Buruli Ulcer in Japan

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1 Epidemiology and Bacteriological and Genomic Features of *M. ulcerans* subsp. *shinshuense*

1.1 Epidemiology

Japan is one of the few countries located in a temperate zone that reports cases of Buruli ulcer (BU). The first case, a 19-year-old female who presented with a chronic and necrotic ulcer on her left elbow, was reported by Mikoshiba et al. in 1982 [1]. This was considered to be an endemic infection due to the lack of a travel history outside the country. A taxonomic study using DNA hybridization assays was later performed on a mycobacterial strain (ATCC 33788) isolated from the skin ulcer lesion of this patient, revealing that this Japanese strain was highly similar to the classical *Mycobacterium ulcerans* strain ATCC 19423. However, the Japanese strain differed from the previously described *M. ulcerans* strain in mycolic acid

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composition and some biological and biochemical characteristics; therefore, it was deemed to belong to a new subspecies of *M. ulcerans* named *M. ulcerans* subsp. *shinshuense* [2]. The disease was not noticed again in Japan until the second case of BU was identified and reported in 2003 [3]. Since then, the number of cases has increased gradually, summing to a total of 60 cases reported from 17 of the country's 47 prefectures till the end of 2016. *M. ulcerans* subsp. *shinshuense* has been isolated from 41 of the cases [4–7].

BU cases are rather sporadically distributed throughout the main and largest island of Japan, Honshu Island, with no particular focus [6, 8]. The Okayama Prefecture, which is localized in the central western regions of Japan facing the Seto Sea, has so far reported most of the cases (ten [17%] cases), followed by the Tottori Prefecture (six [10%] cases) and the Shiga Prefecture (seven [12%] cases). The Akita Prefecture is the most northern prefecture reporting BU cases, where the temperature can be below zero degrees Celsius during the coldest season of the year. All reported cases in Japan are considered to be domestic infections as none of the patients had a history of oversea travel to BU endemic countries before symptom onset. The acquisition of the pathogen from a suspicious aquatic environment remains undetermined in most cases. However, it is interesting to note that most BU cases in Japan were diagnosed in autumn and winter (48 cases), which may indicate a higher incidence of infection during summer considering the expected time lag between infection and symptom onset [9].

1.2   Genome

M. ulcerans and related mycobacterial strains (such as *M. liflandii*) share a characteristic genome structure consisting of a chromosome and a giant plasmid. This genome structure caused some difficulty when analyzing the whole genome sequences. Recently, with the help of next-generation sequencing (NGS), the complete genome sequences of two representative isolates, *M. ulcerans* Agy99 and *M. liflandii* 128FXT, were unraveled [10–12]. The complete genome sequence of *M. ulcerans* subsp. *shinshuense* (ATCC 33728) was also reported by a Japanese group [13]. Comparison of the whole genome of *M. ulcerans* subsp. *shinshuense* with those of the related strains revealed that it bears more similarity with *M. liflandii* 128FXT than with *M. ulcerans* Agy99 (Table 1). *M. ulcerans* subsp. *shinshuense* has a 5,899,681 bp chromosome with 65.64% GC content and a 166,617 bp giant plasmid with 62.76% GC content. The average nucleotide identities were 98.36% to *M. ulcerans* Agy99 and 99.10% to *M. liflandii* 128FXT [13]. The total number of coding DNA sequences (CDSs) in both *M. ulcerans* subsp. *shinshuense* and *M. liflandii* 128FXT were found to be roughly around 5000, approximately 800 CDSs more than in the *M. ulcerans* Agy99 chromosome (Table 1) [10–13]. With respect to insertion sequences (IS), IS*2606* was much more abundant in *M. ulcerans* Agy99 than in *M. ulcerans* subsp. *shinshuense* and *M. liflandii* 128FXT (Table 1) [10–13]. In contrast, more than 200 copies of IS*2404* were observed in *M. ulcerans* subsp. *shinshuense,* which share approximately 99% identity with the classical *M.*

Table 1 Genomic characteristics of *M. ulcerans* subsp. *shinshuense* and related mycobacteria

Characteristics	*M. ulcerans* subsp. *shinshuense* ATCC 33728	*M. ulcerans* Agy99	*M. liflandii* 128FXT
Chromosome			
Size (bp)	5,899,681	5,631,606	6,208,955
G + C (%)	65.64	65.47	65.62
No. of CDSs	5015	4160	4994
No. of pseudogenes	451	771	436
No. of IS*2404*	206	209	224
No. of IS*2606*	1	83	1
Plasmid			
Size (bp)	166,617	174,155	190,588
G + C (%)	62.76	62.5	62.9
No. of CDSs	72	72	95
No. of pseudogenes	7	7	22
No. of IS*2404*	4	4	15
No. of IS*2606*	1	8	3
References	[7], Yoshida et al. (unpublished)	[8]	[9, 10]
GenBank accession no.	AP017624 AP017625	CP000325 BX649209	NC_020133 NC_011355

CDS coding DNA sequence, *IS* insertion sequence

Table 2 Nuclotide substitutions observed in 16S rRNA sequences from *M. ulcerans* subsp. *shinshuense* and related mycobacteria

Species	Differentiating sequence position (underline)		GenBank accession no.
	492	1288	
M. ulcerans subsp. *shinshuense* ATCC 33728	TGG**G**GAA	TAA**G**GCC	AB548733
M. ulcerans Agy99	TGG**A**GAA	TAA**C**GCC	AB548729
M. liflandii 128FXT	TGG**A**GAA	TAA**A**GCC	CP003899
M. marinum ATCC 927	TGG**A**GAA	TAA**A**GCC	AB548717
M. pseudoshottsii JCM 15466	TGG**A**GAA	TAA**A**GCC	AB548713

ulcerans strains and *M. liflandii* [4, 7, 13]. Due to the abundance of IS*2404*, PCR amplification of a partial IS*2404* sequence is currently used for sensitive detection of *M. ulcerans* subsp. *shinshuense*, although differentiation from other closely-related IS*2404*-harboring species requires further investigation. The 16S rRNA sequences in *M. ulcerans* subsp. *shinshuense* were almost identical to those in *M. ulcerans* Agy99 (Table 2), except for two characteristic nucleotide substitutions at base positions 492 and 1288 found exclusively in *M. ulcerans* subsp. *shinshuense* (Table 2) [4, 7]. Including the listed representative isolate ATCC 33728 (Table 2), the same nucleotide substitutions have been found in ten clinical isolates of *M. ulcerans* subsp. *shinshuense* [7].

Comparison of eight pMUM001 gene sequences encoding lipid toxin mycolactone-producing enzymes (including *repA*, *parA*, serine/threonine protein kinase [STPK] gene, loading domain of *mls*, acyltransferase domain of *mls*, *rep* type II thioesterase gene, *rep* type III ketosynthase gene, and *rep* P450 hydroxylase gene) in the giant plasmid of *M. ulcerans* subsp. *shinshuense* and the African *M. ulcerans* strain by PCR amplification revealed the presence of seven pMUM001 gene sequences and the loss of the STPK gene in Japanese isolates [4, 7]. The elucidation of the complete genome sequence of *M. ulcerans* subsp. *shinshuense* further verified the above finding by showing that the STPK gene in *M. ulcerans* subsp. *shinshuense* is truncated, with low similarity to STPK in *M. ulcerans* Agy99 [13]. Despite the deficiency of a functional STPK gene in Japanese clinical isolates, no apparent bacteriological differences were observed, indicating the possibility that some signaling pathways compensate for STPK function in *M. ulcerans* subsp. *shinshuense* [14]. Intriguingly, a Chinese *M. ulcerans* strain has a 16S rRNA gene identical in sequence to that in *M. ulcerans* subsp. *shinshuense*, and also lacks the STPK gene in its giant plasmid like *M. ulcerans* subsp. *shinshuense* [4, 7]. This finding supports the suggestion that the Chinese strain is phylogenetically closest to *M. ulcerans* subsp. *shinshuense* among other *M. ulcerans* strains [15]. However, further characterization of the whole genome sequence of the Chinese strain is required for its classification.

The close resemblance, in addition to distinctive genomic features, between the classical *M. ulcerans* strains and the Japanese strain *M. ulcerans* subsp. *shinshuense* has evoked great interest in the intra-species evolutionary scenario for *M. ulcerans*. Phylogenetic analysis of irreversible genomic changes focusing on insertion-deletion polymorphisms in 12 regions of difference among *M. ulcerans* strains has unambiguously resolved a phylogenetic tree showing that *M. ulcerans* has evolved into at least two distinct lineages since divergence from the *M. marinum* progenitor [15]. In addition to *M. ulcerans* subsp. *shinshuense*, strains from China, South America, and Mexico were shown to belong to a lineage that is more closely related to *M. marinum*, namely the 'ancestral' lineage; while strains from Africa and Australia belong to the 'classical' lineage that has undergone major genomic rearrangement [15]. In agreement with this evolutionary scenario, 26,564 single nucleotide polymorphisms (SNPs) were found in a Japanese strain by a comparison with the reference genome of the classical lineage isolate Agy99 using a high-resolution phylogeny analysis based on genome-wide SNPs [16]. Moreover, calculation of time scales of the evolutionary process by the minimum evolution tree-based approach estimated that the divergence of Ghanaian subtypes (including Agy99) from the *M. marinum* progenitor occurred about 1000–3000 years ago, whereas *M. ulcerans* subsp. *shinshuense* diverged much earlier (about 394,000–529,000 years ago) [16].

1.3   Biochemical Properties

Mycolactone, secreted by *M. ulcerans* in the process of chronic infection, is an essential molecule for the virulence of BU [17]. Mycolactone is also secreted by strains originally designated *M. pseudoshottsii*, *M. liflandii*, and a subset of *M. marinum* strains [18–20]. *M. ulcerans* subsp. *shinshuense* produces mycolactone A/B, among
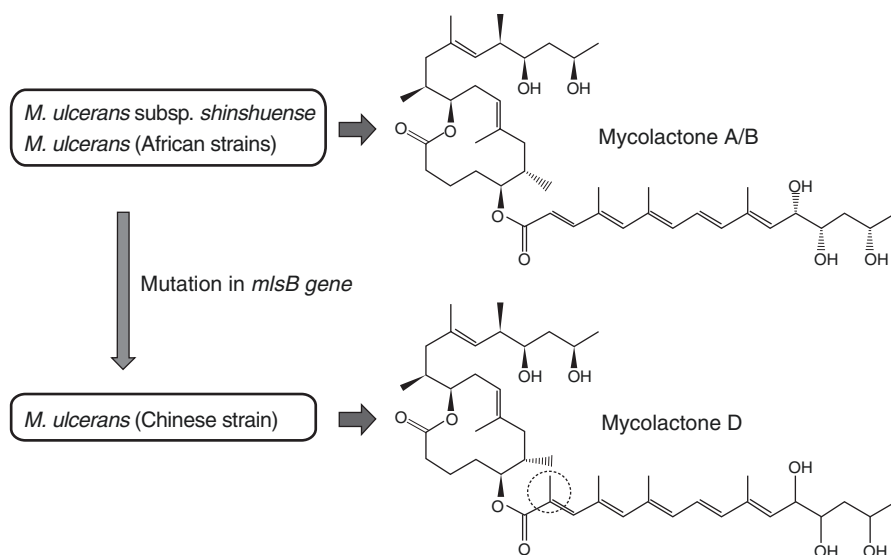
Fig. 1 Structural variation in mycolactones caused by a *mlsB* mutation uniquely found in a Chinese *M. ulcerans* strain. The dashed circle in mycolactone D represents the methyl group that distinctively differentiates it from mycolactone A/B produced by *M. ulcerans* subsp. *shinshuense* and African strains of *M. ulcerans*

five analogs of mycolactone, which was originally found in African *M. ulcerans* strains [12, 17, 18] (Fig. 1). In contrast, the Chinese strain of *M. ulcerans* produces a unique mycolactone D that is not observed in other *M. ulcerans* strains [21]. Mycolactone D possesses an additional methyl group in its acyl side chain, whereas the mycolactone A/B conserves an intact form (Fig. 1) [12, 17, 18, 21]. In the Chinese strain, nonsynonymous substitutions that could cause drastic functional changes have been found in the *mlsB* gene involved in synthesis of acyl side chains, while corresponding mutations were not observed in Japanese *M. ulcerans* subsp. *shinshuense* or the African strains, suggesting that the Chinese strain might have uniquely acquired the ability to modify mycolactone A/B to D through genetic alternations [12].

A variety of glycolipids are present in the mycobacterial cell wall that likely functions as a protective barrier from host immune attack [22, 23]. Mycolate is a type of long chain fatty acid covalently linked to various glycolipids as a major component in the mycobacterial cell wall [22, 23]. Mycobacteria produce three main types of mycolate: alpha-, keto- (which contains additional ketone groups), and methoxy-mycolate (which contains additional methoxy groups) [22, 23]. Tsukamura et al. reported that both *M. ulcerans* subsp. *shinshuense* and other *M. ulcerans* strains share the three types of mycolate, while the average number of carbons in mycolate in *M. ulcerans* subsp. *shinshuense* is higher than that in an Australian *M. ulcerans* strain (ATCC 19423) [2]. However, comprehensive comparison is needed to determine whether the distribution of the three mycolate types differs between *M. ulcerans* subsp. *shinshuense* and other *M. ulcerans* strains, and to unravel their implications in mycobacterial virulence.

As one major glycolipid component in the mycobacterial cell wall, phenolic glycolipids (PGLs) were found in *M. leprae*, *M. tuberculosis*, *M. marinum*, and *M. ulcerans* [24–26]. PGLs vary in their compositions of sugar moieties (including rhamnose, fucose, and glucose), which are highly species-specific [24–26]. Daffe et al. found that 10% of *M. ulcerans* strains produced PGL consisting of monosaccharide (3-*O*-methyl-rhamnose), supporting a close phylogenetic link between *M. ulcerans* and *M. marinum* [27]. However, the rest of the tested strains, including *M. ulcerans* subsp. *shinshuense*, did not produce PGL [27]. In *M. leprae,* PGL is known to induce a strong antibody response and to play a role in invasion into Schwann cells [28, 29]. In *M. tuberculosis*, clinical isolates have been divided into PGL-producing and non-producing groups, and the former is regarded as more virulent, suggesting that PGL plays a role in the pathogenicity of mycobacteria [30]. This suggests that PGL-producing *M. ulcerans* subsp. *shinshuense* strains may be found, albeit less frequently. Analysis of PGL in *M. ulcerans* subsp. *shinshuense* as well as in other *M. ulcerans* strains with reference to the clinical symptoms in each case might offer a new approach to understand the causes of invasive BU cases.

Drug susceptibility tests showed that *M. ulcerans* subsp. *shinshuense* strains were more sensitive to streptomycin (SM), kanamycin (KM), and clarithromycin (CAM) than reference *M. ulcerans* strains (ATCC 19423 and Agy99) [7]. Differing from other antimicrobial drugs, such as rifampicin (RFP) and levofloxacin (LVFX), in the mechanism of action, binding to ribosomal RNA is exclusively required for SM, KM, and CAM to exert their actions to inhibit mycobacterial protein synthesis. The specific nucleotide substitutions found in the 16S rRNA of *M. ulcerans* subsp. *shinshuense* might have affected the conformation of its transcribed ribosomal structure, rendering it more accessible to the drugs [4, 7].

M. ulcerans subsp. *shinshuense* strains and the Chinese strain were all positive for urease activity, whereas the *M. ulcerans* strains from Africa and Australia were negative [7]. This feature may allow scientists to distinguish *M. ulcerans* subsp. *shinshuense* from *M. ulcerans* strains by a simple urease activity test. It has been shown in some mycobacterial species that urease plays a key role in metabolizing urea as a sole nitrogen source under nutrition-limited conditions and in neutralizing the surrounding milieu to inhibit phagolysosomal maturation [31, 32], indicating that urease-positive strains may be more adapted to harsh environments and more capable of achieving intracellular survival. Further comparisons between urease-positive strains with urease-deficient mutants might clarify whether urease activity contributes to the persistence of *M. ulcerans* subsp. *shinshuense* in the host.

2 Clinical Features and Treatment of BU in Japan

2.1 Antimicrobial Treatment

In the BU cases diagnosed in Japan, skin lesions mostly developed on exposed body parts including upper and lower limbs, face, ear, jaw, and clavicle. Although multiple lesions were present in 14 cases, no single lesion larger than 5 cm in diameter

was reported. Therefore, all cases fall into World Health Organization (WHO) category I or II. Advanced BU infection has not been seen in Japan. In contrast to the general impression that BU lesions are typically painless, approximately half of the cases confirmed to be infected by *M. ulcerans* subsp. *shinshuense* have reported pain [5, 8].

In Japan, a variety of antimicrobial drugs have been used to treat BU; e.g., RFP, minocycline (MINO), CAM, LVFX, gatifloxacin (GFXN), norfloxacin (NFX), ethambutol (EB), isoniazid (INH), ethionamide (ETH), and tosufloxacin (TFLX). However, surgical intervention was often unavoidable. Nakanaga et al. tested the drug susceptibilities of *M. ulcerans* subsp. *shinshuense* to major antimicrobial agents and found that it is most susceptible to RFP and CAM, while resistant to EB, ETH, and INH (Table 3) [7]. LVFX, a broad-spectrum fluoroquinolone antibiotic, which also showed satisfactory activity against *M. ulcerans* and *M. ulcerans* subsp. *shinshuense* (Table 3) [7], has been preferentially used for treating skin infections in Japan due to its better transferability to soft tissue and skin. Based on these reasons and in reference to WHO BU guidelines, a triple antimicrobial therapy with RFP (450 mg/day), LVFX (500 mg/day), and CAM (800 mg/day), called RLC therapy, has been recommended and has increased cure rates of BU without surgery (Table 4).

In our latest investigation on the efficacy of BU treatment in Japan, 50 cases (83% of the total reported cases) consisting of 19 male and 31 female patients, aged from 2 to 88 years (average: 45.8 ± 27.63 years) were included. Ten cases were excluded because of the absence of detailed information for treatment. These 50 BU cases were divided in two groups based on their antimicrobial regimens. One group (21 cases) received high-dose RLC (CAM at 800 mg/day). The other 29 cases received other treatments including low-dose RLC (CAM at 400 mg/day) or monotherapy of RFP, CAM or MINO; this group also included cases with interrupted RLC due to side effects and subsequent alternative chemotherapies. Five children received TFLX (12 mg/kg/day) substituted for LVFX, as TFLX (but not LVFX) is covered by medical insurance for children in Japan.

Table 3 Susceptibility testing results of *M. ulcerans* subsp. *shinshuense*

	MIC (µg/mL)	
	M. ulcerans subsp. *shinshuense*	
Antimicrobial agent	ATCC33728	501
SM	0.125	0.25
EB	16	8
KM	0.25	0.25
INH	8	8
RFP	0.06	0.06
LVFX	0.25	0.5
CAM	0.03	0.06
ETH	16	8
AMK	0.5	0.5

AMK amoxicillin, *CAM* clarithromycin, *EB* ethambutol, *ETH* ethionamide, *INH* isoniazid, *LVFX* levofloxacin, *KM* kanamycin, *MIC* minimal inhibitory concentration, *RFP* rifampicin, *SM* streptomycin

Table 4 Efficacy analysis of treatment for BU in Japan

Treatment	No.	Age	Sex (M/F)	Category (I/II)	Ulcer size (mm)	Cured completely without surgery
RLC (high dose)	21	42.1 ± 30.2	11/10	18/3	25.6 ± 23.8	15
Others	29	48.4 ± 26.3	8/21	24/5	27.3 ± 15.7	4
P value		0.43	0.087	1.0	0.79	6.95×10^{-5}

BU Buruli ulcer, *RLC* triple therapy with rifampicin (450 mg/day), levofloxacin (500 mg/day), and clarithromycin (800 mg/day)

Fifteen/twenty-one (71.4%) high-dose RLC-treated patients compared to 4/29 (13.7%) of the other patients had avoided surgery ($P < 0.0001$, Fisher's exact test). The distribution of age, sex, size of skin ulcer, or WHO category did not significantly differ for patients treated with high-dose RLC therapy and the others. Together, these results suggest that high-dose RLC therapy has been most effective among all chemotherapies (Table 4), which is in agreement with a previous investigation that included 40 BU cases in Japan by June 2013 [8]. The duration of high-dose RLC therapy ranged from 8 to 48 weeks (24 weeks in most cases), which was compared to the standard 8-week antibiotic treatment. The failure to determine a significant correlation between the duration of high-dose RLC therapy and the size of skin ulcers ($r = -0.2$, with Pearson correlation coefficient) indicates a tendency of overtreatment for BU in Japan.

The ineffectiveness of low-dose RLC may be attributed to a complex pharmacokinetic interaction between CAM and RFP. It has been reported that RFP induces production of cytochrome P450 3A4, an enzyme involved in the metabolism of CAM [33, 34], which would hasten the elimination of CAM from serum when used together [35, 36]. These reports indicate the need for a higher dose of CAM when used in combination with RFP. With reference to the WHO BU meeting in 2015, we currently recommend a high-dose RLC triple therapy consisting of RFP (10 mg/kg/day), LVFX (500 mg/day), and CAM (800 mg/day) in Japan [37].

2.2 Alternative Treatments of BU

Surgical excision and skin grafting were the only options for BU treatment until 2004 [38]. Since then, apart from chemotherapy also other therapeutic options, such as negative-pressure wound therapy (NPWT), ozone therapy, and hyperbaric oxygen therapy (HBOT) have been used or considered in the treatment of BU. Particularly for patients who have received surgical excision and still present large non-granulating wound areas, NPWT or ozone therapy could be a good choice for the next stage.

Since larger lesions or multiple ulcerative lesions often took a longer time to heal or failed to be cured by chemotherapy alone, surgical excision of the necrotic, ulcerated tissues had been performed in Japan. Among 31 cases that received surgical excision, 21 (67.7%) also received skin grafts (Table 5). Among the 21 patients, NPWT was tentatively performed in one patient for the purpose of wound bed preparation before skin grafting [39]. This patient had an ulcer on the right ankle with a necrotic

Table 5 Treatment for BU in Japan

Treatment	RLC (high dose) (n = 21)	Others (n = 29)	Total (n = 50)
Antimicrobial treatments only	15	4	19
Surgical excision	2	8	10
Surgical excision + skin graft	3	17	20
Surgical excision + NPWT + skin graft	1	0	1

BU Buruli ulcer, *NPWT* negative-pressure wound therapy, *RLC* triple therapy with rifampicin (450 mg/day), levofloxacin (500 mg/day), and clarithromycin (800 mg/day)

bed, and the Achilles tendon and the calcaneal bone were exposed. NPWT with the Vacuum-Assisted Closure (V.A.C.) Therapy System (Kinetics Concepts Inc.; KCI) was started as a pretreatment for a skin graft to be performed 22 days after surgical excision of the necrotic tissue. The vacuum suction was maintained at 125 mmHg. The wound dressings were changed every third day for 24 days. At the end of NPWT, acceptable granulation tissue had covered the ulcer bed and the skin graft was consequently successfully engrafted. The patient was cured without disability.

NPWT has been widely used in Japan for many kinds of ulcerative lesions or for wound treatment after skin grafting, as it can reduce exudate, improve local edema, and activate granulation. It is not to be used for wounds with active infection. Therefore, for BU treatment, NPWT is preferable for use after surgical debridement combined with appropriate antimicrobial chemotherapy. Dressings must be changed every 48–72 h. The absence of active wound infection should be checked each time at dressing. The exact frequency of dressing changes depends on the individual patient's circumstance, but it should not be less than three times a week. There are rental and purchasable NPWT (portable and disposable) models. Rental models include RENASYS by Smith & Nephew and V.A.C. by KCI, and purchasable models include PICO by Smith & Nephew. Otherwise healthy patients often must remain hospitalized for the sole purpose of receiving NPWT because the rental devices are only for hospital use. Thus, an affordable and purchasable NPWT device like PICO may be more cost-effective for an otherwise healthy patient. Nevertheless, its high cost is a large problem for patients in many countries (Table 6).

2.3   Other Suggested Therapies

Ozone therapy has been reported to shorten the course of wound healing and to promote lipid peroxidation and antioxidant protection indices [40]. Briefly, O_3 is topically administered by positioning a bag around the lesion and insufflating an O_2-O_3 mixture at a concentration of 25–30 μg/mL. To avoid gas leakage, the inflated bag is sealed just above the lesion. The bag is positioned and closed with an elastic band to give the gas mixture contact with the wound for about 20 min. When the bag is removed, the wound is covered with a sterile gauze. A case with a skin ulcer in Benin was reported to be healed after the ozone applications in 2 weeks [41].

Table 6 Approximate costs for NPWT per week

| Country | Company and device | | |
| | Smith and Nephew | | KCI |
	RENASYS (US$/week)	PICO (US$/week)	V.A.C. (US$/week)
Japan	620	420	700
South Africa (private)	N/A	N/A	600
South Africa (public)	450	175–300	450
Egypt (private)	290–340	220	N/A
Egypt (public)	145–155	N/A	N/A

Costs shown are approximate costs per week as of January 2017. The weekly costs include the costs for three sets of dressings and canisters and rental fee for the NPWT device (RENASYS and V.A.C.). The costs vary due to the size of lesions. *KCI* Kinetics Concepts Inc., *N/A* not available, *NPWT* negative-pressure wound therapy, *V.A.C.* vacuum-assisted closure therapy system

Compared to surgical treatment, NPWT and ozone therapy are easy to use procedures. NPWT requires only the NPWT device, dressings, and canisters, though the disposable dressings and canisters are expensive. In contrast, ozone therapy does not require such expensive disposable materials. It requires an oxygen tank, an ozone generator, sterile water, and plastic bags, together with a system to drive the gas into contact with the lesions. The oxygen generator is expensive (4000–8000 Euros), but the cost of the disposable items for ozone therapy is much lower than that for NPWT. This simpler and more affordable therapy may offer an effective option for managing BU as an alternative treatment in endemic areas.

HBOT has long been established to treat skin ulcers and wounds such as diabetic foot ulcers, burns, and serious soft tissue infections including gas gangrene and necrotizing fasciitis [42]. HBOT is the application of 100% oxygen at a pressure two or three times of the atmospheric pressure at sea level. This pressure increases arterial and tissue oxygen tension to exert multiple physiological and therapeutic effects [43]. HBOT has been experimentally used to treat mice with *M. ulcerans*-infected footpads [44, 45]. In human patients, Pszolla et al. reported that supportive therapy with HBOT promoted healing after surgical excision of the deep ulcers with osteomyelitis caused by *M. ulcerans* [45]. HBOT requires hyperbaric chambers, which may limit its application in endemic areas. However, if used in conjunction with other therapeutic procedures, HBOT may be an effective therapeutic adjunct in treating BU, and it may contribute to reduce large surgical interventions like amputations and to prevent permanent disabilities [46].

3   *M. ulcerans* subsp. *shinshuense* in the Environment of Japan

3.1   Detection of Environmental *M. ulcerans* subsp. *shinshuense* in Japan

So far, *M. ulcerans* has been detected in the backyard of a family house in a rare case of concurrent familial clustering in Japan [47, 48]. In November, three members of a family living in a rural town in Japan, developed BU symptoms almost

simultaneously. At first, the 2-year-old daughter had a gradually enlarging eruption on her right cheek. Soon after her symptom onset, an asymptomatic indurated eruption appeared on the right forearm of her 5-year-old brother, and a gradually enlarging eruption developed on the right wrist of her 37-year-old mother, both of which showed acid-fast bacilli-positive histopathological features similar to the lesions found in the little girl. The patients were tentatively given oral anti-mycobacterial multi-drug therapy combined with surgical treatment and cured without recurrence. PCR amplification and gene sequencing afterwards determined the strains isolated from the patients' skin biopsy cultures to be *M. ulcerans* subsp. *shinshuense*, thus giving an indubitable diagnosis of BU. The concurrent familial cases strongly suggested the presence of the pathogen in their common living environment, although person-to-person transmission of *M. ulcerans* cannot be excluded. A field study involving environmental sampling was therefore carried out in summer (July and August) and autumn (October) of the following year in an attempt to determine the presence of *M. ulcerans* in the environment around the family's house.

The family was living in an old house surrounded by rich farmland and extensive irrigation channels. Immediate suspicions centered on a one-meter-wide agricultural water channel of stagnant water slowly flowing through the family's backyard, to which the family had routine access and where the children entertained themselves by catching small water creatures. Environmental samples, including water, mud, crayfish, earthworms, a freshwater snail, a hoverfly, a moth, and several kinds of aquatic or semi-aquatic insects, were collected from or near the water channel in the patients' backyard in summer and autumn. Genomic DNA was purified from each sample and subjected to whole genome amplification (WGA) to achieve higher sensitivity in PCR to detect trace amounts of mycobacterial DNA. PCR amplifications and DNA sequencing results showed that the *M. ulcerans* IS*2404* was detected in WGA-amplified DNA from a crayfish that was collected in summer (but not in the crayfish collected in autumn or in any other sample), presenting so far the only piece of evidence that links a contaminated aquatic environment to BU occurrence in Japan.

Intriguingly, *M. ulcerans* IS*2404* was detected in a crayfish but not in the water samples collected from the same channel at the same time [47], indicating that despite the presence of the pathogen in the contaminated aquatic environment, its potential presence in the water was nevertheless below the detection sensitivity of PCR amplification. In an aquatic community, due to biotic interactions between various habitants such as filter feeders, herbivorous, scavengers, and predators, *M. ulcerans* may be widespread across the whole community. In agreement with this point of view, in endemic areas *M. ulcerans* DNA has been detected in a wide variety of environment samples, such as aquatic insects, biofilms, soil, mosquitoes, crustaceans, detritus, fish, frogs, snails, worms, and various small mammals [49]. The transmission of pathogens within an aquatic environment, depending on biodiversity and the number of habitants, can drive the environmental load of the pathogens to be enriched or diluted in certain hosts, carriers, or biofilms. Additionally, certain keystone organisms could play an overwhelming role in the transmission and overall prevalence of *M. ulcerans* in a specific environment [50]. The crayfish (*Procambarus clarkii*) is known to have a wide food range, including plants, small

fish, shrimp, plankton, benthos, and algae, and especially prefers mud and deceased aquatic organisms. Such an omnivorous eating habit may explain an enrichment of *M. ulcerans* in crayfish.

3.2   Seasonal Variation of BU in Japan

In addition to establishing the presence of *M. ulcerans* subsp. *shinshuense* in an aquatic environment in Japan for the first time, the findings of the field work were in line with a noticeable feature of seasonal variation of BU occurrence in Japan. Eighty percent of BU cases in Japan were diagnosed in autumn and winter (from September to February), but only a few in summer (July and August) [6, 8]. Under the assumption that the diagnosis is preceded for several months by *M. ulcerans* infection due to a long incubation period [9] and due to the delay in seeking medical care, it was speculated that the exposure to the pathogen occurred during summer. Such dynamics in *M. ulcerans* infection are putatively driven by a complex interplay of human activities and the prevalence of the pathogen in the environment. Japan has four distinct seasons with a rainy season throughout early summer (typically from early June to mid-July), and there are naturally more chances for bare skin to be exposed to an aquatic environment during the hot and humid summer time, especially in the water-rich countryside.

On the other hand, there was no direct information regarding the prevalence of *M. ulcerans* in the environment throughout the year in Japan before the field work. The results that *M. ulcerans* subsp. *shinshuense* was detected only in July, but not in any of the samples collected from the same water channel in October, hints at potential seasonally changing prevalence of environmental *M. ulcerans* subsp. *shinshuense* with a high peak in summer, which may have contributed to the observed seasonal variation of BU incidence in Japan. However, the scale of this fieldwork alone was far too small to make a solid prediction. Seasonal dynamics in environmental *M. ulcerans* prevalence can be driven by an extremely complex interplay between multiple biotic and abiotic factors, such as topographic factors (distribution and latitude of land cover of watersheds), climatic factors (temperature, precipitation), physical-chemical conditions (pH, dissolved oxygen, salinity) in stagnant and slow flowing ecosystems, and networks of aquatic taxa, as suggested by large-scale field studies conducted in BU endemic countries [51, 52]. In this respect, the seasonal dynamics of the prevalence of *M. ulcerans* in Japan (especially in a high latitude area as the site in the field work) may differ substantially from that reported in tropical/subtropical areas.

3.3   PCR Detection of *M. ulcerans* subsp. *shinshuense* DNA in the Environment

Field studies to detect environmental *M. ulcerans* subsp. *shinshuense* in Japan are still in their infancy compared to studies in BU endemic countries. Presently, the detection of *M. ulcerans* in the environment is mostly based on demonstrating by

PCR the presence of IS*2404*, an insertion sequence with over 200 copies in the whole genome of *M. ulcerans* [10]. IS*2404*-PCR is highly specific and sensitive for testing clinical specimens from human BU lesions; however, its application in the detection of environmental *M. ulcerans* is less straightforward, and the IS*2404*-PCR-based results should be interpreted with caution. IS*2404*-PCR detects the presence of IS*2404* regardless of the pathogenicity of the detected mycobacteria, which alone has little power in predicting endemicity of BU. *M. ulcerans* DNA was detected by IS*2404*-PCR extensively across southern Louisiana, U.S., leading to the conclusion that the distribution of environmental *M. ulcerans* is not restricted to areas where BU is endemic [53].

In a large-scale field study conducted in Benin, IS*2404*-positive samples were detected with similar prevalence in environmental samples in both non-endemic (9/10) and endemic (12/12) villages [49]. However, when IS*2404*-positive samples were further analyzed for a second PCR target, the enoyl reductase (ER) that is required for the synthesis of mycolactone, only 2/10 non-endemic villages had ER-positive samples, whereas 9/12 endemic villages had ER-positive samples. Thus, the PCR for IS*2404* alone falsely predicted 9/10 non-endemic villages to be endemic, and the additional use of the PCR for ER accurately predicted 8/10 non-endemic villages [49]. In a field study, the use of a single PCR target for detection of environmental *M. ulcerans* may easily signal a false alarm to public health authorities. Secondly, the results of conventional IS*2404* PCR analyses are only qualitative and do not reveal the abundance of the pathogen. They are thus inadequate to describe potential seasonal dynamics in the overall environmental load of *M. ulcerans.* Lastly, analysis of many IS*2404*-positive samples in Ghana have revealed the presence of mycolactone-producing mycobacterial species other than the ones commonly associated with BU disease in humans [54]. *Mycobacterial* strains closely related to the human pathogenic *M. ulcerans*, including ecovars originally designated *M. liflandii*, *M. pseudoshottsii*, and mycolactone-producing *M. marinum*, have been found to harbor IS*2404* [55, 56].

Thus, the analyses of *M. ulcerans* that rely solely upon the conventional PCR assay for IS*2404* are flawed. TaqMan Multiplex real-time PCR assays that target two insertion sequences of *M. ulcerans* (IS*2404*, IS*2606*) and a multicopy sequence encoding the ketoreductase B domain [55, 57], in which samples are considered positive only if the detected cycle threshold values are strictly lower than a default value based on external standard curves with serial dilutions of *Mycobacterial* DNA, can quantify the copy number of the targets to allow the differentiation of classical *M. ulcerans* from other IS*2404*-harboring strains in the environment [55, 57].

Moreover, a novel category of variable tandem repeats (VNTRs) called mycobacterial interspersed repetitive units (MIRUs) have been identified in the genomes of *M. ulcerans* and *M. marinum*. Analysis of strain-specific polymorphisms of MIRU loci has been used to tentatively genotype *M. ulcerans*, *M. marinum*, and an *M. marinum*-like organism that is considered a possible missing link between *M. ulcerans* and *M. marinum* [56, 58]. Ideas inspired by many studies conducted in the BU endemic countries, such as the use of multiple targets in quantitative PCR assays and MIRU-VNTR typing, will better equip us to evaluate the environmental

distribution of *M. ulcerans* in future field studies in Japan. Distribution maps of Asian strains will play a key role in understanding the transmission mode of *M. ulcerans* and support control of BU.

4   BU in Asia and Future Perspectives

4.1   BU in China

In addition to BU cases reported from Japan, *M. ulcerans* subsp. *shinshuense* has also been identified as the causative agent of BU in a patient from China [59]. In 1997, a 40-year-old women, who had grown up in China and had been living in Europe, traveled to Shandong Province, China in the summer (July and August). According to the patient's narrative, she received many mosquito bites after walking barelegged in the field. Three months later, the patient noticed a swelling at the front of her left lower leg that afterwards developed a slightly depressed center with no other systemic symptoms. Four months after that, a surgeon excised her lesion. Reportedly, there was no noticeable bleeding during the procedure, and the excised tissue was pale and firm. The lesion did not heal after excision but further developed ulceration. After the patient went back to Europe, a doctor specializing in infectious diseases examined the lesion and suggested that it might be skin tuberculosis. Consequently, a smear of the ulcer base was made by the patient's general practitioner, and Ziehl-Neelsen staining revealed acid-fast bacteria scattered and in clumps. The patient was therefore referred to the Department of Dermatology, Section of Tropical Dermatology of the Academic Medical Center, Amsterdam, where diagnostic procedures showed BU-characteristic clinical, histopathological, and molecular findings [59].

At first examination, it was a single painless lesion (3 × 3.5 cm in diameter) on the lower leg with necrotic ulcer bed and undermined border. Histopathological examination of the biopsy specimens showed thickening and broadening of the epidermis, extensive eosinophilic necrosis in the deeper dermis and the subcutaneous tissue without granulomatous inflammatory reactions. Cultivation tests using a biopsy sample yielded pale-cream to yellow colonies after 40-day incubation on Coletsos medium only at 30°C. PCR analyses using DNA extracts both from ulcer biopsies and culture isolates confirmed the presence of the *M. ulcerans*-specific IS*2404* sequence. Using classical identification schemes the isolate was identified as *M. ulcerans* subsp. *shinshuense*. Treatment began with RFP + CAM and was later switched to ciprofloxacin (CPFX) + rifabutin (RFB) based on the results from drug susceptibility test of the original culture isolate. The patient was cured with an 8-months antibiotic treatment without reported recurrence [59].

The putative infection site of Shandong Province, China is above latitude 30°N. BU occurrence at such a high latitude is rare, and had only been reported in Japan, suggesting that *M. ulcerans* subsp. *shinshuense* is more adapted to environments at higher latitudes than the classical *M. ulcerans* that is observed to be confined to tropical/subtropical regions and temperate climatic areas. Moreover, the infection by *M. ulcerans* subsp. *shinshuense* in the patient from China was highly

suspected to have occurred during her travel in summer, 3–4 months prior to symptom onset, which is in line with the proposed seasonal variation of BU occurrence in Japan. Thus, in contrast to the majority of BU cases that are caused by classical *M. ulcerans*, BU infections by *M. ulcerans* subsp. *shinshuense* have so far been only infrequently found in Japan and China, as apparent from the clinical reports [6, 8].

As stated before, it is possible that extensive reductive genomic changes have enabled the classical lineage to habituate in a more confined environment, resulting in clonal populations within Africa and Australia with genomic rearrangements rendering them more pathogenic and strengthening their ability to infect mammals [15]. In contrast, the ancestral lineage, closer to the *M. marinum* progenitor, may have remained as a largely environmental mycobacterium that only occasionally affects humans. From this point of view, it is reasonable to predict that large-scale field studies in Japan or China may reveal an unexpectedly extensive presence of *M. ulcerans* subsp. *shinshuense* in the environment that does not necessarily lead to an outbreak of BU.

4.2   Toward Detection of BU in Other Asian Countries

It appears that the stably increased number of reported BU cases from Japan is due to increased awareness of the disease among health workers rather than to an actual rise in the number of infections. On the other hand, the extremely low disease reporting rate in China and the absence of BU reporting from the neighboring Asian countries may be indicative of hidden cases. In non-endemic and less resourced Asian countries, awareness and knowledge of BU disease among health workers and the community are lacking, so there is the possibility of misdiagnosis. Moreover, insufficient health infrastructure and geographical challenges can also contribute to the underreporting of cases. Despite little evidence supporting a predictable future outbreak of BU in Asia, the importance of an early diagnosis cannot be overemphasized because of the potential life-long disfigurement and disabilities this disease can cause if left undiagnosed and untreated. Residents of Asian countries should be aware that BU is a mycobacterial infection that is not limited to tropical or subtropical regions.

Education of health workers, in particular dermatologists, in Asian countries is essential for early diagnosis of BU. Also, it is important to provide public health education to familiarize people, especially those living near rural aquatic environments, with BU symptoms. Since international travel has become more popular, the so-called imported tropical diseases may be seen more frequently than before. In addition to early diagnosis, the establishment of a standard treatment guideline optimized for BU infections in Asian countries is also needed to ensure efficacy without unnecessary surgery and overtreatment. Furthermore, a comprehensive comparison of the proteomes of *M. ulcerans* subsp. *shinshuense* with classical *M. ulcerans* may shed new light on the differences in their adaptive biology and response to antibiotic treatment.

For investigating the phylogenetics of *M. ulcerans* subsp. *shinshuense*, various approaches including genomic and biochemical analyses have been carried out [2, 4, 7, 12, 13, 15]. Only one strain of *M. ulcerans* subsp. *shinshuense* has

undergone whole genome sequencing so far [13]. Genomic differences among the sequenced strain of *M. ulcerans* subsp. *shinshuense* and other ecovars of *M. ulcerans* have been found. Further comparative genomic analyses with multiple *M. ulcerans* subsp. *shinshuense* strains, including the Chinese strain, would enable a more precise phylogenetic characterization of *M. ulcerans* subsp. *shinshuense*, which could give information on the spread of these bacteria in Japan as well as in China. However, at the moment, only one Chinese strain has been recognized, so discovery of multiple Chinese strains might further contribute to the above-mentioned phylogenetic resolution. Additionally, it is still unclear whether the clinical features caused by *M. ulcerans* subsp. *shinshuense* differ from those of other *M. ulcerans* strains. Future attempts involving generation of recombinant and mutant strains of *M. ulcerans* subsp. *shinshuense* might clarify the unique characteristics of this *M. ulcerans* subspecies and could provide important clues into the pathogenic mechanisms of BU.

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