Detection of *Mycobacterium ulcerans* by real-time PCR with improved primers

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Abstract

**Background:** Buruli ulcer is a severe skin disease caused by *Mycobacterium ulcerans*. Real-time PCR targeting the IS2404 sequence has been used as a reliable and rapid method for the diagnosis of Buruli ulcer and detection of *M. ulcerans* in the environment. The genome of *M. ulcerans* contains hundreds of IS2404 copies, which have variability in certain sequences. Therefore, the design of new primers specific to conserved IS2404 regions may potentially improve the sensitivity of *M. ulcerans* detection and, consequently, the diagnosis of Buruli ulcer, thus ensuring timely treatment of the disease.

**Results:** In silico analysis indicates that DNA sequences of the IS2404 elements are highly variable within a single strain. As the binding sites of conventional IS2404-specific primers used for *M. ulcerans* detection contain polymorphic sequences, we designed new primers, which enabled the detection of *M. ulcerans* by real-time PCR with higher sensitivity and similar specificity with respect to that of conventional primers. However, the increase in sensitivity with the new primers depended on the *M. ulcerans* strain.

**Conclusions:** The results suggest that real-time PCR based on the new primers could improve Buruli ulcer diagnosis and *M. ulcerans* detection in environmental samples.

**Keywords:** Buruli ulcer, *Mycobacterium ulcerans*, Diagnosis, Real-time PCR

**Background**

Buruli ulcer, a progressive skin disease caused by *Mycobacterium ulcerans*, is prevalent in more than 30 countries, with especially high incidence in West Africa [1–4]. The source of *M. ulcerans* infection is still unknown; however, the bacteria has been detected in aquatic insects [5–8] and the disease mostly occurs in people who live near still water areas, suggesting that contaminated waters may act as a reservoir of *M. ulcerans* [5, 9].

In the early stages of the disease, papules, nodules, plaques, and edema are observed in the skin, followed by progressive ulceration eroding to subcutaneous layers and even bones [10, 11]. In extreme cases, patients may suffer contracture deformity and even amputation [2, 4]; therefore, early diagnosis and treatment is important for Buruli ulcer control. Common diagnostic methods include smear microscopy, histopathology, and culture; however, they have limited sensitivity or are time-consuming [12]. Thus, it is necessary to develop simple and rapid tools that provide sufficient detection sensitivity to confirm the diagnosis of Buruli ulcer.

Several PCR methods for the detection of *M. ulcerans* have been reported; among them, the most widely used is based on targeting the IS2404 repeat sequence, which encodes a transposase and which is unique to *M. ulcerans* genome, where it occurs over 200 times [13–15]. Since other targets such as genes encoding 16S rRNA [16], hsp65 [17] and the ketoreductase domain of mycolactone polyketide synthase [14, 18], or IS2606 [14, 18] have much lower copy numbers than IS2404, the sensitivity of their detection by PCR is lower than that of IS2404 [14, 15, 18]. Moreover, of these sequences, only IS2404 is specific for *M. ulcerans* [14, 15, 18]. IS2404 is also targeted by real-time PCR and loop-mediated isothermal amplification, which are more rapid and sensitive methods than gel-based conventional PCR and which have been recently applied for the detection of *M. ulcerans* [18–20]. Currently,
real-time PCR is the gold standard method to confirm the presence of *M. ulcerans* [14, 15, 21].

Previous studies have used several primer sets for the amplification of IS2404 [14, 15, 22]; however, most of them were designed for gel-based conventional PCR [18] and may not be suitable for a more sensitive real-time PCR. Our in silico analysis indicates that the sequence of the IS2404 elements is highly variable and that the primers commonly used for IS2404 amplification are based on the polymorphic regions. Therefore, in this study, we designed and validated a new set of primers highly specific for stable sequences conserved among IS2404 copies as well as *M. ulcerans* strains with the aim to improve the sensitivity of *M. ulcerans* detection by real-time PCR.

**Methods**

**Software for genetic analysis**

Genetyx version 13 (Genetyx, Shibuya, Tokyo, Japan) was used for homology analysis and primer design. The alignment of IS2404 elements was performed by Genetyx version 13 using the algorithm of the MUSCLE program [23].

**Bacterial strains and culture conditions**

*M. ulcerans* strains used in this study are listed in Table 1. Strains Agy99 and TMC1615 were provided by Dr. Small (University of Tennessee, USA), and GTC16404, GTC16405, and GTC16406 were obtained from the GTC collection of Gifu University (Japan). ATCC strains 19423 and 33728 were purchased from the American Type Culture Collection (Manassas, VA, USA). Other *Mycobacterium* species were provided by Dr. Saito (Shimane University, Japan).

**Table 1** *M. ulcerans* strains and *Mycobacterium* species used in this study

| Species          | Strain   | Country of isolation | Year |
|------------------|----------|----------------------|------|
| *M. ulcerans*    | Agy99    | Ghana                | 1999 |
|                  | TMC1615  | Malaysia             | 1960s|
|                  | ATCC19423| Australia            | 1981 |
|                  | ATCC33728| Japan                | 1980 |
|                  | GTC16404 | Japan                | 2010 |
|                  | GTC16405 | Japan                | 2007 |
|                  | GTC16406 | Japan                | 2011 |
| *M. tuberculosis*| H37Rv    | US                   | 1934 |
| *M. kansasii*    | KHS-001  | Japan                | 2000s|
| *M. avium*       | AVHS-001 | Japan                | 2000s|
| *M. intracellulare* | 4-1974 | Japan                | 2000s|
| *M. abscessus*   | ABHS-001 | Japan                | 2000s|
| *M. scrofulaceum*| CTM35840 | –                    | –    |
| *M. smegmatis*   | ATCC70084| –                    | 1990 |

| En dash: information not available |

*Mycobacteria* were grown in Middlebrook 7H9 broth (BD Biosciences, Sparks, MD, USA) supplemented with 0.05 % (w/v) Tween 80 and 10 % (v/v) OADC Enrichment (BD Biosciences).

**DNA extraction and purification**

Bacteria were collected by centrifugation at 16,200×g for 2 min and resuspended in a solution containing 20 μl of 0.5 M NaOH, 4 μl of 10 % sodium dodecyl sulfate, and 180 μl of distilled water. The cell suspension was heated at 95 °C for 15 min, cooled to room temperature, and thoroughly mixed with 200 μl of phenol/chloroform (1:1). After centrifugation at 16,200×g for 5 min, the aqueous phase was transferred to a new tube and the extraction was repeated. Then, 20 μg of glycogen, 16 μl of 5 M NaCl, and 800 μl of 100 % ethanol were added to the pooled aqueous phases, and the mixture was centrifuged at 16,200×g for 15 min. The pellet was collected, mixed with 500 μl of 70 % ethanol, and the sample was centrifuged for 1 min. The final pellet was resuspended in 50 μl of distilled water.

**Real-time PCR**

Real-time PCR was performed as described by Fyfe et al. [18]. The method is recommended by the World Health Organization (WHO) for *M. ulcerans* detection [14, 21] and is based on primers IS2404TF and IS2404TR and probe IS2404TP [18]. Alternatively, we used our newly designed primers IS2404KF and IS2404KR and probe IS2404KP. Primer and probe sequences are listed in Table 2. The reactions were performed in a total volume of 10 μl containing 5 μl of THUNDERBIRD Probe qPCR Mix (TOYOBO, Osaka, Japan), 0.5 μM of each primer, 0.2 μM of the probe, and 10 ng of purified *M. ulcerans* genomic DNA. The cycling conditions were as follows: 1 cycle of 95 °C for 60 s, and 35 cycles of 95 °C for 15 s and 60 °C for 60 s. The threshold cycle (Ct) for each sample was automatically calculated by the C1000 manager software version 1.0 (Bio-Rad Laboratories, Hercules, CA, USA).

**Table 2** Primers and probes for real-time PCR

| Primer or probe | Sequence (5′-3′) |
|-----------------|-----------------|
| IS2404TF        | AAAGCACACCCGAGCATCT |
| IS2404TR        | AGCGGGAAGTAGGTGAGT |
| IS2404TP        | FAM-CGTCCAACGCGATC-BHQ1 |
| IS2404KF        | TCTCGTGCTGGTGTTC |
| IS2404KP        | TGACGACCTGGATG |
| IS2404TF, IS2404TR, and IS2404TP were described by Fyfe et al. [18] |
| FAM fluorescein amidite, BHQ1 black hole quencher 1, MGB minor groove binder |
Statistical analysis
Statistical analysis was performed using the GraphPad Prism software version 6 (GraphPad Software, LA Jolla, CA, USA). The differences between samples were analyzed by the Student's t test, and differences were considered statistically significant at a $p$ value of 0.05.

Results
IS2404 sequence variations
The genome of $M. ulcerans$ strain Agy99 (gene accession number: CP000325) contains 249 IS2404-like elements [13]. For the purpose of this study, 212 sequences with high homology to the transposase gene of IS2404 (MUL_0099) determined by BLASTN search were retrieved from the Agy99 genome and aligned with the transposase sequence used as a reference.

The results revealed numerous gaps and sequence dissimilarities at the nucleotide level among the compared IS2404 elements (Fig. 1 and Additional file 1: Figure S1); in particular, sequence polymorphism was detected in the regions targeted by the IS2404TF and IS2404TR primers (Fig. 1 and Additional file 1: Figure S1) commonly used for real-time PCR-based detection of $M. ulcerans$ [18]. The high level of sequence variability in the IS2404 elements may affect the accuracy of PCR-based $M. ulcerans$ detection. Thus, our observations suggest that the sensitivity of $M. ulcerans$ detection by real-time PCR may be improved by using a new primer/probe set targeting regions highly conserved among multiple IS2404-like elements.

Sensitivity of the new primer/probe set for $M. ulcerans$ detection
Based on the sequence alignment data, we designed a new set of PCR primers (IS2404KF and IS2404KR) and probe (IS2404KP) (Table 2) specific to stable IS2404 elements.

![Fig. 1](https://example.com/fig1.png)

**Fig. 1** Polymorphism of the IS2404 elements in the genome of $M. ulcerans$ strain Agy99. Two hundred and twelve IS2404-like sequences were compared with the transposase-encoding gene (MUL_0099 gene) used as an IS2404 reference sequence. Red characters indicate polymorphic sites; hyphens/dashes indicate gaps. Arrows and lines indicate positions of primers and probes, respectively; blue and yellow colors mark conventional and new, respectively, primers and probes.
regions (Fig. 1). The IS2404KF primer contained only one variable nucleotide at the 5′-terminus, while IS2404KR and IS2404KP did not have any sequence variability among different IS2404 elements. We performed real-time PCR using the new primers and probe and compared their sensitivity and specificity with those of conventional primers (IS2404TF and IS2404TR) and probe (IS2404TP) (Fig. 2).

The use of the new primer/probe set enabled the detection of genomic DNA from most M. ulcerans strains using a smaller number of cycles than that required with the conventional set. PCR sensitivity was more significantly improved for three Japanese isolates (GTC16404, GTC16405, and GTC16406) than for Agy99 and TMC1615, while no changes were observed for ATCC19423 and ATCC33728. The results suggest that the new primer/probe set could improve the sensitivity of M. ulcerans PCR-based detection; however, the increase in sensitivity varied depending on the strain.

**Specificity of the new primer/probe set**

The detection specificity of real-time PCR based on the new set was evaluated using purified genomic DNA of M. ulcerans and seven other mycobacterial species (Table 1). As shown in Fig. 3, PCR with both conventional and new primer/probe sets detected only M. ulcerans DNA, indicating that the specificity of the new set was similar to that of the conventional set.

**Discussion**

The IS2404-targeting PCR method, which enables rapid and sensitive detection of M. ulcerans, has been established as the gold standard for the diagnosis of Buruli ulcer [15, 18, 21] and is currently recommended by the WHO [21]. IS2404 is a multi-copy insertion sequence encoding a 328-amino acid transposase [14], which is unique to M. ulcerans and is represented by 249 copies in its genome [13]. Because of the high frequency of occurrence of IS2404 in the M. ulcerans genome, this element has been used as a target sequence in PCR-based detection of M. ulcerans infection. However, in this study, we revealed considerable sequence variability among the IS2404 elements of a single M. ulcerans strain (Agy99) (Fig. 1 and Additional file 1: Figure S1); nucleotide polymorphism was also observed in the regions targeted by commonly used primers, which could affect the accuracy of Buruli ulcer diagnosis. Therefore, to increase the detection sensitivity of M. ulcerans, we designed a new primer/probe set specific for the regions highly conserved among IS2404 copies. Compared with the conventionally used set, the new set provided an increased sensitivity and similar specificity of real-time PCR detection for most tested M. ulcerans strains (Figs. 2 and 3). Detection sensitivity with the new primer/probe set was particularly high for three Japanese isolates (GTC16404, GTC16405, and GTC16406); on the other hand, no changes were observed for the ATCC19423 and ATCC3372 strains. The results suggest that the new set could lead to better PCR-based detection of M. ulcerans than that with conventional primers, although the advantage may be strain dependent. In this study, we used only seven M. ulcerans isolates; more of them should be tested to comprehensively evaluate strain-specific differences in detection sensitivity using the new primer/probe set. In addition, it should be noted that clinical and environmental specimens could contain PCR inhibitors and contaminating DNA; therefore, the new set should be validated using a panel of clinical and environmental samples.
Since we observed sequence variability among IS2404 copies of the same *M. ulcerans* strain, we hypothesized that the difference may also exist among the strains. As described in results, PCR sensitivity was more significantly improved by new primers for three Japanese isolates. The result suggests that the Japanese strains could have more sequence diversity in the binding regions of conventional primers than other strains. In this study, we used *M. ulcerans* isolates from geographically distant areas. Interestingly, for the strains from Africa (Agy99), Southeast Asia (TMC1615), and Australia (ATCC19423), which belong to the classical lineage [24], the new primer/probe set provided only moderate or no improvement of detection sensitivity, while for most Japanese strains, a significant increase in sensitivity was observed. Weihong et al. [25] demonstrated higher frequency of large chromosomal rearrangements in a Japanese strain compared to the classical lineage strains [25]. Since the IS2404-encoded transposase may be closely involved in genomic rearrangements, Japanese strains might harbor the IS2404 elements carrying different types of polymorphisms compared to the classical lineage strains. To clarify why the new primers improved the sensitivity of *M. ulcerans* PCR detection, further analysis, including whole genome sequencing of each strain may be required. On the other hand, there is possibility that particular *M. ulcerans* isolates may escape IS2404-targeting PCR detection. Therefore, to provide sensitive and robust detection of *M. ulcerans*, it might be useful to perform multiplex PCR, which would target, along with IS2404, several other *M. ulcerans* sequences such as IS2606 or ketoreductase domain in the genes encoding mycolactone polyketide synthase, as reported by Fyfe et al. [18].

Further sequence analysis of the IS2404 elements is necessary to develop more sensitive methods for *M. ulcerans* diagnosis.

**Conclusions**

The results of our study suggest that the new primer/probe set is more sensitive for PCR-based detection of *M. ulcerans* than the conventionally used set, suggesting that its application can improve the diagnosis of Buruli ulcer.

**Additional file**

Additional file 1: Figure S1. Sequence alignment of the IS2404 elements from the genome of *M. ulcerans* strain Agy99. (PDF 1040 kb)

**Abbreviations**

BHQ1, black hole quencher 1; FAM, fluorescein amidite; MGB, minor groove binder

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**Availability of data and materials**

The datasets supporting the conclusion of this study are included within the article and the supplemental data.

**Authors’ contributions**

NS designed the study, analyzed the data, and wrote the manuscript. HN read and approved the final manuscript. MW assisted with the study design, data analysis, and manuscript preparation. All authors read and approved the final manuscript.

**Competing interests**

The authors declare that they have no competing interests.

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