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 IS2404 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 IS2404, 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 IS2404 (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 GCCCATTGGTGCTCGGTCA	IS2404 (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	<i>rpoB</i> (341)	11
TB11 TB12	ACCAACGATGGTGTGTCCAT CTTGTCGAACCGCATACCCT	<i>hsp65</i> (441)	30
RepAF RepAR	CTACGAGCTGGTCAGCAATG ATCGACGCTCGCTACTTCTG	<i>repA</i> in pMUM001 (413)	26
ParAF ParAR	GCAAGCTGGGCAATGTTTAT GTCCGGTCCTTGATAGGTCA	<i>parA</i> in pMUM001 (501)	26
MUP11F MUP11R	ACCACCCAAGAGTGGAAGT TGTCGTGTCGAGGTATGTGG	Serine/threonine protein kinase in pMUM001 (479)	26
MLSloadF MLSloadR	GGGCAATCGTCCTCACTG CAAGGGCAGTCTTGATTAGG	<i>mls</i> load in pMUM001 (560)	26
MLSAT(II)F MLSAT(II)R	AACGTTGAATCCCCTTTTGT GCACCACAAAGGAACGTCTAA	<i>mlsAT(II)</i> 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.

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 pseudoshottii* 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. pseudoshottii* 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. pseudoshottii*.

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. pseudoshottii* 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.

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. IS2404 detection in 19 cases of BU in Japan

Case no.	Yr of diagnosis	Origin (region)	Sample type			Isolation period ^c
			Tissue sample ^a	Paraffin section ^b	Isolate	
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 IS2404 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 <i>Escherichia coli</i> 16S rRNA gene sequence position(s):								
		95	487–488	492	969	1007	1215	1247	1288	1449–1451 ^a
<i>M. ulcerans</i> subsp. <i>shinshuense</i>										
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
<i>M. ulcerans</i>										
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
<i>M. marinum</i>										
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
<i>M. pseudoshottsii</i>										
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 mycobacte-

rium 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 :							
		<i>repA</i>	<i>parA</i>	STPK	<i>mls</i> (load)	<i>mlsAT(II)</i>	TEII	KSIII	P450
<i>M. ulcerans</i> subsp. <i>shinshuense</i>									
ATCC 33728	Japan	+	+	—	+	+	+	+	+
JATA753	Japan	+	+	—	+	+	+	+	+
0401	Japan	+	+	—	+	+	+	+	+
0501	Japan	+	+	—	+	+	+	+	+
0701	Japan	+	+	—	+	+	+	+	+
0702	Japan	+	+	—	+	+	+	+	+
0703	Japan	+	+	—	+	+	+	+	+
0801	Japan	+	+	—	+	+	+	+	+
0901	Japan	+	+	—	+	+	+	+	+
1001	Japan	+	+	—	+	+	+	+	+
<i>M. ulcerans</i>									
ITM 98-912	China	+	+	—	+	+	+	+	+
ATCC 19423 ^T	Australia	+	+	+	+	+	+	+	+
Agy99	Ghana	+	+	+	+	+	+	+	+
<i>M. pseudoshottsii</i> 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 under-

went 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 IS2404 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 <i>M. marinum</i> ^b
		Growth rate	Colony morphology	Pigment in dark	Urease activity	Tween 80 hydrolysis	PZase ^a activity	MPB64 production	
<i>M. ulcerans</i> subsp. <i>shinshuense</i>									
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	+	—	—	—	+
<i>M. ulcerans</i>									
ITM 98-912	China	Low	Rough	Yellow	+	—	—	—	+
ATCC 19423 ^T	Australia	Low	Rough	None	—	—	—	—	+
Agy99	Ghana	Low	Rough	Yellow	—	—	—	—	+
<i>M. marinum</i> 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:			
	<i>M. ulcerans</i> subsp. <i>shinshuense</i>		<i>M. ulcerans</i>	
	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.

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 IS2404 would prevent misidentification. IS2404 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*

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).....	
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.

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-

ever, the Japanese patients received both surgery and a large array of antimycobacterial drugs (Table 7). In addition, in Africa, most patients who had lesions with cross-sectional diameters of ≤ 10 cm showed excellent healing without surgery (17). Although the *in vitro* susceptibilities of the Japanese isolates to streptomycin, kanamycin, and clarithromycin are higher than those of the *M. ulcerans* strains from West Africa (Table 6), treatment has been fairly aggressive in Japan. It is speculated that because the majority of doctors and patients in Japan have not experienced and cannot recognize Buruli ulcer disease, they might fear the progression and recurrence of disease. Especially when patients complain of pain (9 patients in this study [47%] experienced pain [Fig. 4]), their doctors and family members are willing to initiate aggressive treatment, even in the absence of an immunodeficiency risk factor. Public information campaigns about the disease are needed, as is the establishment of guidelines for the treatment of Buruli ulcer in Japan. Clarification of the mode of transmission is also important. However, the occurrence of cases has been very sporadic, and none could be linked to an aquatic environment. Thus, the source and route of the infection remain unclear.

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