A Genotypic Approach for Detection, Identification, and Characterization of Drug Resistance in Mycobacterium ulcerans in Clinical Samples and Isolates from Ghana

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Abstract. Standardized antimycobacterial therapy is considered the treatment of choice for Buruli ulcer disease. To assess the prevalence of drug resistance among clinical Mycobacterium ulcerans isolates in Ghana, we conducted a sequence-based approach to detect mutations associated with drug resistance. We subjected clinical samples to direct DNA sequencing of rpoB and rpsL genes and compared culture and whole-genome extracts regarding the efficiency of sequence analysis; 99.1% (rpoB) and 100% (rpsL) of the patients harbored M. ulcerans wild type. In one isolate (0.9%), a point mutation of the rpoB gene at codon Ser522 leading to an amino acid change was detected. Culture extracts yielded a significantly higher sequencing efficiency than whole-genome extracts. Our data suggest a low level of drug resistance in Ghana. However, mutations associated with drug resistance do occur and require monitoring. Improved techniques are necessary to enhance the efficiency of sequence analysis of whole-genome extracts.

INTRODUCTION

Buruli ulcer disease (BUD), caused by Mycobacterium ulcerans, is the third most common mycobacterial infection in humans after tuberculosis and leprosy, and it has been reported from more than 30 countries worldwide, with dominant endemic foci in West Africa. BUD involves the skin and the subcutaneous adipose tissue. The disease starts as a painless nodule, papule, plaque, or edema and evolves into a painless ulcer with characteristically undermined edges. If left untreated, severe disability may occur. Previously, BUD was treated by wide surgical excision, and the World Health Organization (WHO) recommended antimycobacterial treatment of 56 days with streptomycin (SM) and rifampicin (RMP), if necessary, followed by surgical excision in 2004.12

Most West African countries implemented the standardized antibiotic therapy in 2006. However, non-standardized regimens of RMP and/or SM were also used as concomitant antibiotic therapy in 2006.3–5

Although effective and in many aspects advantageous over surgery, introduction of antimycobacterial treatment poses new challenges for the management of BUD.1

As is well-known from tuberculosis and leprosy, antimycobacterial treatment is prone to the development of drug resistance. Risk factors hereby encompass a lack of patients’ compliance as well as irregular and inadequate treatment regimens in terms of duration, dosage, and drug combination. The WHO estimates the current global level of drug resistance of Mycobacterium tuberculosis complex (MTBC) to RMP and SM, as determined by the proportion method, to be 6.3% and 12.6%, respectively.α Genotypic drug-resistance testing constitutes a reliable and expedient alternative to predict phenotypic drug resistance of M. ulcerans to RMP and SM. In 2007, WHO also emphasized the importance of systematic global drug resistance surveillance for leprosy and implemented a genotypic drug-resistance surveillance system in 2009. So far, high concordance between conventional drug-susceptibility testing (mouse footpad technique) and DNA sequencing methods has been observed. In a large-scale study on sequence-based detection of drug resistance in human leprosy, rifampicin-resistant M. leprae strains were detected among 2% of new cases and 8% of relapses in Southeast Asia.2–11

The majority of M. tuberculosis strains expressing phenotypic resistance to rifampicin shows mutations within the highly conserved 81-bp RMP resistance-determining region (RRDR) of the rpoB gene comprising codons 507–533, with codons Ser531 and His526 being involved most frequently.2–11 For M. leprae, rpoB gene mutations leading to phenotypic rifampicin resistance were likewise detected within the RRDR comprising codons 401–427 (equivalent to codons 507–533 in M. tuberculosis), predominantly affecting codon Ser425 (equivalent to Ser531 in M. tuberculosis). Resistance of M. tuberculosis to SM was most frequently associated with a single mutation in codon 43 of the rpsL gene and less commonly with mutations of the rrs gene.12–15

To date, clinical M. ulcerans strains resistant to RMP or SM have not yet been reported. However, mutations of the rpoB gene in codons Ser416 and His420 (Ser522 and His526 in M. tuberculosis numbering, respectively) of three M. ulcerans strains causing RMP-resistant phenotypes have been detected after RMP monotherapy of experimentally infected mice.16

With respect to the irregular use of antimycobacterial drugs before introduction of standardized antimycobacterial treatment, we conducted a pilot study in Ghana to evaluate the potential use of molecular tools, specifically gene sequencing, to obtain baseline data on the prevalence of mutations possibly associated with resistance of M. ulcerans to RMP and SM.

MATERIALS AND METHODS

Ethics statement. The study was approved by the Committee of Human Research Publication and Ethics,
School of Medical Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana (CHRPE/07/01/05).

For all patients who were enrolled in a drug trial, written informed consent was obtained from the study participants and/or their legal representatives if aged under 18 years. For patients who underwent routine surgical treatment (with or without concomitant drug therapy), written consent or in the case of the participants who were illiterate, verbal consent, according to the requirements of the ethics committee, for all treatment-related procedures including the collection of diagnostic specimens was obtained in the treatment centers and documented in the medical records of the respective patients.

**Patients.** One hundred sixty-two laboratory-confirmed BUD patients with ulcerative (N = 99) and non-ulcerative (N = 63) lesions seeking treatment in five treatment centers in Ghana (Agogo Presbyterian Hospital, N = 116; Nkwie Government Hospital, N = 30; Dunkwa Government Hospital, N = 12; Global Evangelic Mission Hospital, N = 3; Goaso Hospital, N = 1) were enrolled in the study.

Between 2004 and 2007, 80 patients (49.4%) were treated by surgical excision with or without concomitant antimycobacterial treatment. At the time of collection of clinical samples, 29 of those patients (36.3%) had not received any antimycobacterial drugs (ST). Thirty-two patients (40.0%) had received either a combination of RMP and SM (N = 30) or RMP alone (N = 2; ST+). Fourteen patients were treated for ≤7 days (short-term; ST+S), and two of those patients received RMP only. Twelve patients received 8–28 days of drug treatment (intermediate; ST+IN), and six were treated for 29–42 days (long term; ST+L). Nineteen patients (23.8%) had received antimycobacterial treatment; however, detailed information on the drugs applied and duration of treatment was not available (ST+NK).

The mean duration of treatment before surgical excision and collection of clinical samples was 13.1 days (Table 1).

Between 2005 and 2007, according to the clinical records, 82 patients (50.6%) were treated with antimycobacterial drugs alone (DT). Of these, 44 patients were enrolled in a randomized controlled drug trial. By the time of collection of clinical samples, 81 (98.8%) of these patients had not received antibiotics; one recurrent case, however, had received a full course of RMP and SM before the trial (Table 1).

Clinical samples for follow-up analysis were available from seven patients (antimycobacterial treatment only, N = 6; antibiotics plus surgery, N = 1). At the time of collection of follow-up samples, five patients had received full-term treatment with RMP and SM for 56 days, and two patients were treated with

### Table 1

<table>
<thead>
<tr>
<th>Treatment group*</th>
<th>Patients†</th>
<th>Culture extracts‡</th>
<th>Swab extracts§</th>
<th>Tissue extracts¶</th>
<th>Results per patient**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>rpoB gene results††</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>WT MUT NR</td>
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</tr>
<tr>
<td>DT</td>
<td>82</td>
<td>50</td>
<td>37</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>ST</td>
<td>29</td>
<td>14</td>
<td>14</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>ST+S</td>
<td>14</td>
<td>1</td>
<td>9</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>ST+IN</td>
<td>12</td>
<td>1</td>
<td>4</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>ST+L</td>
<td>6</td>
<td>5</td>
<td>2</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>ST+NK</td>
<td>19</td>
<td>16</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Total†††</td>
<td>162</td>
<td>87</td>
<td>68</td>
<td>88</td>
<td></td>
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<td>108</td>
<td>1</td>
<td></td>
<td></td>
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</table>

<table>
<thead>
<tr>
<th>rpsL gene results†††</th>
<th>WT</th>
<th>MUT</th>
<th>NR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>53 (32.7%)</td>
<td>0</td>
<td>51 (40.8%)</td>
</tr>
</tbody>
</table>

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Table 1 provides sequencing results of *M. ulcerans* isolates from 162 PCR-confirmed BUD patients. Two hundred forty-three DNA extracts were analyzed from these patients, and one final consensus result per patient is shown. From seven follow-up patients, sequencing results of isolates from visit one are listed only.

* Patients were divided into different treatment groups as follows. DT = drug treatment [patients received a full course of rifampicin (RMP) and streptomycin (SM) for 56 days after specimen collection; one recurrent case, however, had received a full term of antimycobacterial treatment at the time of specimen collection, (long term; ST+L)]. Nineteen patients (23.8%) had received antimycobacterial treatment; however, detailed information on the drugs applied and duration of treatment was not available (ST+NK).

† Number of patients per treatment group.

‡ Number of analyzed culture extracts from swab and/or tissue specimens.

§ Number of sequenced whole-genome extracts from swab specimens.

¶ Number of sequenced whole-genome extracts from tissue specimens.

|| Final sequencing result per patient. Percentages are given in brackets.

** rpoB is the gene for RNA polymerase β subunit (partial sequence = 342 bp).

†† rpsL is the gene for ribosomal protein S12 (complete sequence = 375 bp).

†‡ Wild type. The analyzed sequence corresponds 100% to the WT nucleotides of *M. ulcerans* strain Agy99 (accession number CP000325).

†§ MUT = mutation. One isolate showed a point MUT at Ser522 of the rpoB gene. Species identification by sequence analysis of genes for 16S rRNA, rpsL, hsp65, and the ITS allowed the distinct allocation to *M. ulcerans* strain Agy99 (accession number CP000252) by 100% nucleotide concordance.

†¶ NR = no result. Sequences were non-analyzable because of mixed sequences, deviation > 3% from *M. ulcerans* wild type, or inability to amplify.

†‖ From 24 patients, a swab and a tissue extract were sequenced; additionally, 12 culture extracts were analyzed from these patients. For 45 patients, a swab or tissue extract was available with a corresponding culture extract inoculated from the respective clinical specimen.
RMP and SM for 21 days. Analysis of these specimens was done separately and is not provided in the attached tables.

**Clinical samples and laboratory confirmation.** Clinical samples for laboratory confirmation were collected according to standardized procedures. Hereby, diagnostic swabs and tissue specimens from surgical patients were collected at the time of surgery, and diagnostic swabs and punch biopsies were collected from patients receiving antimycobacterial treatment before onset of treatment. As described elsewhere, the clinical samples were subjected to a dry-reagent based *IS2404* polymerase chain reaction (DRB-PCR) and culture on Loewenstein-Jensen media at the Kumasi Center for Collaborative Research in Tropical Medicine, Kumasi, Ghana (KCCR) according to standardized procedures, including external quality assurance by standard *IS2404* PCR. Mycobacterial cultures were subjected to a confirmatory *IS2404* PCR.3,5,18-21

**Samples subjected to sequence analysis.** Suspicions of *IS2404* PCR confirmed *M. ulcerans* cultures (*N* = 87) dissolved in 700 μL Cell Lysis Solution (Qiagen, Hilden, Germany) followed by inactivation at 80°C for 20 minutes, and *IS2404* PCR-positive whole-genome extracts (total of 156 genome extracts) derived from swab (*N* = 68) and tissue samples (*N* = 88) were subjected to sequence analysis of *rpoB* and *rpsL* genes at the Department of Infectious Diseases and Tropical Medicine, University of Munich (DITM). Samples were stored at −20°C before shipment and on arrival at DITM, and shipment (courier service) was conducted at ambient temperature. Briefly, DNA was prepared using the Puregene DNA isolation kit (Gentra Systems) as described elsewhere (Table 1).19

**Amplification of *rpoB* and *rpsL* genes.** A partial sequence of the mycobacterial *rpoB* gene (342 bp) encompassing the RRDR was amplified by *Mycobacterium* genus-specific primers as described by Kim and others.22

A set of *Mycobacterium* genus-specific primers (rpsL-F: 5′-AAC AGC GAG AAC GAA GGC C-3′; rpsL-R: 5′-TCA CCA GTT GGC TGA CCA G-3′) was used to amplify a sequence, including the entire *rpsL* gene (375 bp). The thermal-cycling protocol consisted of initial denaturation at 95°C (7 minutes) followed by 37 repeats [95°C (20 seconds), 52°C (25 seconds), and 72°C (45 seconds)] and a final extension at 72°C (5 minutes). Because of the very low frequency of *rrs* gene mutation reported from phenotypic SM-resistant isolates of the MTBC, we did not analyze this region in *M. ulcerans*.14,15

**Gel extraction of PCR products.** Amplicons were electrophoresed in a 1.2% agarose gel prepared with Tris-Acetate EDTA (TAE) buffer light (Roth, Karlruhe, Germany). Positive bands where cut out with a single sterile scalpel for each amplion. Purification was carried out using the Millipore Ultrafree DA kit (Roth).

**DNA sequencing.** Cycle sequencing was performed according to the manufacturer’s protocol on an ABI3730 automatic sequencer (Applied Biosystems, Darmstadt, Germany) at Helmholtz Research Center, Neuherberg, Germany. For each gene, a forward and a reverse sequence were generated.

**Species identification.** In case deviant nucleotide sequences from *M. ulcerans* wild type (Agy99, complete genome; accession number CP000325) were detected, verification of the species *M. ulcerans* or identification of a contamination by another species was attempted by sequence analysis of the following genes using *Mycobacterium* genus-specific primers: 16S *rRNA* gene (924 bp), 65 kDa heat shock protein (*HSP*) gene (644 bp), and 16S-23S *rRNA* internal transcribed spacer gene (ITS; 220 bp) in accordance with the authors’ protocols. With respect to the observations regarding the inaccuracy of 16S *rRNA* sequencing results of non-tuberculous mycobacteria made by Turenne and others,24 sequencing of the two additional regions of the *M. ulcerans* genome served as a quality-assurance measure.23-26

**Sequence analysis.** Sequences were analyzed using DNASIS Max software (MiraiBio, San Francisco, CA) and aligned with the *M. ulcerans* wild-type sequence (Agy99) of the respective gene. BLASTn analysis was performed on entries of GenBank. Quality assurance of 16S *rRNA* and ITS results was performed within ribosomal differentiation of microorganisms (RIDOM).24

Definitive sequences were defined as wild type (WT; 100% nucleotide concordance with *M. ulcerans*, Agy99) or mutation (MUT; <3% nucleotide deviation from the WT for the respective gene and positive species identification for *M. ulcerans*). No result (NR) subsumes non-analyzable sequences [i.e., negative (non-amplifiable) and contaminated (mixed) sequences as well as sequences deviating >3% from the WT]. For each patient, the sequencing results of different specimens were aligned, and the consensus result (WT, MUT, or NR) is shown in Table 1.

**Comparison of whole-genome extracts with culture extracts.** The efficiency of sequencing was defined as the number of analyzable sequences divided by the number of extracts subjected to sequencing. The overall efficiencies of sequencing of *rpoB* and *rpsL* genes from all whole-genome extracts analyzed in this study were compared with those from all available culture extracts of clinical specimens from all patients and types of lesions (Table 2).

**Comparison of whole-genome extracts with corresponding culture extracts.** From 45 patients (ulcerative and non-ulcerative lesions), whole-genome extracts and culture material derived from corresponding swab and/or tissue samples obtained from the same patients were available for comparison. The efficiencies of sequencing of *rpoB* and *rpsL* genes from whole-genome extracts were directly compared with those of the corresponding culture extracts (Table 3).

**Statistical analysis.** For all statistical analyses, approximate tests (χ² tests) and exact tests (Fisher’s exact tests) were conducted using Stata software (version 9.0; Stata Corp., College Station, TX). The results of statistical analyses were presented by means of *P* values, whereby significant differences were defined as *P* values below 0.05. *P* values did not serve only for hypothesis testing but as base for discussion. The study was cross-sectional, and no specific selection or randomization of study participants was performed.

**RESULTS**

**Laboratory confirmation.** From all 162 patients (100%), the *IS2404* DRB-PCR result was positive. Of those, positive culture results (confirmed by standard *IS2404* PCR were obtained from 87 patients (53.7%).

**RpoB sequencing results per patient.** Definite sequencing results of the *rpoB* gene (obtained from culture isolates and/or whole-genome extracts from swab and/or tissue samples) were retrieved from 109 of 162 laboratory-confirmed cases (67.3%). The *rpoB* WT sequence of *M. ulcerans* was detected in 108 patients (99.1%; Table 1). The isolate of one patient (0.9%; treatment group S+NK) showed a mutation at codon Ser522
Among these, five strains showed highly deviated strain Agy99. Identification, however, did not allow the allocation to a distinct mycobacterial species; therefore, a contamination with closely related species could not be excluded for these cases.

**RpsL sequencing results per patient.** Definite sequencing results of the *rpsL* gene (obtained from culture isolates and/or whole-genome extracts from swab and/or tissue samples) were retrieved for 74 of 125 laboratory-confirmed patients (59.2%). All of these showed the *M. ulcerans* WT *rpsL* sequence. For 51 patients (40.8%), no results could be obtained (NR; Table 1).

**Comparison of whole-genome extracts with culture extracts.** Among all clinical specimens analyzed in this study, the overall efficiency of *rpoB* sequencing of culture extracts (91.7%) was significantly higher (*P* < 0.01) than the efficiency of sequencing of whole-genome extracts (35.3%). Likewise, the overall efficiency of *rpsL* sequencing of culture extracts (83.6%) was significantly higher (*P* < 0.01) than the efficiency of sequencing of whole-genome extracts (36.1%).

Among the whole-genome extracts, 64.7% of the *rpoB* sequences (63.4% negative and 36.6% mixed) and 63.9% of the *rpsL* sequences (68.4% negative and 31.6% mixed) were non-analyzable. Among the culture extracts, 83.3% of the *rpoB* sequences (75% negative and 25% mixed) and 16.4% of the *rpsL* sequences (81.8% negative and 18.2% mixed) were non-analyzable (Table 2).

**Comparison of whole-genome extracts with corresponding culture extracts.** Among clinical samples from patients with corresponding culture extracts and whole-genome extracts, the efficiency of *rpoB* sequencing of culture extracts (97.8%) was significantly higher (*P* < 0.01) than that of whole-genome extracts (44.2%). Likewise, the efficiency of *rpsL* sequencing from culture extracts (91.1%) was significantly higher (*P* < 0.01) than the efficiency of sequencing of whole-genome extracts (35.6%; Table 3).

### Table 2

**Comparison of *rpoB* and *rpsL* gene-sequencing results of whole-genome extracts and culture extracts**

<table>
<thead>
<tr>
<th>Sequenced genes</th>
<th>Culture extracts</th>
<th>Whole-genome extracts</th>
<th><em>P</em> value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Swab extracts</td>
<td>Tissue extracts</td>
<td>Total</td>
</tr>
<tr>
<td><em>rpoB</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total analyzed</td>
<td>39</td>
<td>57</td>
<td>96</td>
</tr>
<tr>
<td>Definite sequences†</td>
<td>35 (89.7%)</td>
<td>53 (93.0%)</td>
<td>88 (91.7%)</td>
</tr>
<tr>
<td>Non-analyzable</td>
<td>4 (10.3%)</td>
<td>4 (7.0%)</td>
<td>8 (8.3%)</td>
</tr>
<tr>
<td>Negative**</td>
<td>3 (75%)</td>
<td>3 (75%)</td>
<td>6 (75%)</td>
</tr>
<tr>
<td>Mixed††</td>
<td>1 (25%)</td>
<td>1 (25%)</td>
<td>2 (25%)</td>
</tr>
</tbody>
</table>

|                 | Swab extracts    | Tissue extracts       | Total    |
| *rpsL*          |                  |                       |          |
| Total analyzed  | 26               | 41                    | 67       |
| Definite sequences† | 23 (88.5%) | 33 (80.5%)           | 56 (83.6%) | <0.01 |
| Non-analyzable  | 3 (11.5%)        | 8 (19.5%)             | 11 (16.4%) |
| Negative**      | 2 (66.7%)        | 7 (87.5%)             | 9 (81.8%) |
| Mixed††         | 1 (33.3%)        | 1 (12.5%)             | 2 (18.2%) |

Table 2 shows *rpoB* and *rpsL*-sequencing results from whole-genome extracts and culture extracts of 162 IS2404 PCR-confirmed BUD patients.

* DNA was extracted from mycobacterial cultures that were inoculated from swab or tissue specimens.
† DNA was extracted from tissue or swab specimens as whole-genome extracts.
‡ DNA was extracted from cultured strains that were inoculated from swab or tissue specimens.
§ DNA was extracted from mycobacterial cultures that were inoculated from swab or tissue specimens.
¶ DNA was extracted from tissue or swab specimens as whole-genome extracts from ulcerative lesions.
†† Mixed sequence is the number of extracts that could not be analyzed, because amplified sequences were contaminated.
‡‡ Number of extracts with non-analyzable sequences (the result is a mixed or non-amplifiable sequence in PCR).
** Number of extracts with non-amplifiable sequences (negative).

### Table 3

**Comparison of corresponding whole-genome extracts and culture extracts**

<table>
<thead>
<tr>
<th>Sequenced genes</th>
<th>Corresponding specimens*</th>
<th><em>P</em> value†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Culture extracts</td>
<td>Whole-genome extracts</td>
</tr>
<tr>
<td><em>rpoB</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total analyzed</td>
<td>45</td>
<td>45</td>
</tr>
<tr>
<td>Definite sequences†</td>
<td>44 (97.8%)</td>
<td>19 (42.2%)</td>
</tr>
<tr>
<td>Non-analyzable</td>
<td>1 (2.2%)</td>
<td>26 (57.8%)</td>
</tr>
<tr>
<td>Negative§</td>
<td>0</td>
<td>12 (46.2%)</td>
</tr>
<tr>
<td>Mixed††</td>
<td>1 (100%)</td>
<td>14 (53.8%)</td>
</tr>
</tbody>
</table>

| *rpsL*          |                          |          |
| Total analyzed  | 45                       | 45       |
| Definite sequences† | 41 (91.1%) | 16 (35.6%) | <0.01 |
| Non-analyzable  | 4 (8.9%)                 | 29 (64.4%) |
| Negative§       | 4 (100%)                 | 19 (65.5%) |
| Mixed††         | 0                        | 10 (34.5%) |

Table 3 shows *rpoB* and *rpsL*-sequencing results of corresponding whole-genome and culture extracts.

* From 45 patients, culture extracts (inoculated from swab or tissue specimens) were analyzed, and results are compared with the corresponding whole-genome extract.
† DNA was extracted from cultured strains that were inoculated from swab or tissue specimens (for ulcerative lesions) or solely from tissue specimens of non-ulcerative lesions. Per patient, either a swab or a tissue specimen was analyzed corresponding to the specimen of which a mycobacterial culture was inoculated and a positive confirmatory IS2404 PCR result was obtained.
‡ DNA is the complete sequence of the gene encoding the ribosomal protein S12 (375 bp).
one patient and could not be amplified from specimens of the second patient (NR).

DISCUSSION

Conventional in vitro resistance testing according to the proportion method constitutes the most widespread technique within the WHO global surveillance system of antimycobacterial drug resistance of tuberculosis. Because of the inability to cultivate \textit{M. leprae}, sequence-based detection of drug resistance has successfully been applied for leprosy. According to WHO recommendations, \textit{M. leprae} isolates with an amino acid change in a drug resistance-determining region that have been confirmed by mouse footpad testing to confer phenotypic drug resistance are scored as resistant. Sequence-based resistance testing was also proven to be a rapid and reliable alternative to conventional resistance testing of \textit{M. tuberculosis}.

\textit{M. ulcerans} strains expressing phenotypic and genotypic resistance were generated under RMP monotherapy in a mouse model, but systematic drug resistance surveillance in BUD patients treated by surgery and/or antimycobacterial drugs. At the time of the study, conventional susceptibility testing was also proven to be a rapid and reliable alternative to conventional drug resistance testing according to the \textit{in vitro} method. Therefore, monitoring of drug resistance will facilitate individual clinical management decisions, especially in recurrences, defaulters, and patients with non-healing lesions.

With respect to the long generation time of \textit{M. ulcerans} and the limited sensitivity of the method, especially in pre-treated patients, cultures alone are not ideal for genotypic drug resistance testing. Especially for clinical management decisions, whole-genome extracts constitute a better diagnostic target to obtain rapid results. In our study, analysis of cultured isolates yielded definite sequencing results for \textit{rpoB} and \textit{rpsL} genes in > 80\%, whereas the number of definite results obtained from whole-genome extract was significantly lower (<40\% for \textit{rpoB} and \textit{rpsL} genes). The percentage of non-analyzable sequences among whole-genome extracts was > 60\%. Among these, the respective sequences of 63.4\% of the \textit{rpoB} and 68.4\% of the \textit{rpsL} genes were non-amplifiable. In general, the presence of only a low amount of mycobacterial DNA in clinical samples often hampers diagnostic procedures and may also constitute a source of error in the present study. Amplification of mixed sequences from related species existing as commensals on the human skin represents another challenge in sequencing of whole-genome extracts.

According to the results of this study, the authors consider it essential to apply refined techniques in further studies. The design of more specific primers can improve the efficiency of sequencing of whole-genome extracts. Optimization of extraction procedures (e.g., mechanical homogenization by zirconium beads followed by enzymatic lysis) can augment the yield of \textit{M. ulcerans} DNA recovered from swab and tissue samples. Immunomagnetic separation, developed and successfully applied in environmental studies by Marsollier and others, may be a promising tool to concentrate and purify \textit{M. ulcerans} from clinical samples; however, this technique is currently restricted to BUD reference laboratories or collaborative research programs.

From 12\% of the study subjects, documentation of previous antimycobacterial treatment could not be retrieved from the clinical records. These partially incomplete sets of data, therefore, constitute a weakness of this study. With respect to correct interpretation of results, further studies on drug resistance in \textit{M. ulcerans} should aim at obtaining complete and detailed patient-related information. WHO provides a form (BU01) for the recording of clinical, epidemiological, and treatment data, which, in general, is available in all endemic countries. A consequent use of the BU01 form will facilitate the collection of relevant information.

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