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Comparison of Two Assays for Molecular Determination of Rifampin Resistance in Clinical Samples from Patients with Buruli Ulcer Disease

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This study evaluates a novel assay for detecting rifampin resistance in clinical *Mycobacterium ulcerans* isolates. Although highly susceptible for PCR inhibitors in 50% of the samples tested, the assay was 100% *M. ulcerans* specific and yielded >98% analyzable sequences with a lower limit of detection of 100 to 200 copies of the target sequence.

Buruli ulcer disease (BUD), caused by *Mycobacterium ulcerans*, involves the skin, subcutaneous fatty tissue, and bones and predominantly affects children <15 years of age. If left untreated, contractures may cause severe functional limitation. Standardized antimycobacterial treatment consists of rifampin (RMP) and streptomycin administered for 8 weeks. An oral regimen combining RMP and clarithromycin is currently under clinical evaluation (1–3). Notwithstanding the efficiency of chemotherapy, treatment failures and various types of secondary lesions have been reported, suggesting the need for customized clinical management strategies (4–6).

Tuberculosis and leprosy studies have shown that RMP treatment is prone to the development of drug resistance due to missense mutations within the RMP resistance-determining region (RRDR) of the mycobacterial *rpoB* gene (7, 8). RRDR mutations in *M. ulcerans* have been described in a mouse model (9); data on drug resistance among clinical isolates, however, are scarce. A pilot study on molecular drug resistance testing conducted by our group from 2004 through 2007 in Ghana revealed a low level (0.9%) of RMP resistance. However, the overall test efficiency of the assay applied in the pilot study (referred to here as assay A) was low (35%) (10). Therefore, the aim of this study was to develop an improved sequencing assay (referred to here as assay B).

The study was approved by the National Togolese Ethics Committee (14/2010/CRBS) and the Ghanaian Kwame Nkrumah University of Science and Technology Ethics Committee (CHRPE/91/10).

The primers MuB-F and MuB-R were designed to amplify a 606-bp region encompassing the RRDR by alignment of (myco)bacterial *rpoB* genes as retrieved from GenBank (PubMed, NCBI) using DNASIS Max (MiraiBio, San Francisco, CA) (see Table S1 in the supplemental material). MuB-F specifically binds a polymorphic region of the mycobacterial *rpoB* gene (11); the sequencing primer Bseek-F binds downstream of primer MuB-F (Table 1). Amplification was conducted using the *Thermococcus kodakaraensis*-derived KOD Hot Start polymerase (Merck, Darmstadt, Germany) followed by performing agarose gel electrophoresis, purification of PCR products, cycle sequencing, and sequence analysis, as previously described (10) (Table 1; see also PCR Protocol S2 and S3 in the supplemental material).

PCR standards were generated by exact quantification of whole-genome DNA from two *M. ulcerans* cultures from Ghana by IS*2404* quantitative real-time PCR (12, 13). The limits of detection for the two assays were determined by testing 10-fold serial dilutions of PCR standards. The analytical sensitivity of assay B was 10 times higher than that of assay A (100 to 200 and 1,000 to 2,000 copies of the *rpoB* gene, respectively).

The specificity of assay B was assessed with DNA extracts of 18 closely related human-pathogenic mycobacterial species and five bacterial species frequently colonizing human skin (12, 14–16) (Table 2). Besides *M. ulcerans*, only *M. marinum* was amplified. As *rpoB* wild-type sequences of these two species can be differentiated in two nucleotides by sequencing, assay B was considered *M. ulcerans* specific.

To determine the performances of the two assays on clinical specimens, 133 whole-genome extracts from IS*2404*-positive samples collected before the onset of treatment (swab samples, $n = 63$; fine-needle aspirates [FNA], $n = 40$; 3-mm punch biopsy specimens, $n = 30$) from 91 BUD patients from Togo (17, 18) were assessed. P values of <0.05 were considered significant.

Due to initial *rpoB* PCR inhibition, significantly more DNA extracts had to be diluted when subjected to assay B (54.1%, 72/133) than when subjected to assay A (7.5%, 10/133) ($P < 0.01$). With a P value of 0.39, the overall *rpoB* PCR positivity rate (i.e., the proportion of positive *rpoB* PCR results among all samples tested) was not significantly different in assay A (56.4%, 75/133) and assay B (51.1%, 68/133). However, the *rpoB* PCR positivity rate of swab samples was significantly higher in assay A (50.8%, 32/63) than in

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TABLE 1 Primer sequences and nucleotide positions of *rpoB* PCR and sequencing primers[a]

Assay	Primer[b]	Sequence (5′ to 3′)[c]	Nucleotide position[d]	Amplicon size, bp[e]
A[f]	MF	CGA CCA CTT CGG CAA CCG	785041–785058	351
	MR	TCG ATC GGG CAC ATC CGG	785374–785391	
B[g]	MuB-F	CAT CAC CAG CTC GAC GCT	784912–784929	606
	MuB-R	TGG ATC TCG TCG GAA ACG	785500–785517	
	Bseek-F	AAT ACC TGG TCC GCT TGC	784959–784976	

[a] Shown are the primer sequences, nucleotide positions within the *M. ulcerans* genome, and corresponding amplicon sizes. The *rpoB* gene encodes the beta subunit of (myco)bacterial RNA polymerases. Significant sequence concordances of primers with human or other (myco)bacterial DNA were excluded by Primer BLAST (PubMed, NCBI).
[b] F, forward primer; R, reverse primer. Primers MF and MR were used in assay A for the amplification of a 351-bp fragment of the *M. ulcerans rpoB* gene (including the RRDR) encompassing the region sequenced by primer MF. Primers MuB-F and MuB-R were used in assay B for the amplification of a 606-bp fragment of the *M. ulcerans rpoB* gene (including the RRDR) encompassing the region sequenced by primer Bseek-F.
[c] Primer sequence from the 5′ to the 3′ end.
[d] Nucleotide positions are provided for the respective amplicon in *M. ulcerans* strain Agy99 (GenBank accession no. CP000325 [PubMed, NCBI]).
[e] Amplicon sizes for *rpoB* PCR of assay A or B, respectively.
[f] For assay A, final concentrations of PCR reagents per 20-μl reaction: 0.5 μM each primer (TIB-Molbiol, Berlin, Germany); 2.5 mM $MgCl_2$, 0.8 mM deoxynucleoside triphosphates (dNTPs), 0.05 U/μl AmpliTaq Gold DNA polymerase, 1× PCR buffer II (Applied Biosystems, Foster City, CA); template DNA, 2 μl; run protocol, 95°C for 5 min, 37 cycles at 95°C for 15 s, 56°C for 15 s, and 72°C for 30 s, and final extension at 72°C for 5 min.
[g] For assay B, final concentrations of PCR reagents per 20-μl reaction: 0.3 μM each primer (TIB-Molbiol); 1.5 mM $MgSO_4$, 0.8 mM dNTPs, 0.02 U/μl KOD Hot Start polymerase, 1× PCR buffer for KOD (Merck, Darmstadt, Germany); template DNA, 2 μl; run protocol, 95°C for 2 min and 39 cycles at 95°C for 20 s, 63°C for 10 s, and 70°C for 15 s.

assay B (30.2%, 19/63) ($P = 0.02$). Among all of the samples with a positive *rpoB* PCR result in both assays, the proportion of samples yielding a definite sequencing result (overall *rpoB* sequencing positivity rate) was significantly higher for assay B (98.0%, 48/49) than for assay A (85.7%, 42/49) ($P = 0.03$). Among all the samples tested, the proportion of samples yielding a definite *rpoB* sequencing result (overall test efficiency) was 39.8% (53/133) for assay A and 48.9% (65/133) for assay B ($P = 0.14$). Following stratification of the overall test efficiencies into different sample types, the test efficiency for FNA samples was significantly higher in assay B (70.0%, 28/40) than in assay A (35.0%, 14/40) ($P < 0.01$) (Table 3).

TABLE 2 Specificity of assay B[a]

Bacterial species	Source[b]	Isolate origin	Result[c]
Mycobacterium abscessus	NRZ	Human[d]	−
Mycobacterium africanum	NRZ	Human[d]	−
Mycobacterium avium	NRZ	Human[d]	−
Mycobacterium bovis	NRZ	Cattle[d]	−
Mycobacterium chelonae	NRZ	Human[d]	−
Mycobacterium fortuitum	NRZ	Human[e]	−
Mycobacterium gordonae	NRZ	Human[e]	−
Mycobacterium gordonae	DITM	Human[e]	−
Mycobacterium kansasii	NRZ	Human[d]	−
Mycobacterium leprae	DITM	Human[d]	−
Mycobacterium malmoense	NRZ	Human[e]	−
Mycobacterium marinum	NRZ	Human[d]	+
Mycobacterium microti	NRZ	Mouse[d]	−
Mycobacterium scrofulaceum	NRZ	Human[d]	−
Mycobacterium smegmatis	NRZ	Human[d]	−
Mycobacterium szulgai	NRZ	Human[d]	−
Mycobacterium tuberculosis	NRZ	Human[d]	−
Mycobacterium ulcerans	DITM	Human[d]	+
Mycobacterium xenopi	NRZ	Human[e]	−
Escherichia coli	MVP	Human[e]	−
Propionibacterium acnes	MVP	Human[d]	−
Staphylococcus aureus	MVP	Human[e]	−
Staphylococcus epidermidis	MVP	Human[e]	−
Streptococcus pyogenes	MVP	Human[d]	−

[a] Shown are (myco)bacterial species commonly contaminating human skin and the results of the specificity testing of sequencing assay B.
[b] DNA extracts that were not available at the Department of Infectious Diseases and Tropical Medicine, University Hospital, Ludwig-Maximilians-University, Munich, Germany (DITM) were provided by the National Reference Center for Mycobacteria, Borstel, Germany (NRZ), and the Max von Pettenkofer-Institut, Ludwig-Maximilians-University, Munich, Germany (MVP).
[c] Results of DNA extracts subjected to assay B: +, positive PCR result; −, negative PCR result.
[d] The primary patient isolates were considered pathogenic bacteria.
[e] The primary patient isolates were considered commensals/contaminants of clinical samples.

The *Mycobacterium* genus-specific primers applied in assay A resulted in 30.7% (23/75) coamplification of DNA from other bacterial species (e.g., *Corynebacterium* species), resulting in non-analyzable mixed *rpoB* sequences in these cases. In contrast, assay B did not detect any species other than *M. ulcerans* strain Agy99 (data not shown).

Furthermore, 12 IS*2404* PCR-confirmed whole-genome extracts from the pretreatment samples of 10 BUD patients from Ghana which had yielded contaminated sequences ($n = 9$) or no sequencing results ($n = 3$) in assay A (10) were reexamined with assay B. Out of these, 11 (91.7%) rendered definite *M. ulcerans rpoB* wild-type sequences, and one sample remained negative in assay B.

The current level of RMP resistance among clinical *M. ulcerans* isolates in countries where BUD is endemic is unknown. Although there is no evidence for, and therefore no general concern about, person-to-person transmission of drug-resistant *M. ulcerans* strains, individual treatment outcomes of BUD patients may be compromised by drug resistance evolving under chemotherapy. Slow-healing lesions related to drug resistance may negatively influence the manifestation of disabilities; nonhealers and recurrences caused by resistant strains may benefit from timely surgical intervention in default of alternative antimycobacterial drugs. Therefore, rapid molecular assays for the detection of drug resistance constitute valuable tools for supporting individual clinical management decisions and prerequisites for new drug trials.

Although the overall *rpoB* PCR positivity rates and overall test efficiencies did not differ significantly in the two assays we compared, 20% more *rpoB* amplicons were generated from swab samples in assay A. However, due to the high specificity of sequencing assay B, out of those swab samples with a positive *rpoB* PCR in both assays, 100% yielded analyzable sequences in assay B, in con-

TABLE 3 Comparison of the results from clinical samples of Togolese BUD patients subjected to assays A[a] and B[b,c]

Test, parameter, and sample collection type	% (no. of samples with a positive result/no. of samples tested) (95% confidence interval)			
	PCR A	PCR B	PCR A and PCR B (concordance rate)[d]	McNemar's *P*[e]
rpoB PCR				
Positivity rate[f]				
All	56.4 (75/133) (48.0–64.8)	51.1 (68/133) (42.6–59.6)	66.2 (88/133) (58.1–74.2)	0.39
Swabs	50.8 (32/63) (38.4–63.1)	30.2 (19/63) (18.8–41.5)	57.1 (36/63) (44.9–69.4)	0.02[g]
Punch biopsy	73.3 (22/30) (57.5–89.2)	66.7 (20/30) (49.8–83.5)	86.7 (26/30) (74.5–98.8)	0.58
FNA	52.5 (21/40) (37.0–68.0)	72.5 (29/40) (58.7–86.3)	65.0 (26/40) (50.2–79.8)	0.06
Initial inhibition[h]				
All	07.5 (10/133) (03.0–12.0)	54.1 (72/133) (45.7–62.6)	48.9 (65/133) (40.4–57.4)	<0.01[g]
Swabs	09.5 (6/63) (02.3–16.8)	65.1 (41/63) (53.3–76.9)	41.3 (26/63) (29.1–53.4)	<0.01[g]
Punch biopsy	06.7 (2/30) (00.0–15.6)	30.0 (9/30) (13.6–46.4)	76.7 (23/30) (61.5–91.8)	0.02[g]
FNA	05.0 (2/40) (00.0–11.8)	55.0 (22/40) (39.6–70.4)	40.0 (16/40) (24.8–55.2)	<0.01[g]
rpoB-Sequencing				
Positivity rate[i]				
All	85.7 (42/49) (75.9–95.5)	98.0 (48/49) (94.0–100)	87.8 (43/49) (78.6–96.9)	0.03[g,j]
Swabs	75.0 (9/12) (50.5–99.5)	100 (12/12) (NA[k])	75.0 (9/12) (50.5–99.5)	0.07[j]
Punch biopsy	100 (19/19) (NA[k])	100 (19/19) (NA[k])	100 (19/19) (NA[k])	NA[k]
FNA	77.8 (14/18) (58.6–97.0)	94.4 (17/18) (83.9–100)	77.8 (14/18) (58.6–97.0)	0.15[j]
Efficiency[l]				
All	39.8 (53/133) (31.5–48.2)	48.9 (65/133) (40.4–57.4)	74.4 (99/133) (67.0–81.8)	0.14
Swabs	28.6 (18/63) (17.4–39.7)	27.0 (17/63) (16.0–37.9)	73.0 (46/63) (62.1–84.0)	0.84
Punch biopsy	70.0 (21/30) (53.6–86.4)	66.7 (20/30) (49.8–83.5)	90.0 (27/30) (79.3–100)	0.78
FNA	35.0 (14/40) (20.2–49.8)	70.0 (28/40) (55.8–84.2)	65.0 (26/40) (50.2–79.8)	<0.01[g]

[a] Assay A as applied in the pilot study.
[b] The newly developed assay B.
[c] Shown are PCR and sequencing results of all clinical samples subjected to assays A and B.
[d] Proportion of samples with concordant results in assays A and B out of all samples tested by both assays.
[e] *P* value of McNemar chi-square test for matched pairs of samples with categorical test results.
[f] Overall *rpoB* PCR positivity rate: the proportion of positive *rpo*B PCR results among all samples tested (assay A or B, respectively).
[g] *P* values of <0.05 were considered significant.
[h] Proportion of samples which led to PCR inhibition if tested undiluted out of all samples tested by PCR in assay A or B, respectively.
[i] Overall *rpoB*-sequencing positivity rate: proportion of samples yielding a definite sequencing result among all samples with a positive *rpoB* PCR result in both assays.
[j] Fisher's exact test (at least one cell, $n < 5$).
[k] NA, not applicable.
[l] Overall test efficiency: the proportion of samples yielding a definite sequencing result (assay A and/or B) among all samples tested.

trast to 75% in assay A. In general, assay A resulted in 30% mixed sequences, while assay B yielded a significantly higher proportion of definite sequencing results with 98% analyzable sequences. However, the overall susceptibility of assay B to the presence of PCR inhibitors was considerable, as 50% of all clinical samples had to be diluted, compared to <10% of them in assay A. In turn, dilution leads to decreased *M. ulcerans* DNA yields, certainly affecting the *rpoB* PCR positivity rate of assay B. Furthermore, for FNA samples, the overall test efficiency was significantly higher in assay B (70%), suggesting FNAs as the most appropriate sample type for molecular drug resistance testing, which is compatible with current WHO recommendations for sample collection (19).

In conclusion, assay B constitutes a sensitive and 100% *M. ulcerans*-specific molecular tool for determining rifampin resistance with the highest efficiency in FNA samples. In combination with a recently described 16S rRNA-based viability assay (12), molecular drug resistance testing would also allow a reliable differentiation of individuals harboring viable drug-resistant organisms opposed to mycobacterial DNA residues detectable in secondary lesions (4). Furthermore, novel real-time PCR high-resolution melt analysis assays without allele-specific primers or probes may constitute a promising tool for screening clinical isolates in future studies (20).

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