol* 2000; **151**: 135–142.
5 Harrison PM, Gerstein M. Studying genomes through the aeons: protein families, pseudogenes and proteome evolution. *J Mol Biol* 2002; **318**: 1155–1174.
6 Liu Y, Harrison PM, Kunin V, Gerstein M. Comprehensive analysis of pseudogenes in prokaryotes: widespread gene decay and failure of putative horizontally transferred genes. *Genome Biol* 2004; **5**: R64.
7 Nakata N, Matsuoka M, Kashiwabara Y, Okada N, Sasakawa C. Nucleotide sequence of the *Mycobacterium leprae katG* region. *J Bacteriol* 1997; **179**: 3053–3057.
8 Madan Babu M. Did the loss of sigma factors initiate pseudogene accumulation in *M. leprae*? *Trends Microbiol* 2003; **11**: 59–61.
9 Cole ST, Supply P, Honoré N. Repetitive sequences in *Mycobacterium leprae* and their impact on genome plasticity. *Lepr Rev* 2001; **72**: 449–461.
10 Lawrence JG, Hendrix RW, Casjens S. Where are the pseudogenes in bacterial genomes? *Trends Microbiol* 2001; **9**: 535–540.
11 Vissa VD, Brennan PJ. The genome of *Mycobacterium leprae*: a minimal mycobacterial gene set. *Genome Biol* 2001; **2**: REVIEWS1023.
12 Eiglmeier K, Parkhill J, Honore N *et al.* The decaying genome of *Mycobacterium leprae*. *Lepr Rev* 2001; **72**: 387–398.
13 Akama T, Suzuki K, Tanigawa K *et al.* Whole-genome tiling array analysis of *Mycobacterium leprae* RNA reveals high expression of pseudogenes and noncoding regions. *J Bacteriol* 2009; **191**: 3321–3327.
14 Akama T, Tanigawa K, Kawashima A, Wu H, Ishii N, Suzuki K. Analysis of *Mycobacterium leprae* gene expression using DNA microarray. *Microb Pathog* 2010; **49**: 181–185.
15 Nakamura K, Akama T, Bang PD *et al.* Detection of RNA expression from pseudogenes and non-coding genomic regions of *Mycobacterium leprae*. *Microb Pathog* 2009; **47**: 183–187.
16 Suzuki K, Nakata N, Bang PD, Ishii N, Makino M. High-level expression of pseudogenes in *Mycobacterium leprae*. *FEMS Microbiol Lett* 2006; **259**: 208–214.
17 Monot M, Honore N, Baliere C *et al.* Are variable-number tandem repeats appropriate for genotyping *Mycobacterium leprae*? *J Clin Microbiol* 2008; **46**: 2291–2297.
18 Monot M, Honore N, Garnier T *et al.* On the origin of leprosy. *Science* 2005; **308**: 1040–1042.
19 Monot M, Honore N, Garnier T *et al.* Comparative genomic and phylogeographic analysis of *Mycobacterium leprae*. *Nat Genet* 2009; **41**: 1282–1289.

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20 Han XY, Seo YH, Sizer KC *et al.* A new *Mycobacterium* species causing diffuse lepromatous leprosy. *Am J Clin Pathol* 2008; **130**: 856–864.
21 Matsuo E. Leprosy as a challenge to science on the ability to decode its enigma. A hypothesis on how to respond. *Jpn J Infect Dis* 2005; **58**: 59–64.
22 Alford PL, Lee DR, Binhazim AA, Hubbard GB, Matherne CM. Naturally acquired leprosy in two wild-born chimpanzees. *Lab Anim Sci* 1996; **46**: 341–346.
23 Gormus BJ, Wolf RH, Baskin GB *et al.* A second sooty mangabey monkey with naturally acquired leprosy: first reported possible monkey-to-monkey transmission. *Int J Lepr Other Mycobact Dis* 1988; **56**: 61–65.
24 Hubbard GB, Lee DR, Eichberg JW. Diseases and pathology of chimpanzees at the southwest foundation for biomedical research. *Am J Primatol* 1991; **24**: 273–282.
25 Hubbard GB, Lee DR, Eichberg JW, Gormus BJ, Xu K, Meyers WM. Spontaneous leprosy in a chimpanzee (Pan troglodytes). *Vet Pathol* 1991; **28**: 546–548.
26 Leininger JR, Donham KJ, Meyers WM. Leprosy in a chimpanzee. Postmortem lesions. *Int J Lepr Other Mycobact Dis* 1980; **48**: 414–421.
27 Leininger JR, Donham KJ, Rubino MJ. Leprosy in a chimpanzee. Morphology of the skin lesions and characterization of the organism. *Vet Pathol* 1978; **15**: 339–346.
28 Meyers WM, Gormus BJ, Walsh GP, Baskin GB, Hubbard GB. Naturally acquired and experimental leprosy in nonhuman primates. *Am J Trop Med Hyg* 1991; **44**: 24–27.
29 Meyers WM, Walsh GP, Brown HL *et al.* Leprosy in a mangabey monkey – naturally acquired infection. *Int J Lepr Other Mycobact Dis* 1985; **53**: 1–14.
30 Suzuki K, Udono T, Fujisawa M, Tanigawa K, Idani G, Ishii N. Infection during infancy and long incubation period of leprosy suggested in a case of a chimpanzee used for medical research. *J Clin Microbiol* 2010; **48**: 3432–3434.
31 Truman R. Leprosy in wild armadillos. *Lepr Rev* 2005; **76**: 198–208.
32 Walsh GP, Meyers WM, Binford CH, Gerone PJ, Wolf RH, Leininger JR. Leprosy – a zoonosis. *Lepr Rev* 1981; **52** (Suppl 1): 77–83.
33 Truman RW, Singh P, Sharma R *et al.* Probable zoonotic leprosy in the southern United States. *N Engl J Med* 2011; **364**: 1626–1633.
34 Davey TF, Rees RJ. The nasal discharge in leprosy: clinical and bacteriological aspects. *Lepr Rev* 1974; **45**: 121–134.
35 McDougall AC, Rees RJ, Weddell AG, Kanan MW. The histopathology of lepromatous leprosy in the nose. *J Pathol* 1975; **115**: 215–226.
36 Job CK, Jayakumar J, Kearney M, Gillis TP. Transmission of leprosy: a study of skin and nasal secretions of household contacts of leprosy patients using PCR. *Am J Trop Med Hyg* 2008; **78**: 518–521.
37 Krutzik SR, Ochoa MT, Sieling PA *et al.* Activation and regulation of Toll-like receptors 2 and 1 in human leprosy. *Nat Med* 2003; **9**: 525–532.
38 Tanigawa K, Suzuki K, Kimura H *et al.* Tryptophan aspartate-containing coat protein (CORO1A) suppresses Toll-like receptor signalling in *Mycobacterium leprae* infection. *Clin Exp Immunol* 2009; **156**: 495–501.
39 Ferrari G, Langen H, Naito M, Pieters J. A coat protein on phagosomes involved in the intracellular survival of mycobacteria. *Cell* 1999; **97**: 435–447.
40 Suzuki K, Takeshita F, Nakata N, Ishii N, Makino M. Localization of CORO1A in the macrophages containing *Mycobacterium leprae*. *Acta Histochem Cytochem* 2006; **39**: 107–112.
41 Barker LP. *Mycobacterium leprae* interactions with the host cell: recent advances. *Indian J Med Res* 2006; **123**: 748–759.
42 Chan J, Fujiwara T, Brennan P *et al.* Microbial glycolipids: possible virulence factors that scavenge oxygen radicals. *Proc Natl Acad Sci USA* 1989; **86**: 2453–2457.
43 Tanigawa K, Suzuki K, Nakamura K *et al.* Expression of adipose differentiation-related protein (ADRP) and perilipin in macrophages infected with *Mycobacterium leprae*. *FEMS Microbiol Lett* 2008; **289**: 72–79.
44 Mattos KA, Lara FA, Oliveira VG *et al.* Modulation of lipid droplets by *Mycobacterium leprae* in Schwann cells: a putative mechanism for host lipid acquisition and bacterial survival in phagosomes. *Cell Microbiol* 2011; **13**: 259–273.
45 Bleharski JR, Li H, Meinken C *et al.* Use of genetic profiling in leprosy to discriminate clinical forms of the disease. *Science* 2003; **301**: 1527–1530.
46 Mira MT, Alcais A, Nguyen VT *et al.* Susceptibility to leprosy is associated with PARK2 and PACRG. *Nature* 2004; **427**: 636–640.
47 Santos AR, Suffys PN, Vanderborght PR *et al.* Role of tumor necrosis factor-alpha and interleukin-10 promoter gene polymorphisms in leprosy. *J Infect Dis* 2002; **186**: 1687–1691.
48 Croft RP, Richardus JH, Nicholls PG, Smith WC. Nerve function impairment in leprosy: design, methodology, and intake status of a prospective cohort study of 2664 new leprosy cases in Bangladesh (The Bangladesh Acute Nerve Damage Study). *Lepr Rev* 1999; **70**: 140–159.
49 Mahajan PM, Jogaikar DG, Mehta JM. A study of pure neuritic leprosy: clinical experience. *Indian J Lepr* 1996; **68**: 137–141.
50 Suneetha S, Arunthathi S, Chandi S, Kurian N, Chacko CJ. Histological studies in primary neuritic leprosy: changes in the apparently normal skin. *Lepr Rev* 1998; **69**: 351–357.
51 Van Brakel WH, de Soldenhoff R, McDougall AC. The allocation of leprosy patients into paucibacillary and multibacillary groups for multidrug therapy, taking into account the number of body areas affected by skin, or skin and nerve lesions. *Lepr Rev* 1992; **63**: 231–246.
52 Ridley DS, Jopling WH. Classification of leprosy according to immunity. A five-group system. *Int J Lepr Other Mycobact Dis* 1966; **34**: 255–273.
53 Ishikawa A, Ishikawa S, Hirakawa M. Osteoporosis, bone turnover and hypogonadism in elderly men with treated leprosy. *Lepr Rev* 2001; **72**: 322–329.
54 Boldsen JL. Leprosy in the early medieval Lauchheim community. *Am J Phys Anthropol* 2008; **135**: 301–310.
55 Boldsen JL, Mollerup L. Outside St. Jorgen: leprosy in the medieval Danish city of Odense. *Am J Phys Anthropol* 2006; **130**: 344–351.
56 Aufderheide AC, Rodríguez-Martín C. *The Cambridge Encyclopedia of Human Paleopathology*. Cambridge University Press, Cambridge 1998.
57 Ortner DJ. *Identification of Pathological Conditions in Human Skeletal Remains*, 2nd edn. Academic Press, London 2003.
58 Roberts C, Manchester K. *The Archaeology of Disease*, 3rd edn. Cornell University Press, New York 2007.
59 Suzuki K, Takigawa W, Tanigawa K *et al.* Detection of *Mycobacterium leprae* DNA from archaeological skeletal remains in Japan using whole genome amplification and polymerase chain reaction. *PLoS ONE* 2010; **5**: e12422.
60 Courtright P, Daniel E, Sundarrao *et al.* Eye disease in multibacillary leprosy patients at the time of their leprosy diagnosis: findings from the Longitudinal Study of Ocular Leprosy (LOSOL) in India, the Philippines and Ethiopia. *Lepr Rev* 2002; **73**: 225–238.
61 WHO. *Expert Committee on Leprosy*. 7th report 1998: 1–13.
62 Report of the International Leprosy Association Technical Forum. Paris, France, 22–28 February 2002. *Int J Lepr Other Mycobact Dis* 2002; **70**: S1–S62.
63 Sugita Y, Koseki M, Narita M, Kim S, Ishii N, Nakajima H. Suitability of a skin-smear examination needle for leprosy screening by PCR. *Int J Lepr Other Mycobact Dis* 1992; **60**: 658–659.
64 Buhrer-Sekula S. PGL-I leprosy serology. *Rev Soc Bras Med Trop* 2008; **41** (Suppl 2): 3–5.
65 Kampirapap K. Assessment of subclinical leprosy infection through the measurement of PGL-1 antibody levels in residents of a former leprosy colony in Thailand. *Lepr Rev* 2008; **79**: 315–319.
66 Mwanatambwe M, Yajima M, Etuaful S *et al.* Phenolic glycolipid-1 (PGL-1) in Buruli ulcer lesions. First demonstration by immuno-histochemistry. *Int J Lepr Other Mycobact Dis* 2002; **70**: 201–205.
67 Burki T. Old problems still mar fight against ancient disease. *Lancet* 2009; **373**: 287–288.
68 Nicholls PG, Ross L, Smith WC. Promoting early detection in leprosy – a literature review to identify proven and potential interventions addressing patient-related delay. *Lepr Rev* 2006; **77**: 298–310.
69 WHO Technical Advisory Group on Leprosy Control. *Report of the Tenth Meeting of the WHO Technical Advisory Group on Leprosy Control*. World Health Organization Regional Office for South-East Asia, New Delhi 2009.
70 Kaimal S, Thappa DM. Relapse in leprosy. *Indian J Dermatol Venereol Leprol* 2009; **75**: 126–135.

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71 Levy L, Moon N, Murray LP, O'Neill SM, Gustafson LE, Evans MJ. Studies of the mouse foot pad technic for cultivation of Mycobacterium leprae. 1. Fate of inoculated organisms. *Int J Lepr Other Mycobact Dis* 1974; **42**: 165–173.
72 Ishii N, Sugita Y, Sato I, Nakajima H. Sparfloxacin in the treatment of leprosy patients. *Int J Dermatol* 1997; **36**: 619–621.
73 Kai M, Matsuoka M, Nakata N *et al.* Diaminodiphenylsulfone resistance of *Mycobacterium leprae* due to mutations in the dihydropteroate synthase gene. *FEMS Microbiol Lett* 1999; **177**: 231–235.
74 Williams DL, Spring L, Harris E, Roche P, Gillis TP. Dihydropteroate synthase of *Mycobacterium leprae* and dapsone resistance. *Antimicrob Agents Chemother* 2000; **44**: 1530–1537.
75 Honore N, Cole ST. Molecular basis of rifampin resistance in *Mycobacterium leprae*. *Antimicrob Agents Chemother* 1993; **37**: 414–418.
76 Becx-Bleumink M, Berhe D. Occurrence of reactions, their diagnosis and management in leprosy patients treated with multidrug therapy; experience in the leprosy control program of the All Africa Leprosy and Rehabilitation Training Center (ALERT) in Ethiopia. *Int J Lepr Other Mycobact Dis* 1992; **60**: 173–184.
77 Kumar B, Dogra S, Kaur I. Epidemiological characteristics of leprosy reactions: 15 years experience from north India. *Int J Lepr Other Mycobact Dis* 2004; **72**: 125–133.
78 Scollard DM, Smith T, Bhoopat L, Theetranont C, Rangdaeng S, Morens DM. Epidemiologic characteristics of leprosy reactions. *Int J Lepr Other Mycobact Dis* 1994; **62**: 559–567.
79 Britton WJ. The management of leprosy reversal reactions. *Lepr Rev* 1998; **69**: 225–234.
80 Croft RP, Nicholls PG, Richardus JH, Smith WC. Incidence rates of acute nerve function impairment in leprosy: a prospective cohort analysis after 24 months (The Bangladesh Acute Nerve Damage Study). *Lepr Rev* 2000; **71**: 18–33.
81 Manandhar R, LeMaster JW, Roche PW. Risk factors for erythema nodosum leprosum. *Int J Lepr Other Mycobact Dis* 1999; **67**: 270–278.
82 Lockwood DN. The management of erythema nodosum leprosum: current and future options. *Lepr Rev* 1996; **67**: 253–259.
83 Jakeman P, Smith WC. Thalidomide in leprosy reaction. *Lancet* 1994; **343**: 432–433.
84 Moreira AL, Kaplan G, Villahermosa LG *et al.* Comparison of pentoxifylline, thalidomide and prednisone in the treatment of ENL. *Int J Lepr Other Mycobact Dis* 1998; **66**: 61–65.
85 Senior K. Stigma, chemoprophylaxis, and leprosy control. *Lancet Infect Dis* 2009; **9**: 10.
86 Kazen RO. Management of plantar ulcers in leprosy. *Lepr Rev* 1999; **70**: 63–69.
87 Cross H, Newcombe L. An intensive self care training programme reduces admissions for the treatment of plantar ulcers. *Lepr Rev* 2001; **72**: 276–284.
88 Seboka G, Saunderson P, Currie H. Footwear for farmers affected by leprosy. *Lepr Rev* 1998; **69**: 182–183.
89 Nicholls PG. Guidelines for social and economic rehabilitation. *Lepr Rev* 2000; **71**: 422–465.

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Future Microbiology

Special Report

Chimpanzees used for medical research shed light on the pathoetiology of leprosy

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Leprosy is a chronic infectious disorder caused by *Mycobacterium leprae*, which mainly affects skin and peripheral nerves. It is classified as either paucibacillary or multibacillary based upon clinical manifestations and slit-skin smear results. It is speculated that leprosy develops after a long latency period following *M. leprae* infection. However, the actual time of infection and the duration of latency have never been proven in human patients. To date, four cases of spontaneous leprosy have been reported in chimpanzees who were caught in West Africa in infancy and used for medical research in the USA and Japan. One of these chimpanzees was extensively studied in Japan, and single-nucleotide polymorphism analysis for the *M. leprae* genome was conducted. This analysis revealed that the chimpanzee was infected with *M. leprae* during infancy in West Africa and the pathognomonic signs of leprosy appeared after at least 30 years of incubation. Analysis of leprosy in chimpanzees can contribute not only to medical research but also to the understanding of the pathoetiology of leprosy.

Leprosy is caused by a chronic infection with *Mycobacterium leprae* and has afflicted humans for millennia. Leprosy is a systemic disease that primarily affects the skin, nerves and eyes. The diverse clinical manifestations of the disease are produced by variations in host immune responses [1]. *M. leprae* is an obligate intracellular parasite that cannot be cultivated *in vitro*. The inability to cultivate *in vitro* and the lack of animal models have been major disadvantages for leprosy research. The genome sequence of *M. leprae* has revealed that only half of the small genome contains protein-coding genes, while the remainder consists of pseudogenes and noncoding regions [2,3]. However, analyses have demonstrated that some of these pseudogenes and noncoding regions are highly expressed at the RNA level. In clinical samples, these RNAs show varying expression patterns among patients, which suggests they have yet unknown functions [4–7]. The analysis of single-nucleotide polymorphisms (SNPs) revealed four primitive subtypes of *M. leprae*, but the number is increasing as the analysis progresses [8–10].

Multibacillary (MB) patients excrete bacilli from their nasal mucosa and skin [11], making close and repeated contact with these patients, directly and/or indirectly, a potential source of transmission. It is believed that clinical manifestations are only apparent after many years of incubation [12,13]. Although serum antibodies against phenolic glycolipid (PGL)-I have been widely evaluated in diagnosis and community surveys, there have been some arguments for their specificity [14–16]. Therefore, there is no definitive method that can be used to prove the existence of subclinical infection in humans.

The *M. leprae* bacterium was first described in modern literature in 1873, prior to the first description of *Mycobacterium tuberculosis* in 1882. Nevertheless, despite the passing of more than 130 years since its discovery, methods for *in vitro* cultivation of *M. leprae* have still not been established, and there is no effective animal model for the human disease. Therefore, the processes of infection, dormancy and disease activation of *M. leprae* remain unclear.

Since primates are humans' closest relatives, studying human diseases in primates is sometimes helpful when trying to understand the nature of diseases. Naturally acquired leprosy cases have been reported in mangabey monkeys and cynomologus macaques [17–20]. Among primates, the chimpanzee is considered to be an anthropoid as it is genetically known to be very similar to humans. In fact, the genetic difference between humans and chimpanzees is only 1.23% of genomic DNA [21]. For this reason, many infectious diseases that humans acquire are infectious in chimpanzees as well. Many of those infectious diseases are contagious from humans to chimpanzees and *vice versa*. For example, HIV, HBV

Keywords

■ animal models ■ chimpanzee ■ latency ■ leprosy ■ *Mycobacterium leprae* ■ SNPs

future medicine part of fsg

and HCV are infectious diseases that are common in both humans and chimpanzees [22–26]. With this genetic similarity in mind, and keeping ethical considerations of using chimpanzees as subjects for animal research in mind, it can be argued that it is unfortunate for chimpanzees to be used as experimental animal models because they are so very similar to humans.

Cases of naturally acquired leprosy in chimpanzees

There have only been four reports of leprosy in chimpanzees in the literature (Table 1). All four chimpanzees were brought from Africa when they were infants for the purpose of being used for medical studies. The first three cases of leprosy were diagnosed solely by clinical and pathological evaluation, and *M. leprae* DNA was not identified.

The first reported case was a male chimpanzee who was captured in Sierra Leone, West Africa [20,27–29]. He was one of eight chimpanzees being used for experiments on bovine leukemia virus infections. After 2 months of virus infection in 1975, a leprosy-like skin lesion (macular rash) appeared and was followed by a progressive maculopapular rash with a crust that covered his abdomen and the medial aspect of his thighs. The other seven chimpanzees used for the same experiment did not develop gross or microscopic lesions. After that, his entire trunk and limbs were covered with a rash and nodular thickening of the ear margins appeared. Lepromatous leprosy was diagnosed due to acid-fast bacteria that were identified in biopsy specimens of the granulomas. The infection progressed with appearance of nodular lesions in the lower lip, nostrils, nasal septum, eyebrows, carpus and scrotum. No specific treatment for leprosy was administered. The chimpanzee died inadvertently following sedation with phencyclidine and ketamine 33 months after the first appearance of the lesions. A necropsy was performed 2 h after death [29]. Marked atrophy of skeletal muscles, alopecia and diffuse thickening of the skin of the hands, feet and digits were observed as gross lesions. Microscopically, there were diffuse and multifocal infiltrations of foamy histiocytes with acid-fast bacilli in the dermis, nasal mucosa, epiglottis, lung interstitium and parenchyma, liver, spleen, kidneys, lymphnodes, peripheral nerves, both eyes (especially the sclera, cornea, ciliary body and iris) and testicular tunics.

In 1989, the second and third chimpanzees were diagnosed with leprosy after they had both been held in research facilities in the USA for many years [30–33]. The second case was a male who was brought from Africa when he was approximately 2-years-old [31]. The chimpanzee began self-mutilating his digits at 7 years. At 9 years, he had a positive reaction to an intradermal tuberculin test and was treated with antibiotics. However, after several examinations, it was revealed that there was no evidence of tuberculosis. At 13 years, his tuberculin test results again showed positive, but no evidence of active tuberculosis was found. At 18 years, he developed nodular and papular eruptions of his eyelids, face, ear margins, lips, distal portion of the penis and scrotum. Histologic evaluation of the cutaneous lesions revealed granulomatous dermatitis consisting predominantly of foamy histiocytes containing acid-fast bacilli. A diagnosis of borderline leprosy was made on the bases of clinical and histopathological findings and bacterial indices. Retrospective evaluations of tissue sections of an amputated finger revealed leprosy with neural involvement. Serum PGL-I antibodies were above baseline. A multidrug treatment (MDT) regimen was started as recommended by the WHO study group for human patients, and was continued for 4 years [34]. A total of 6 months after the start of treatment, a severe leprae reaction developed with the subject manifesting pain and marked impairment of locomotion. Although treatment with prednisone and aspirin restored quadrupedal locomotion and some climbing activity, he sustained permanent neurologic and musculoskeletal dysfunction.

The third case was a male chimpanzee who was imported to the USA when he was approximately 3 years old [31–33]. He had three episodes of ulcerative gingivitis of unknown origin. When he was 26-years-old, he initially developed a persistent clear nasal discharge and chronic areas of epidermal erosions, which were followed by a development of coalescent nodules in the skin of the supraorbital area, lips, chin, ear and scrotum. He was diagnosed as having subpolar lepromatous to borderline leprosy by histological examination. Intracellular aggregates of acid-fast bacilli were found in the liver histiocytes. Antibodies against lipoarabinomannan (LAM) and PGL-I were markedly elevated. He was treated with MDT, but died suddenly 1.5 years later when recovering from anesthesia with ketamine. Necropsy revealed that the immediate cause of death was heart failure secondary to acute, severe myocardial necrosis and hemorrhage.

Gormus *et al.* measured serum anti-PGL-I and anti-LAM antibodies in 160 chimpanzees housed in two research facilities in the USA [30].

Table 1. Reported leprosy cases in wild-born chimpanzees.

Case number	Place of birth	Sex	Name	Place of diagnosis	Age of onset (years)	Locations of affected lesions	Histological diagnosis	Acid-fast bacilli	Anti-PGL-I	*M. leprae* DNA	Treatment	Purpose of import	Remarks	Ref.
1	Sierra Leone, West Africa	Male	Unknown	USA	5–7	Nostrils, nasal septum, nares, lower lip, eyebrows, scrotum and carpus	Lepromatous type leprosy	Yes	NT	NT	None	Bovine leukemia virus innoculation	Died 33 months after diagnosis	[20,27–29]
2	Africa	Male	Kevin	USA	7 or 18	Eyelids, face, ear margins, lips, hands, feet, arms, legs, penis and scrotum	Borderline lepromatous leprosy	Yes	Positive	NT	MDT	Not known	Self-multilation of digits, hyperglobulinemia	[30,31]
3	Africa	Male	Brian	USA	28	Multiple skin lesions	Lepromatous type leprosy	Yes	Positive	NT	MDT	Isoniazid pharmacology study, administration of SV40 peptide	Ulcerative gingivitis of unknown origin. Died from heart failure after anesthesia	[30–33]
4	Sierra Leone, West Africa	Female	Haruna	Japan	31	Eyebrows, lips, abdomen, forearms and crus	Lepromatous type leprosy	Yes	Positive	Positive	MDT	Hepatitis research	Symptoms developed after 30-year incubation period	[35]

MDT: Multidrug treatment; NT: Not tested; PGL: Phenolic glycolipid.

This includes the cases 2 and 3 as described earlier and other wild-born adults, captive-born adults and adolescents, and colony-born infants, juveniles and adolescents. Among the chimpanzee cases with anti-PGL-I positive serum, there were four with IgG and five with IgM antibodies. Samples from cases 2 and 3 were the only cases that were sera positive for both IgG and IgM anti-PGL-I. On the other hand, among the anti-LAM positive serum samples there were three with IgG and three with IgM antibodies, but only the serum from case 2 was positive for both IgG and IgM antibodies.

A case of leprosy in a chimpanzee in Japan

Haruna is a female chimpanzee who was imported to Japan from West Africa for use in medical research in March 1980 when she was approximately 2 years old [35], and was used for HBV and HCV research. In March 2002, at 21 years she was healthy and was retired to live in a primate sanctuary. In January 2009 at 30 years, her caretaker noticed swelling and nodes on her face. In April, swelling of her eyelids and lips were observed, but no decrease in appetite or other symptoms were observed. Lesions on her face were notable and leontiasis developed. Whole body examination, blood tests, skim smears and biopsies were conducted under anesthesia. The tuberculin reaction was negative, but staining of a nasal swab and a skin smear from a nodule on the left forearm demonstrated acid-fast bacilli. Numerous foamy histiocytes were found in tissue sections of skin biopsies, and diagnosis of lepromatous leprosy was made. PCR analysis for *M. leprae Hsp-70* DNA using skin tissue was positive, but *M. tuberculosis* DNA was not detected. 16S RNA sequencing revealed a 100% match with *M. leprae* genetics. Therefore, it was concluded that the pathogenic bacteria was the same *M. leprae* that causes human leprosy.

On June 1 2009, the MDT for MB regimen was administered as suggested by the WHO by mixing the drug with fruit or juice. After 2 months of MDT, her skin hives disappeared, and after 5 months nasal swab staining for acid-fast bacillus became negative. The treatment was continued for 1 year, as recommended by the WHO [34]. Anti-PGL-I antibody in the preserved sera was negative between 2001 and 2004. In 2008 a false positive was recorded, and the sera turned positive after the onset of symptoms in 2009. After the treatment, the sera again turned negative and it has remained negative for anti-PGL-I antibodies since then. In general, the specificity of anti-PGL-I antibody was uncertain, since the antibody has been detected in some household contacts and even in some healthy individuals in addition to MB patients [15]. However, in Haruna's case, the antibody response against *M. leprae* correlated with acid-fast bacteria detected in her body. Since skin lesions were first noticed in January 2009, the false-positive result of anti-PGL-I in 2008 suggests that *M. leprae* started to grow well before clinical manifestations were evident.

In order to determine the possible origin of the *M. leprae* found in Haruna, SNPs for three reported loci in the *M. leprae* genome were studied [9,10]. PCR amplification followed by direct sequencing identified the SNP type 4 *M. leprae* genotype. This genotype was identified to have originated in West Africa and been introduced to parts of the Caribbean islands and South America (most probably by the slave trade), but has not been found in any other areas. Therefore, infection of Haruna is highly unlikely to have taken place in Japan, particularly given the strict biosafety standards of primate housing facilities in experimental laboratories and the very low prevalence of leprosy in Japan. Evidence strongly suggests that Haruna was infected with *M. leprae* when she was in West Africa by the age of 2 years, and she developed leprosy after an incubation period of at least 30 years [35].

All of the other 13 chimpanzees imported into Japan at the same time lived together in the same cage as Haruna. They have all tested negative for anti-PGL-I antibody. Also, nasal swab staining for acid-fast bacilli and PCR detection of *M. leprae* DNA for 32 other chimpanzees in Japan have all been negative. A survey was conducted at zoos and other facilities which have chimpanzees in Japan, but no other chimpanzees were reported to have had skin symptoms like Haruna. At the sanctuary, no human caretakers have reported skin hives with decreased consciousness [35].

Routes of *M. leprae* infection in chimpanzees

There is a possibility that *M. leprae* might be transmitted among chimpanzees in Africa [30]. Another possibility is that contact with a human patient with *M. leprae* occurred during the 2–3 month period the chimpanzees were housed in outdoor cages while awaiting shipment after capture [27]. In addition, possible transmission from the environment cannot be excluded. There have been some reports of African wild chimpanzees with nasal discharge thought to

be caused by infectious diseases, including leprosy [12]. However, it is difficult to make a diagnosis of leprosy by just observing and without performing a close examination of wild animals. If peripheral neuritis or ulcerations of extremities exist, survival in the wild must be quite difficult.

M. leprae infection is thought to occur when a subject is exposed to a certain number of bacilli either directly or indirectly [12,13]. Although the disease develops after a long incubation period, this progression of disease has never been proven since it is not possible to identify the infection period in human subjects. The life expectancy of chimpanzees is 40–50 years, and the four chimpanzees with leprosy in the present report are considered to be elderly. Although it is not clear what triggered the initiation of active growth of *M. leprae* after the long incubation period, it was speculated in that Haruna's case a recent change in her social group might have resulted in stress that may have transiently impaired her immune system [35].

Conclusion & future perspective

A total of four leprosy cases have been reported in chimpanzees imported from Africa for the purpose of medical experiments. With the most recent case in Japan, the existence of *M. leprae* was proven with DNA analysis. Furthermore, by identification of *M. leprae* SNPs, it was shown that the chimpanzee was infected by the age of 2 years in West Africa, and that the symptoms appeared after an incubation period of at least 30 years. In this case, anti-PGL-I antibody levels were negative before the onset of the disease, turned positive after the onset, and returned again to negative along with the improvement of the symptoms.

These cases also suggest that isolated clinical leprosy in the wild may exist [17,36,37], yet have escaped detection due to reduced fitness and shorter lifespans in the wild. Since the habitat of chimpanzees and other apes is becoming increasingly restricted, contact with humans may become more frequent, increasing the risks of many zoonoses [20,23,36]. Although humans appear to be the major reservoir of *M. leprae* infection, naturally occurring infection has been reported in wild animals, including the nine-banded armadillo and several species of primates [17,19,20,28,29,31–33,35–37]. A recent study found that the same genotypic strain of *M. leprae* was detected at high incidence in wild armadillos and leprosy patients in the southern USA, suggesting that leprosy may be a zoonotic in regions in which armadillos serve as a reservoir [38]. Thus, leprosy is clearly a common disease among humans and other animals. Even after worldwide efforts to reduce the disease burden of leprosy were successfully completed, isolated leprosy cases in wild animals may still exist, which may serve as potential sources of human infection. It will be imperative, therefore, to reconsider the relationship between humans and nonhuman primates in the wild. Careful attention needs to be paid, as both have the possibility to transmit infectious diseases to each other.

Since infection of the four chimpanzees is unlikely to have taken place in the USA or in Japan, they seem to be infected in Africa and disease manifested 5–30 years after infection. However, risk of infection and the clinical manifestations of leprosy depend on multiple factors, but are not restricted to infection during infancy via the nasopharynx. Large numbers of prospective cohort studies of human leprosy patients and their contacts suggest that the risk of leprosy is associated with consanguinity, BCG vaccination and the clinical form of the transmitting cases [39–41]. It was long thought that leprosy might have a strong host genetic component. Genome-wide association studies and searches for susceptibility loci have provided a list of genes associated with host immune response in human leprosylesions [42–45]. Since emergence from dormancy, as well as leprae reaction, appear to be related to stress and changes of immunological status in humans [46], this is possibly also the case with chimpanzees. Research on the role of various stressors in the onset of leprosy and leprae reactions is needed.

Chimpanzees are the closest genetic relatives to humans that have been used for medical research, including infectious diseases research [21–26]. Although chimpanzees and humans have very similar immune systems, fewer genes on the chimpanzee Y chromosome are responsible for immune function [47]. The innate immune systems could also differ, as the living environments of chimpanzees and humans are quite different. Whether these immune system differences affect the occurrence of mycobacterial diseases in chimpanzees is not clear.

The parents of these four chimpanzees must have been killed in order to capture them during their infancy before they were brought to the USA or Japan. Since 1980, approximately 150 chimpanzees have been imported to Japan. They have been used to test the safety of HBV vaccines. During this time, the development of vaccines from human virus carrier plasma was innovative and untested, and the vaccines were

tested on chimpanzees in order to eliminate the possibility of the vaccines containing the active virus. The chimpanzees introduced in this article have contributed not only to the medical experiments for which they were originally intended, but have also unexpectedly contributed to leprosy research. The fourth case discussed in this report, Haruna, fortunately does not have any notable peripheral neuropathy or leprae reactions. She is living her life in the sanctuary peacefully. We truly wish her peace for the rest of her life there.

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Executive summary

Leprosy

- Leprosy is a chronic infectious disorder, caused by *Mycobacterium leprae*, that primarily affects the skin and peripheral nerves.
- Leprosy is suspected to develop after a long period of latency following infection with *M. leprae*, but definitive proof is lacking.
- There is no definitive method that can be used to prove the existence of subclinical infection in humans.

Chimpanzees

- Chimpanzees, as well as great apes including gorillas and orangutans, are members of the Hominoidea superfamily of primates and are humans' closest genetic relatives.
- They are considered the best models for a handful of human diseases, but their status as members of an endangered species and ethical considerations for these highly sentient animals have justifiably limited their use in research.
- They were transported from Africa for the purpose of medical experiments.

Spontaneous leprosy cases in chimpanzees

- Four spontaneous leprosy cases were reported in chimpanzees.
- These chimpanzees have contributed not only to the medical experiments for which they were originally intended, but have also unexpectedly contributed to leprosy research.

Bibliography

Papers of special note have been highlighted as:
- ▪ of interest
- ▪▪ of considerable interest

1. Pinheiro RO, de Souza Salles J, Sarno EN, Sampaio EP. *Mycobacterium leprae*-host-cell interactions and genetic determinants in leprosy: an overview. *Future Microbiol.* 6(2), 217–230 (2011).
2. Cole ST, Eiglmeier K, Parkhill J *et al.* Massive gene decay in the leprosy bacillus. *Nature* 409(6823), 1007–1011 (2001).
3. Singh P, Cole ST. *Mycobacterium leprae*: genes, pseudogenes and genetic diversity. *Future Microbiol.* 6(1), 57–71 (2011).
4. Akama T, Suzuki K, Tanigawa K *et al.* Whole-genome tiling array analysis of *Mycobacterium leprae* RNA reveals high expression of pseudogenes and noncoding regions. *J. Bacteriol.* 191(10), 3321–3327 (2009).
5. Akama T, Tanigawa K, Kawashima A, Wu H, Ishii N, Suzuki K. Analysis of *Mycobacterium leprae* gene expression using DNA microarray. *Microb. Pathog.* 49(4), 181–185 (2010).
6. Nakamura K, Akama T, Bang PD *et al.* Detection of RNA expression from pseudogenes and non-coding genomic regions of *Mycobacterium leprae. Microb. Pathog.* 47(3), 183–187 (2009).
7. Suzuki K, Nakata N, Bang PD, Ishii N, Makino M. High-level expression of pseudogenes in *Mycobacterium leprae. FEMS Microbiol. Lett/* 259(2), 208–214 (2006).
8. Monot M, Honore N, Baliere C *et al.* Are variable-number tandem repeats appropriate for genotyping *Mycobacterium leprae*? *J. Clin. Microbiol.* 46(7), 2291–2297 (2008).
9. Monot M, Honore N, Garnier T *et al.* On the origin of leprosy. *Science* 308(5724), 1040–1042 (2005).
10. Monot M, Honore N, Garnier T *et al.* Comparative genomic and phylogeographic analysis of *Mycobacterium leprae. Nat. Genet.* 41(12), 1282–1289 (2009).
11. Job CK, Jayakumar J, Kearney M, Gillis TP. Transmission of leprosy: a study of skin and nasal secretions of household contacts of leprosy patients using PCR. *Am. J. Trop. Med. Hyg.* 78(3), 518–521 (2008).
12. Davey TF, Rees RJ. The nasal discharge in leprosy: clinical and bacteriological aspects. *Lepr. Rev.* 45(2), 121–134 (1974).
13. McDougall AC, Rees RJ, Weddell AG, Kanan MW. The histopathology of lepromatous leprosy in the nose. *J. Pathol.* 115(4), 215–226 (1975).
14. Buhrer-Sekula S. PGL-I leprosy serology. *Rev. Soc. Bras. Med. Trop.* 41(Suppl. 2) 3–5 (2008).
15. Kampirapap K. Assessment of subclinical leprosy infection through the measurement of PGL-1 antibody levels in residents of a former leprosy colony in Thailand. *Leprosy Rev.* 79(3), 315–319 (2008).
16. Mwanatambwe M, Yajima M, Etuaful S *et al.* Phenolic glycolipid-1 (PGL-1) in Buruli ulcer lesions. First demonstration by immunohistochemistry. *Int. J. Lepr. Other Mycobact. Dis.* 70(3), 201–205 (2002).
17. Meyers WM, Gormus BJ, Walsh GP, Baskin GB, Hubbard GB. Naturally acquired and experimental leprosy in nonhuman primates. *Am. J. Trop. Med. Hyg.* 44(4 Pt 2), 24–27 (1991).
18. Meyers WM, Walsh GP, Brown HL *et al.* Leprosy in a mangabey monkey–naturally

acquired infection. *Int. J. Lepr. Other Mycobact. Dis.* 53(1), 1–14 (1985).

19. Gormus BJ, Wolf RH, Baskin GB *et al.* A second sooty mangabey monkey with naturally acquired leprosy: first reported possible monkey-to-monkey transmission. *Int. J. Lepr. Other Mycobact. Dis.* 56(1), 61–65 (1988).
20. Walsh GP, Meyers WM, Binford CH, Gerone PJ, Wolf RH, Leininger JR. Leprosy – a zoonosis. *Lepr. Rev.* 52(Suppl. 1) 77–83 (1981).
21. Varki A, Altheide TK. Comparing the human and chimpanzee genomes: searching for needles in a haystack. *Genome Res.* 15(12), 1746–1758 (2005).
22. Francis DP, Feorino PM, Broderson JR *et al.* Infection of chimpanzees with lymphadenopathy-associated virus. *Lancet* 2(8414), 1276–1277 (1984).
23. Keele BF, Van Heuverswyn F, Li Y *et al.* Chimpanzee reservoirs of pandemic and nonpandemic HIV-1. *Science* 313(5786), 523–526 (2006).
24. Maynard JE, Berquist KR, Krushak DH, Purcell RH. Experimental infection of chimpanzees with the virus of hepatitis B. *Nature* 237(5357), 514–515 (1972).
25. Tabor E, Gerety RJ, Drucker JA *et al.* Transmission of non-A, non-B hepatitis from man to chimpanzee. *Lancet* 1(8062), 463–466 (1978).
26. Yoshizawa H, Itoh Y, Iwakiri S *et al.* Demonstration of two different types of non-A, non-B hepatitis by reinjection and cross-challenge studies in chimpanzees. *Gastroenterology* 81(1), 107–113 (1981).
27. Donham KJ, Leininger JR. Spontaneous leprosy-like disease in a chimpanzee. *J. Infect. Dis.* 136(1), 132–136 (1977).
   - **The first leprosy case report in chimpanzees.**
28. Leininger JR, Donham KJ, Rubino MJ. Leprosy in a chimpanzee. Morphology of the skin lesions and characterization of the organism. *Vet. Pathol.* 15(3), 339–346 (1978).
29. Leininger JR, Donham KJ, Meyers WM. Leprosy in a chimpanzee. Postmortem lesions. *Int. J. Lepr. Other Mycobact. Dis.* 48(4), 414–421 (1980).
30. Gormus BJ, Xu KY, Alford PL *et al.* A serologic study of naturally acquired leprosy in chimpanzees. *Int. J. Lepr. Other Mycobact. Dis.* 59(3), 450–457 (1991).
   - **Results of serological screening of anti-phenolic glycolipid-1 and anti-lipoarabinomannan in chimpanzees in the USA.**
31. Alford PL, Lee DR, Binhazim AA, Hubbard GB, Matherne CM. Naturally acquired leprosy in two wild-born chimpanzees. *Lab. Anim. Sci.* 46(3), 341–346 (1996).
32. Hubbard GB, Lee DR, Eichberg JW, Gormus BJ, Xu K, Meyers WM. Spontaneous leprosy in a chimpanzee (*Pan troglodytes*). *Vet. Pathol.* 28(6), 546–548 (1991).
33. Hubbard GB, Lee DR, Eichberg JW. Diseases and pathology of chimpanzees at the southwest foundation for biomedical research. *Am. J. Primatol.* 24, 273–282 (1991).
34. No authors listed. Chemotherapy of leprosy for control programmes. *World Health Organ. Tech. Rep. Ser.* 675, 1–33 (1982).
35. Suzuki K, Udono T, Fujisawa M, Tanigawa K, Idani G, Ishii N. Infection during infancy and long incubation period of leprosy suggested in a case of a chimpanzee used for medical research. *J. Clin. Microbiol.* 48(9), 3432–3434 (2010).
   - ▪▪ **A case report with extensive genetic analysis of *Mycobacterium leprae*.**
36. Meyers WM, Gormus BJ, Walsh GP. Nonhuman sources of leprosy. *Int. J. Lepr. Other Mycobact. Dis.* 60(3), 477–480 (1992).
   - **Good review of leprosy in wild animals.**
37. Truman R. Leprosy in wild armadillos. *Lepr. Rev.* 76(3), 198–208 (2005).
38. Truman RW, Singh P, Sharma R *et al.* Probable zoonotic leprosy in the southern United States. *N. Engl. J. Med.* 364(17), 1626–1633 (2011).
39. Fine PE, Sterne JA, Ponnighaus JM *et al.* Household and dwelling contact as risk factors for leprosy in northern Malawi. *Am. J. Epidemiol.* 146(1), 91–102 (1997).
40. Moet FJ, Pahan D, Schuring RP, Oskam L, Richardus JH. Physical distance, genetic relationship, age, and leprosy classification are independent risk factors for leprosy in contacts of patients with leprosy. *J. Infect. Dis.* 193(3), 346–353 (2006).
41. Sales AM, Ponce de Leon A, Duppre NC *et al.* Leprosy among patient contacts: a multilevel study of risk factors. *PLoS Negl. Trop. Dis.* 5(3), e1013 (2011).
42. Bleharski JR, Li H, Meinken C *et al.* Use of genetic profiling in leprosy to discriminate clinical forms of the disease. *Science* 301(5639), 1527–1530 (2003).
43. Mira MT, Alcais A, Nguyen VT *et al.* Susceptibility to leprosy is associated with PARK2 and PACRG. *Nature* 427(6975), 636–640 (2004).
44. Santos AR, Suffys PN, Vanderborght PR *et al.* Role of tumor necrosis factor-α and interleukin-10 promoter gene polymorphisms in leprosy. *J. Infect. Dis.* 186(11), 1687–1691 (2002).
45. Cardoso CC, Pereira AC, de Sales Marques C, Moraes MO. Leprosy susceptibility: genetic variations regulate innate and adaptive immunity, and disease outcome. *Future Microbiol.* 6(5), 533–549 (2011).
46. Mohanty KK, Joshi B, Katoch K, Sengupta U. Leprosy reactions: humoral and cellular immune responses to *M. leprae*, 65kDa, 28kDa, and 18 kDa antigens. *Int. J. Lepr. Other Mycobact. Dis.* 72(2), 149–158 (2004).
47. Kuroki Y, Toyoda A, Noguchi H *et al.* Comparative analysis of chimpanzee and human Y chromosomes unveils complex evolutionary pathway. *Nat. Genet.* 38(2), 158–167 (2006).

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Nineteen Cases of Buruli Ulcer Diagnosed in Japan from 1980 to 2010▽

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The etiology, clinical manifestations, and treatment of 19 sporadic cases of Buruli ulcer (BU) in Japan are described. The cases originated in different regions of Honshu Island, with no evidence of patient contact with an aquatic environment. The majority (73.7%) of cases occurred in females, with an average age of 39.1 years for females and 56.8 years for males. All patients developed ulcers on exposed areas of the skin (e.g., face, extremities). Most ulcers were <5 cm in diameter (category I), except in one severe progressive case (category II). Pain was absent in 10 of the 19 cases. Fourteen ulcers were surgically excised, and nine patients needed skin grafting. All cases were treated with various antibiotic regimens, with no reported recurrences as of March 2011. *Mycobacterium ulcerans*-specific IS*2404* was detected in all cases. Ten isolates had identical 16S rRNA gene sequences, which were similar to those of *M. ulcerans*. However, the *rpoB* gene showed a closer resemblance to *Mycobacterium marinum* or *Mycobacterium pseudoshottsii*. PCR identified pMUM001 in all isolates but failed to detect one marker. DNA-DNA hybridization misidentified all isolates as *M. marinum*. The drug susceptibility profile of the isolates also differed from that of *M. ulcerans*. Sequence analysis revealed "*Mycobacterium ulcerans* subsp. *shinshuense*" as the etiologic agent of BU in Japan. Clinical manifestations were comparable to those of *M. ulcerans* but differed as follows: (i) cases were not concentrated in a particular area; (ii) there was no suspected connection to an aquatic environment; (iii) drug susceptibility was different; and (iv) bacteriological features were different.

Buruli ulcer (BU) was first reported in 1935 as a series of unusual painless ulcers in a patient from southeast Australia (2). Thirteen years after the first report, the etiological agent of the ulcer was determined to be *Mycobacterium ulcerans*, a previously unknown mycobacterium (5, 14). During the 1960s, many *M. ulcerans* infections were reported in Uganda, especially in Buruli County, for which this disease was eventually named (3, 32). It is a necrotizing disease of the skin that mostly affects children, producing massive ulcers and permanent, disabling scars. At present, the disease is found primarily in West and Central Africa and in humid tropical areas: BU has been reported in 32 countries, and *M. ulcerans* infection is the third most common mycobacterial infection, after tuberculosis and leprosy. Treatment of progressive cases is difficult and generally requires surgery, usually accompanied by skin grafting and prolonged courses of antibiotics (21, 34).

The first reported case of BU in Japan occurred in 1980 in a 19-year-old woman who had never been abroad (15). The causative agent was isolated and classified as "*Mycobacterium ulcerans* subsp. *shinshuense*" because it was closely related to *M. ulcerans* (31). The disease was not seen again until a 37-year-old woman was affected in 2003 (10). The number of cases increased gradually, until 19 cases had been detected by December 2010 (K. Nakanaga, Y. Hoshino, and N. Ishii, presented at the WHO Annual Meeting on Buruli Ulcer, Geneva, Switzerland, 22 to 24 March 2010). We conducted a comprehensive study using these 19 clinical samples and/or isolated bacteria. Etiology, differential diagnosis, clinical manifestations, and treatments are discussed in this report.

(The preliminary results of this study were presented by K.N. and R. R. Y. in the WHO Annual Meeting on Buruli Ulcer, Geneva, Switzerland, 28 to 30 March 2011.)

MATERIALS AND METHODS

Patients. The research protocol was approved by the institutional review board of the National Institute of Infectious Diseases, Japan. The BU diagnostic criteria were established prior to this study. The primary characteristic was the presence of a clinical lesion, which usually started as a painless subcutaneous nodule, and which secondarily ulcerated with characteristic undermined edges. Other preulcerative forms consisted of papules affecting only the skin, plaques (large, firm, painless, and raised lesions), and edema (a severe form of the disease). Apart from the clinical lesions, at least one of the following criteria must be included for a diagnosis of BU: (i) detection of acid-fast bacilli in a smear from a swab or a biopsy specimen after Ziehl-Neelsen staining, (ii) growth on 7H11 or Ogawa medium, (iii) histopathological confirmation, or (iv) PCR amplification of IS*2404*, an *M. ulcerans*-specific repetitive element. This article is a summary of all BU cases diagnosed to date in Japan. Some have already been published elsewhere as case reports in Japanese and/or English (6, 7, 10, 12, 16, 28, 35).

PCR, sequencing, and phylogenetic analyses. All PCRs targeting IS*2404* (18) were performed on extracted DNA from one or more of the following: fresh skin biopsy specimens, a thin section of formalin-fixed, paraffin-embedded skin, and bacteria isolated from a skin lesion. Briefly, the PCR product, amplified using forward primer PU4F and reverse primer PU7Rbio (Table 1), was electrophoresed on a 2% agarose gel and was stained with ethidium bromide.

The sequences of the internal transcribed spacer between the 16S and 23S rRNA genes (ITS region) and of the 16S rRNA, *rpoB*, and *hsp65* genes were analyzed with the primers listed in Table 1. Amplified PCR products (sizes shown in Table 1) were directly sequenced using the ABI Prism 310 PCR genetic analyzer (Applied Biosystems, Foster City, CA) (16). Sequences were obtained for 1,475- or 1,478-bp (16S rRNA gene), 272-bp (ITS region), 315-bp (*rpoB*), and

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TABLE 1. Primer sequences

Primer	Sequence (5′–3′)	PCR target (fragment size [bp])	Reference
PU4F PU7Rbio	GCGCAGATCAACTTCGCGGT GCCCGATTGGTGCTCGGTCA	IS*2404* (154)	18
8F16S 1047R16S	AGAGTTTGATCCTGGCTCAG TGCACACAGGCCACAAGGGA	16S rRNA gene (1,515 or 1,518)	24
830F16S 1542R16S	GTGTGGGTTTCCTTCCTTGG AAGGAGGTGATCCAGCCGCA		
ITSF ITSR	TTGTACACACCGCCCGTC TCTCGATGCCAAGGCATCCACC	16S–23S ITS region (ca. 340)	23
MF MR	CGACCACTTCGGCAACCG TCGATCGGGCACATCCGG	*rpoB* (341)	11
TB11 TB12	ACCAACGATGGTGTGTCCAT CTTGTCGAACCGCATACCCT	*hsp65* (441)	30
RepAF RepAR	CTACGAGCTGGTCAGCAATG ATCGACGCTCGCTACTTCTG	*repA* in pMUM001 (413)	26
ParAF ParAR	GCAAGCTGGGCAATGTTTAT GTCCGGTCCTTGATAGGTCA	*parA* in pMUM001 (501)	26
MUP11F MUP11R	ACCACCCAAGAGTGGAACTG TGTCGTGTCGAGGTATGTGG	Serine/threonine protein kinase in pMUM001 (479)	26
MLSloadF MLSloadR	GGGCAATCGTCCTCACTG CAAGGGCAGTCTTGATTAGG	*mls* load in pMUM001 (560)	26
MLSAT(II)F MLSAT(II)R	AACGTTGAATCCCGTTTTTG GCACCACAAAGGAACGTCTAA	*mlsAT(II)* in pMUM001 (504)	26
TEIIF TEIIR	ATTCAAACGGATGCGAACTG ACATTGCTGGACAAACGACA	Type II thioesterase in pMUM001 (500)	26
MUP045F MUP045R	CAGCAAGTAACGGTGGAACA ACGTGGCCCATTTGTCTTAG	Type III ketosynthase in pMUM001 (496)	26
P450F P450R	CCCACCTCGTCGTTAGTCAT GTGCTCGGTGATCCAGAAGT	P450 in pMUM001 (500)	26

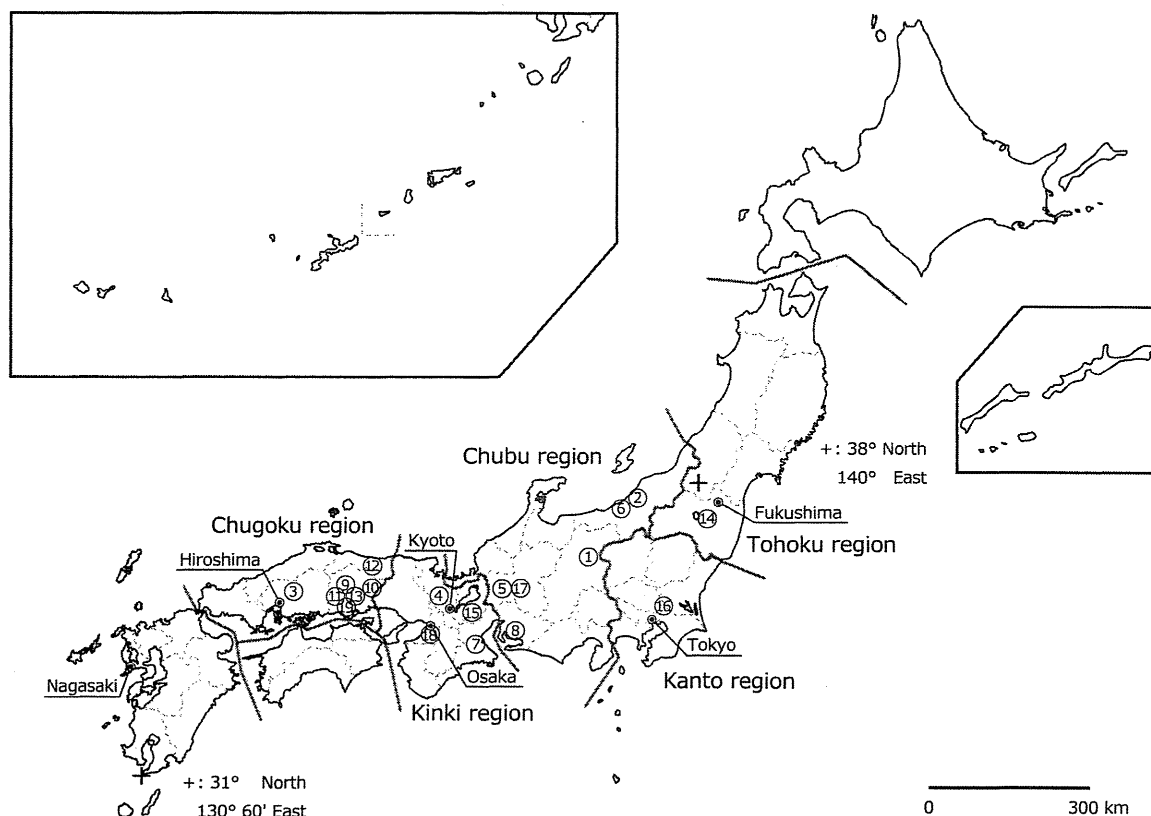

FIG. 1. Distribution of BU patients in Japan. Most of the patients lived in a typical temperate region, and all lived on the island of Honshu. The two plus signs on the map indicate 38°N, 140°E, and 31°N, 130°60′E, placing most of the island in the temperate zone.

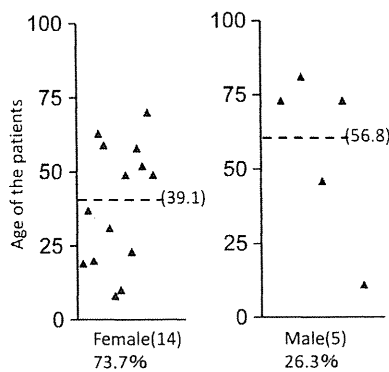

FIG. 2. Ages and genders of BU patients in Japan.

401-bp (*hsp65*) fragments. Ten clinical isolates were compared to six reference strains: *M. ulcerans* ITM 98-912, *M. ulcerans* ATCC 19423^T, *M. ulcerans* Agy99 (25), *Mycobacterium marinum* ATCC 927^T, *M. marinum* clinical isolate LRC 112509, and *Mycobacterium pseudoshottsii* JCM 15466^T. A similarity search was also performed with other mycobacterial reference strains and the 10 clinical strains using the DNA Data Bank of Japan (DDBJ) (8). Phylogenetic analyses were performed using the MEGA software package, version 4.0.2 (build 4028) (29). A tree was constructed using the neighbor-joining method with Kimura's two-parameter distance correction model with 1,000 bootstrap replications.

Finally, primers for eight pMUM001 sequences that encode toxic lipid mycolactone-producing enzymes (26) were used to compare the PCR products of the 10 clinical isolates, *M. ulcerans* ITM 98-912, *M. ulcerans* ATCC 19423^T, *M. ulcerans* Agy99, and *M. pseudoshottsii* JCM 15466^T.

DNA-DNA hybridization assay. A commercially available DNA-DNA hybridization method (DDH Mycobacteria kit; Kyokuto Pharmaceutical Industrial, Tokyo, Japan) was used to identify mycobacterial species isolated from patients (13). The 18 strains in the *Mycobacterium* reference panel included *M. marinum* but not *M. ulcerans*, *M. ulcerans* subsp. *shinshuense*, or *M. pseudoshottsii*.

Growth characteristics and biochemical assay. Culture growth characteristics were determined, and identification was performed, as described previously (16) for 10 of the 11 mycobacterial isolates recovered from patients.

Assay for susceptibility to antimycobacterial drugs. The susceptibilities of the clinical isolates to antibiotics *in vitro* were determined by microdilution (33) using the BrothMIC NTM kit (Kyokuto Pharmaceutical Industrial Co. Ltd., Tokyo, Japan), with modification of the incubation temperature (32°C) and period (2 to 3 weeks). MIC testing was performed in triplicate on different days, with two of three matching MICs used as the criterion for MIC determination.

Nucleotide sequence accession numbers. The DNA sequences of the 16S rRNA (1,475-bp), *hsp65* (401-bp), *rpoB* (315-bp), and ITS (272-bp) fragments from the reference strains (*M. ulcerans* ITM 98-912, *M. ulcerans* ATCC 19423^T, *M. ulcerans* Agy99, *M. marinum* ATCC 927^T, *M. marinum* clinical isolate LRC 112509, and *M. pseudoshottsii* JCM 15466^T) and 10 clinical isolates have been deposited in the International Nucleotide Sequence Database (INSD) through the DDBJ under accession numbers AB548711 to AB548734 and AB624260 to AB624295.

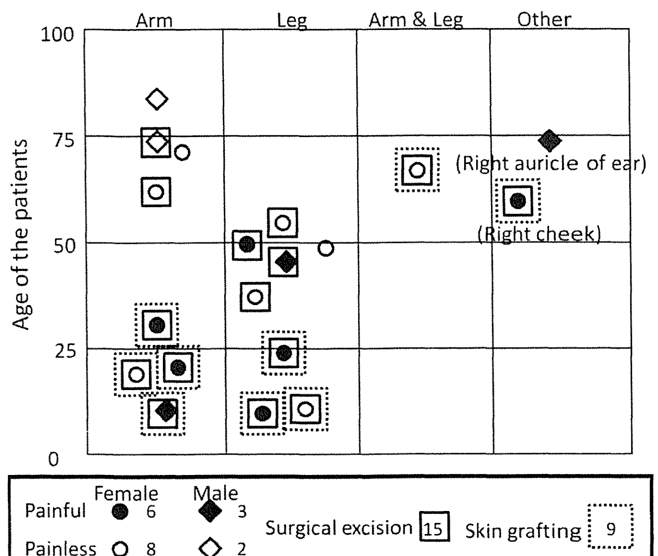

FIG. 4. Localization, pain, and surgical treatment of ulcer lesions by age and gender.

RESULTS

Epidemiology. Nineteen BU cases from Japan have been reported to the WHO BU committee as of December 2010. Many of the *M. ulcerans*-related reports of BU have originated in tropical wetlands. However, Japan is located in eastern Asia, and the majority of the country is covered by mountainous terrain. The 19 cases were distributed between latitudes 34°N and 38°N, in a typical temperate region of Japan.

There was no geographic focal point in the distribution of the BU cases. However, all of the patients lived on Honshu, the largest island of Japan. Seven cases were found in the Chugoku region (western Honshu), 6 in the Chubu region (central Honshu), 4 in the Kinki region (between Chugoku and Chubu), 1 in the Tohoku region (northern Honshu), and 1 in the Kanto region (eastern Honshu) (Fig. 1).

Fourteen (73.7%) subjects were female, and 5 (26.3%) were male. The average age was 39.1 years (range, 8 to 70 years) for the females and 56.8 years (range, 11 to 81 years) for the males (Fig. 2). Despite careful and precise patient interviews, none of the cases could be linked to an aquatic environment.

The affected areas were on exposed sites, such as arms (8

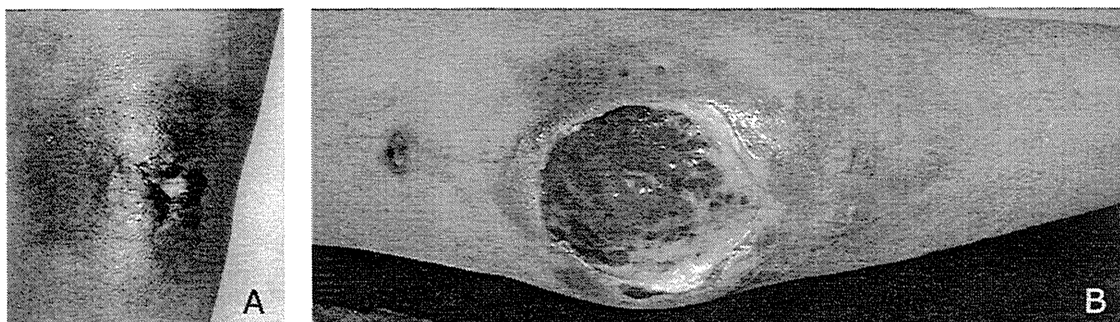

FIG. 3. (A) Buruli ulcer case 8: a category I ulcer on the right forearm. (B) Buruli ulcer case 3: a category II ulcer on the right elbow extensor surface.

TABLE 2. IS*2404* detection in 19 cases of BU in Japan

Case no.	Yr of diagnosis	Origin (region)	Sample type: Tissue sample[a]	Sample type: Paraffin section[b]	Sample type: Isolate	Isolation period[c]
1	1980	Chubu	NT	NT	P	4 wk
2	2004	Chubu	NT	NT	P	S
3	2006	Chugoku	P	P	P	11 wk
4	2005	Kinki	NT	NT	P	6 wk
5	2007	Chubu	P	P	P	8 wk
6	2007	Chubu	NT	NT	P	S
7	2007	Kinki	NT	NT	P	S
8	2008	Chubu	P	NT	P	11 mo
9	2008	Chugoku	P	NT	NT	NT
10	2009	Chugoku	P	NT	NT	NT
11	2009	Chugoku	P	P	NT	NT
12	2009	Chugoku	P	P	NT	NT
13	2009	Chugoku	NT	P	P	12 wk
14	2009	Tohoku	P	NT	NT	NT
15	2010	Kinki	P	NT	NT	6 wk
16	2010	Kanto	P	P	NT	NT
17	2010	Chubu	P	P	P	5 wk
18	2010	Kinki	P	P	NT	NT
19	2010	Chugoku	P	P	NT	NT

[a] Frozen or fresh skin biopsy sample. NT, not tested; P, positive.
[b] Sliced from a formalin-fixed, paraffin-embedded skin biopsy sample.
[c] S, isolation was successful, but the incubation period was uncertain.

cases), legs (8 cases), the right auricle of the ear (1 case), the right cheek (1 case), and both arms and legs (1 case). While skin ulcer lesions were present in all cases, most were smaller than 5 cm in diameter and were classified as category I (Fig. 3A) (36). In one severe case, the patient presented with a progressive ulcer larger than 10 cm in diameter on the extensor surface of the right elbow, which fell into category II (Fig. 3B). Nine patients (47%) experienced pain, although in many reported cases, BU is painless or only slightly painful (Fig. 4).

Genotypic analysis. PCR screening to detect IS*2404* gave a positive result for at least one of three sample types in all 19 cases. We should note that fresh tissue samples were the source of the template for 13 cases, while formalin-fixed, paraffin-embedded specimens were also used for 9 cases, and all were positive (Table 2). Mycobacteria were successfully isolated in 11 of the 19 cases; however, further bacteriological tests, including genotypic analysis, were performed on 10 available isolates.

The 16S rRNA gene sequences (1,475 bp) of these isolates were identical to each other but partially different from those of *M. ulcerans*, *M. marinum*, and *M. pseudoshottsii* (Table 3). The *hsp65* (401-bp), *rpoB* (315-bp), and internal transcribed spacer (ITS) (272-bp) sequences were also identical among isolates. Sequence analysis identified *M. ulcerans* subsp. *shinshuense* as the bacterium in the clinical samples. Phylogenetic trees based on 16S rRNA and *hsp65* gene sequences showed a close relationship between *M. ulcerans* subsp. *shinshuense* and *M. ulcerans* (Fig. 5A and B). A phylogenetic analysis of the 16S–23S intergenic spacer region showed no differences between *M. ulcerans* subsp. *shinshuense*, *M. marinum*, and *M. ulcerans* and found that *M. pseudoshottsii* is a close relative (Fig. 5C). In contrast, the tree based on the *rpoB* gene showed a closer relationship of *M. ulcerans* subsp. *shinshuense* to *M. marinum* and *M. pseudoshottsii* than to *M. ulcerans*, supporting the premise that *M. ulcerans* subsp. *shinshuense* is distinct from *M. ulcerans* (Fig. 5D).

Next, amplification of eight pMUM001-associated genes was used to determine whether these isolates had genes that encode toxic lipid mycolactone-producing enzymes. All isolates

TABLE 3. Comparison of 16S rRNA gene sequences of 10 *M. ulcerans* subsp. *shinshuense* isolates and related mycobacterial strains

Strain	Country	Nucleotide(s) at the following *Escherichia coli* 16S rRNA gene sequence position(s): 95	487–488	492	969	1007	1215	1247	1288	1449–1451[a]
M. ulcerans subsp. *shinshuense*										
ATCC 33728	Japan	T	GG	G	A	G	T	G	G	ACCC---TTTG
JATA753	Japan	T	GG	G	A	G	T	G	G	ACCC---TTTG
0401	Japan	T	GG	G	A	G	T	G	G	ACCC---TTTG
0501	Japan	T	GG	G	A	G	T	G	G	ACCC---TTTG
0701	Japan	T	GG	G	A	G	T	G	G	ACCC---TTTG
0702	Japan	T	GG	G	A	G	T	G	G	ACCC---TTTG
0703	Japan	T	GG	G	A	G	T	G	G	ACCC---TTTG
0801	Japan	T	GG	G	A	G	T	G	G	ACCC---TTTG
0901	Japan	T	GG	G	A	G	T	G	G	ACCC---TTTG
1001	Japan	T	GG	G	A	G	T	G	G	ACCC---TTTG
M. ulcerans										
ITM 98-912	China	T	GG	G	A	G	T	G	G	ACCC---TTTG
ATCC 19423^{T}	Australia	T	GG	A	A	G	T	G	C	ACCC---TTTG
Agy99	Ghana	T	GG	A	A	G	T	G	C	ACCCTTTTTTG
M. marinum										
ATCC 927^{T}	United States	T	GG	A	A	G	T	A	A	ACCC---TTTG
112509	Japan	T	GG	A	A	G	T	A	A	ACCC---TTTG
M. pseudoshottsii JCM 15466^{T}	United States	C	GA	A	G	T	C	A	A	ACCC---TTTG

[a] Hyphens indicate gaps.

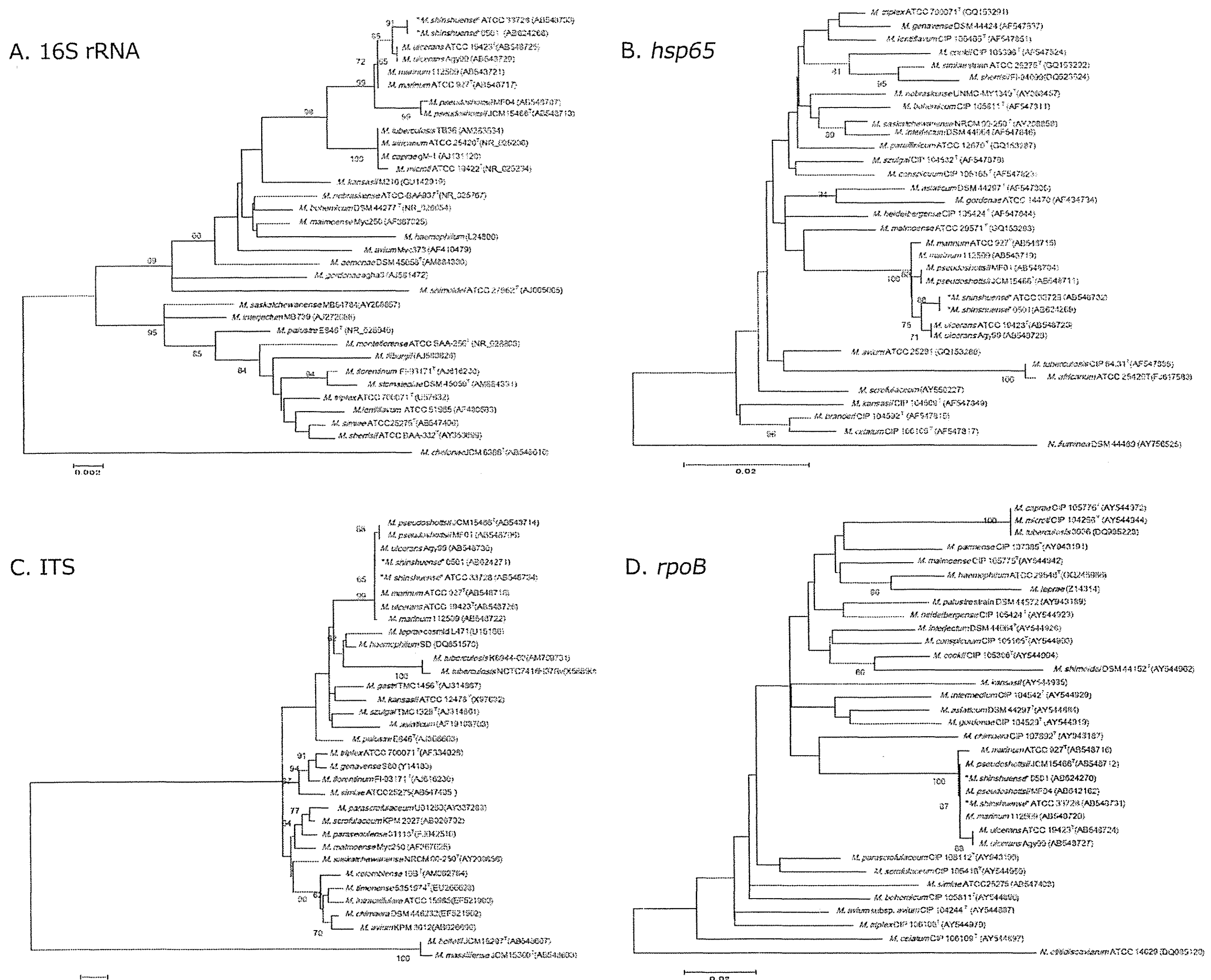

FIG. 5. Phylogenetic analyses of *M. ulcerans* subsp. *shinshuense* based on the 16S rRNA gene (A), the *hsp65* gene (B), the 16S–23S intergenic spacer region (C), and the *rpoB* gene (D).

showed positive results, but as previously reported, the band representing the serine/threonine protein kinase (STPK) gene was absent in *M. ulcerans* subsp. *shinshuense* strains (16). However, this phenomenon was also observed with one strain of *M. ulcerans*, ITM 98-912, that was isolated in China (4). All eight bands were detected in the *M. ulcerans* strains isolated from Australia and Ghana. *M. pseudoshottsii* lacked the band representing P450, but the other seven bands were successfully amplified (Table 4).

A commercially available DNA-DNA hybridization assay was used to verify species identity. The kit contained a reference panel of 18 mycobacterial strains that included *M. marinum* but not *M. ulcerans*, *M. ulcerans* subsp. *shinshuense*, or *M. pseudoshottsii*. All 10 isolates showed clear positive signals for *M. marinum* (Table 5, rightmost column).

Biochemical characteristics. The 10 isolates exhibited the same characteristics: rough colonies and yellow pigmentation, even when grown in the dark. The slowly growing mycobacterium formed visible colonies at 25°C and 32°C on a 2% Ogawa egg slant, but not at 37°C or 42°C. No growth was seen on a medium supplemented with 500 μg/ml *p*-nitrobenzoic acid or 5% NaCl. The isolates were negative for niacin, nitrate reduction, arylsulfatase (3 days), Tween 80 hydrolysis, pyrazinamidase, and iron uptake but were positive for semiquantitative catalase and 68°C catalase and urease. Comparisons between *M. ulcerans* subsp. *shinshuense*, *M. ulcerans*, and *M. marinum* are summarized in Table 5. These results were in accordance with those of a previous report (22) except for the positive result of *M. ulcerans* subsp. *shinshuense* on the urease test.

Drug susceptibility assays. Table 6 shows the results of testing of the susceptibilities of *M. ulcerans* subsp. *shinshuense* ATCC 33728 and *M. ulcerans* subsp. *shinshuense* clinical isolate 0501 to antimicrobial agents. These isolates exhibited high susceptibilities to streptomycin, kanamycin, levofloxacin, and clarithromycin. Notably, *M. ulcerans* subsp. *shinshuense* was more susceptible to streptomycin, kanamycin, and clarithromy-

TABLE 4. PCR detection of eight pMUM001-associated genes in 10 *M. ulcerans* subsp. *shinshuense* isolates and related mycobacterial strains

Strain	Country	Presence or absence of the following pMUM001 marker gene[a]:							
		repA	*parA*	STPK	*mls* (load)	*mlsAT(II)*	TEII	KSIII	P450
M. ulcerans subsp. *shinshuense*									
ATCC 33728	Japan	+	+	−	+	+	+	+	+
JATA753	Japan	+	+	−	+	+	+	+	+
0401	Japan	+	+	−	+	+	+	+	+
0501	Japan	+	+	−	+	+	+	+	+
0701	Japan	+	+	−	+	+	+	+	+
0702	Japan	+	+	−	+	+	+	+	+
0703	Japan	+	+	−	+	+	+	+	+
0801	Japan	+	+	−	+	+	+	+	+
0901	Japan	+	+	−	+	+	+	+	+
1001	Japan	+	+	−	+	+	+	+	+
M. ulcerans									
ITM 98-912	China	+	+	−	+	+	+	+	+
ATCC 19423[T]	Australia	+	+	+	+	+	+	+	+
Agy99	Ghana	+	+	+	+	+	+	+	+
M. pseudoshottsii JCM 15466[T]	United States	+	+	+	+	+	+	+	−

[a] +, present; −, absent. STPK, serine/threonine protein kinase; TEII, type II thioesterase; KSIII, type III ketosynthase.

cin than the *M. ulcerans* reference strains. Like the *M. ulcerans* reference strains, *M. ulcerans* subsp. *shinshuense* was susceptible to amikacin but resistant to ethambutol, isoniazid, and ethionamide.

Treatment. The 19 patients were treated with various antibiotic regimens. Clarithromycin was effective for many of the Japanese patients (12 cases). Rifampin was successful in the first case and was used thereafter in 9 cases. Attempts at treatment with other medications, alone and in combinations, were also made (Table 7). In 2 cases, the initial choice of antibiotics was ineffective, and they were changed. In 2 other cases, the antibiotic treatment was discontinued due to adverse effects. In addition to antibiotic treatment, 13 patients underwent surgical excision, and 9 needed skin grafting (Fig. 4). No relapses had been reported as of March 2011.

DISCUSSION

This is the first report that comprehensively analyzes both the genotypic and the biochemical profiles of a causative agent of Buruli ulcer in Japan. It is noteworthy that BU in Japan was induced by *Mycobacterium ulcerans* subsp. *shinshuense*, not by *M. ulcerans*. We compared certain characteristics of *M. ulcerans* and *M. ulcerans* subsp. *shinshuense* by several analyses. They are relatively similar; detection of IS*2404* by PCR was the most important test for early diagnosis and differential diag-

TABLE 5. Bacteriological characteristics of 10 *M. ulcerans* subsp. *shinshuense* isolates and closely related mycobacterial strains

Strain	Country	Biochemical characteristic							Identification of *M. marinum*[b]
		Growth rate	Colony morphology	Pigment in dark	Urease activity	Tween 80 hydrolysis	PZase[a] activity	MPB64 production	
M. ulcerans subsp. *shinshuense*									
ATCC 33728	Japan	Low	Rough	Yellow	+	−	−	−	+
JATA753	Japan	Low	Rough	Yellow	+	−	−	−	+
0401	Japan	Low	Rough	Yellow	+	−	−	−	+
0501	Japan	Low	Rough	Yellow	+	−	−	−	+
0701	Japan	Low	Rough	Yellow	+	−	−	−	+
0702	Japan	Low	Rough	Yellow	+	−	−	−	+
0703	Japan	Low	Rough	Yellow	+	−	−	−	+
0801	Japan	Low	Rough	Yellow	+	−	−	−	+
0901	Japan	Low	Rough	Yellow	+	−	−	−	+
1001	Japan	Low	Rough	Yellow	+	−	−	−	+
M. ulcerans									
ITM 98-912	China	Low	Rough	Yellow	+	−	−	−	+
ATCC 19423[T]	Australia	Low	Rough	None	−	−	−	−	+
Agy99	Ghana	Low	Rough	Yellow	−	−	−	−	+
M. marinum ATCC 927[T]	United States	Medium	Smooth	None	+	+	+	−	+

[a] PZase, pyrazinamidase.
[b] By use of the DDH Mycobacteria kit (Kyokuto Pharmaceutical Industrial, Tokyo, Japan).

TABLE 6. Drug susceptibility test results

Antimycobacterial drug[a]	MIC (μg/ml) for:			
	M. ulcerans subsp. *shinshuense*		*M. ulcerans*	
	ATCC 33728	0501	ATCC 19423^T	Agy99
SM	0.125	0.25	1	4
EB	16	8	16	128
KM	0.25	0.25	1	1
INH	8	8	>32	>32
RFP	0.06	0.06	0.06	0.06
LVFX	0.25	0.5	0.5	8
CAM	0.03	0.06	0.25	0.125
TH	16	8	16	16
AMK	0.5	0.5	0.5	0.5

[a] SM, streptomycin; EB, ethambutol; KM, kanamycin; INH, isoniazid; RFP, rifampin; LVFX, levofloxacin; CAM, clarithromycin; TH, ethionamide; AMK, amikacin.

TABLE 7. Antibiotic treatment regimens for BU cases

Regimen[a]	No. of cases
Single drug	
CAM	2
MINO	1
RFP	1
Two drugs	
CAM, RFP	2
ITZ, MINO	1
LVFX, MINO	1
Three drugs	
CAM, LVFX, RFP	3
CAM, CFPN-PI, NFLX	1
CFPN-PI, LVFX, MINO	1
CAM, MINO, NFLX	1
GRNX, LVFX, MINO	1
Four drugs (EB, LVFX, RFP, SM)	1
Six drugs	
AZM, CAM, CPFX, LVFX, MINO, RFP	1
CAM, EB, GFLX, INH, RFP, SM	1
CAM, CPFX, LVFX, MINO, PZFX, RFP	1

[a] AZM, azithromycin; CAM, clarithromycin; CFPN-PI, cefcapene-pivoxil; CPFX, ciprofloxacin; EB, ethambutol; GFLX, gatifloxacin; GRNX, garenoxacin; INH, isoniazid; ITZ, itraconazole; LVFX, levofloxacin; MINO, minocycline; NFLX, norfloxacin; RFP, rifampin; SM, streptomycin; PZFX, pazufloxacin.

nosis for distinguishing both *M. ulcerans* subsp. *shinshuense* and *M. ulcerans* infections from *M. marinum* infection. Although the DDH Mycobacteria kit could not distinguish *M. ulcerans* and *M. ulcerans* subsp. *shinshuense* from *M. marinum* (Table 5), simultaneous detection of IS*2404* would prevent misidentification. IS*2404* was well amplified from clinical samples and/or isolates in all 19 cases (Table 2). The 16S rRNA gene sequences of *M. ulcerans* subsp. *shinshuense* and *M. ulcerans* are similar, but conserved sites that were different in *M. ulcerans* subsp. *shinshuense* versus *M. ulcerans* were seen (Table 3); these matched perfectly with the sequences reported by Portaels et al. (20) and subsequently found to be useful in discrimination (6, 16). PCR targeting of pMUM001 revealed that all *M. ulcerans* subsp. *shinshuense* isolates lack the band representing the STPK gene, suggesting a small but conservative mutation(s) in *M. ulcerans* subsp. *shinshuense* versus *M. ulcerans* sequences. This PCR test was also applied for detection of a virulent plasmid and for differential diagnosis of *M. ulcerans* versus *M. ulcerans* subsp. *shinshuense* (16). The DNA sequence of the ITS region and the 16S rRNA and *hsp65* genes showed similarity between the *M. ulcerans* subsp. *shinshuense* isolates and *M. ulcerans*. However, the *rpoB* gene showed more similarity to *M. marinum* and *M. pseudoshottsii* than to *M. ulcerans* (Fig. 5). These data were suggestive of the evolutionary paths of these related mycobacterial species (9).

It is noteworthy that *M. ulcerans* subsp. *shinshuense* was identified in all of the isolates from Japanese patients diagnosed with BU. *M. ulcerans* subsp. *shinshuense*, not *M. ulcerans*, could be the primary etiological agent of BU in eastern Asia. It has been reported that the STPK gene was not amplified from the isolate of a BU patient in China (26). While there might be a taxonomical reason, this isolate was finally classified as *M. ulcerans* (4). A more precise genotypic examination might have revealed this to be a case of *M. ulcerans* subsp. *shinshuense* infection. If so, this finding would suggest that *M. ulcerans* subsp. *shinshuense* is distributed not only in Japan, but also in other areas of eastern Asia. Thorough field work and increased vigilance on the part of dermatologists and physicians are needed to determine the predominant cause of BU in eastern Asia. Because disease severity and susceptibility to antibacterial drugs are significantly different for *M. ulcerans* versus *M. ulcerans* subsp. *shinshuense*, they must be identified and distinguished in clinical settings.

The Japanese *M. ulcerans* subsp. *shinshuense* isolates and the Chinese strain of *M. ulcerans* presumably belong to the same cluster, based on genetic analyses such as microarray-based comparative genomic hybridization (9) and comparative sequence analysis of polymorphic variable-number tandem repeats (VNTR) (27). Their genomes were distinctly different from those of *M. ulcerans* strains that originated in other geographic regions. However, one of the VNTR loci can be used to distinguish between the Chinese and Japanese strains (1). Pidot et al. described the clear difference between the two strains by analyzing virulent plasmid genes and the resulting mycolactone production, noting that the Japanese strain produces mycolactone A/B, while the Chinese strain produces a unique mycolactone D (19). Further study is needed to elucidate the evolution and distribution of *M. ulcerans*, and its relation to *M. ulcerans* subsp. *shinshuense*, in Asia.

It is notable that most of the biochemical characteristics (Table 5) and drug susceptibilities (Table 6) of the isolates were the same as those found in a previous report (22), with the exception of the urease test. Interestingly, the Japanese *M. ulcerans* subsp. *shinshuense* isolates, the Chinese strain of *M. ulcerans*, and the related species *M. marinum* were all urease positive, though other strains of *M. ulcerans* originating from Ghana and Australia were urease negative. The urease test is a simple method with clear results that would be useful in distinguishing between *M. ulcerans* and *M. ulcerans* subsp. *shinshuense*.

Clinical manifestation of BU in Japan was essentially similar to that of BU in other countries, but distinct differences in management were observed. Ulcerated areas were usually smaller for Japanese (Fig. 3) than for African patients; how-