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Untersuchungen zur Epidemiologie, Früherkennung, Diagnostik und Behandlung der Buruli-Ulkus-Erkrankung in Westafrika

vorgelegt von

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Diese Arbeit ist meinem Vater Wilfried Beißner gewidmet.

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Die Zusammenfassung dieser Arbeit steht unter cc-by-nc-nd

I) Zusammenfassung und Bedeutung der eigenen Arbeiten für das Fachgebiet

I.1 Einleitung

Die Buruli Ulkus Erkrankung („Buruli ulcer disease“, BUD) wird durch eine Infektion der Haut und des subkutanen Fettgewebes mit *Mycobacterium ulcerans* hervorgerufen. BUD zählt zu den derzeit 20 vernachlässigten Tropenkrankheiten und wurde aus 33 Ländern Afrikas, Südamerikas, Asiens und des West-Pazifiks gemeldet. Mit Ausnahme von Australien, China und Japan treten die meisten Fälle in tropischen bzw. subtropischen Regionen auf. Seit 2002 wurden der WHO weltweit über 55.000 Fälle gemeldet, wobei die Dunkelziffer nicht registrierter Fälle weitaus höher liegen wird. Die Endemiegebiete befinden sich zumeist fokussiert in ländlichen Regionen mit stehenden bzw. langsam fließenden Gewässern in West- und Zentralafrika, v.a. in der Elfenbeinküste, in Ghana, Togo, Benin, Kamerun und der Demokratischen Republik Kongo. Hauptsächlich sind Kinder unter 15 Jahren, die vorwiegend unter Armutsbedingungen leben, von der Erkrankung betroffen. Exakte globale Daten zur Inzidenz und Prävalenz des Buruli Ulkus sind aufgrund mangelnder Kapazitäten für eine Laborbestätigung klinischer Verdachtsfälle nicht bekannt. Jedoch wurden 2018 aus den Endemiegebieten Afrikas 86 % der weltweit neu gemeldeten Fälle registriert. Die Ergebnisse bisheriger Transmissions-Studien schließen eine Infektion aus der belebten Umwelt nicht aus. Der exakte Transmissionsweg ist jedoch gegenwärtig nicht geklärt. BUD beginnt typischerweise mit nicht-ulzerativen Hauteffloreszenzen wie einem Nodulus, einer Papel oder Plaque aus denen unbehandelt ein Ulkus mit typischen unterminierten Rändern entstehen kann. Ohne adäquate Behandlung führt die Erkrankung bei geringer Mortalität zu entstellenden Narben und Kontrakturen, die mit erheblichen Funktionseinschränkungen der betroffenen Gelenke einhergehen können. Es existieren bislang keine ausreichend belegten Daten zu primären Präventionsstrategien. Die Kontrolle der Erkrankung zur Vermeidung von Spätkomplikationen besteht daher in der klinischen Früherkennung und der Laborbestätigung zur zeitnahen Einleitung einer antimykobakteriellen Therapie. Zur Sicherung der klinischen Diagnose stehen bakteriologische, molekularbiologische und histopathologische Untersuchungsmethoden zur Verfügung. Prinzipiell fordert die WHO die Laborbestätigung eines BUD Verdachtsfalles vor der Einleitung der achtwöchigen Kombinationstherapie mit Rifampicin (RMP) und Clarithromycin (CLR) *per os* [WHO 2017[1], WHO 2017[2], WHO 2019].

Im Rahmen von zwei durch die Europäische Kommission geförderten Forschungsprojekten im FP6 („Project No. INCO-CT-2005-015476-BURULICO“) und im FP7 („Agreement No. 241500 – BuruliVac“) – Antragstellerin und Projektleiterin: Frau Prof. Dr. G. Bretzel – sowie weiteren Projekten unserer Arbeitsgruppe, die durch Drittmittel von der Deutschen Lepra- und Tuberkulosehilfe (DAHW), der Friedrich-Baur Stiftung, dem Deutschen Zentrum für Infektionsforschung (DZIF) und der Foundation for Innovative New Diagnostics (FIND) gefördert werden, konnten die im Folgenden beschriebenen Studien zur Epidemiologie, Früherkennung, Diagnostik und Behandlung der Buruli Ulkus Erkrankung in Westafrika von mir in Teilen als „Co-Investigator“ und in Teilen als „Principal Investigator“ durchgeführt werden.

I.2 Klinisches Bild

Die BUD tritt durch unterschiedliche klinische Manifestationen in Erscheinung und umfasst nicht-ulzerative sowie ulzerative Formen. Das erste Symptom kann ein schmerzloser und verschieblicher Nodulus unter der Haut oder eine Papel sein. Andere Manifestationsformen können sich als Plaque oder Ödem (letzteres auch in Abwesenheit anderer Hautefflorescenzen) darstellen. Im weiteren Verlauf treten indolente Ulzerationen der Dermis auf, die zunächst typischerweise unterminierte Ränder aufweisen. Dieser Übergang kann sich innerhalb von wenigen Tagen bis Wochen manifestieren. Die Knochen können ebenfalls befallen sein. Klinisch werden die Läsionen in drei Kategorien eingeordnet: Zur Kategorie I zählen einzelne Läsionen unter 5 cm, Kategorie II umfasst einzelne Läsionen zwischen 5-15 cm und in die Kategorie III werden alle Läsionen über 15 cm, Osteomyelitiden, Hauterscheinungen mit Gelenkbeteiligung sowie multiple Läsionen und solche an einer kritischen Lokalisation (Genitale, Gesicht) eingeteilt [WHO 2017[1], WHO 2019].

I.3 Diagnostik zur Laborbestätigung

Zur Diagnostik der BUD stehen vier etablierte Methoden zur Verfügung: Die mikroskopische Untersuchung zur Detektion säurefester Stäbchenbakterien (MIK), wie z. B. Ziehl-Neelsen gefärbter Ausstriche aus Wundabstrichen ulzerativer Läsionen oder von Feinnadelaspiraten (FNA) nicht-ulzerativer Läsionen, der DNA Nachweis von *M. ulcerans* mittels PCR sowie die Kultur des Erregers aus den gesamten Untersuchungsmaterialien und die histopathologische Untersuchung von 3 mm Stanzbiopsien oder exzidiertem Gewebe.

1) Die MIK stellt eine schnelle, kostengünstige und einfach durchzuführende Methode dar. Die Beurteilung hängt wesentlich von der Qualität des Ausstriches sowie der Erfahrung des Untersuchers ab. Der positive prädiktive Wert (PPW) der Untersuchung liegt bei 97 % und die diagnostische Sensitivität wird in verschiedenen Studien mit 40-70 % angegeben. Eine kontinuierliche Qualitätssicherung muss gegeben sein.

2) Die Polymerase-Kettenreaktion (PCR) zur Amplifikation einer 100 % erregerspezifischen Insertionssequenz (IS*2404*) aus klinischen Proben stellt mit einem PPW von 100 % und einer diagnostischen Sensitivität von 79-95 % den empfindlichsten und spezifischsten Test zur Laborbestätigung dar. Die Methode liefert in technisch entsprechend ausgestatteten nationalen Referenz- bzw. Forschungslaboren mit gut geschultem Laborpersonal verlässliche Ergebnisse in kurzer Zeit. Die PCR ist kostenaufwendig und bedarf ebenfalls einer kontinuierlichen internen und externen Qualitätssicherung.

3) *In vitro* gelingt die Kultur von *M. ulcerans* (KUL) bei einem Temperaturoptimum von 29-33 °C auf verschiedenen Nährmedien (z. B. Löwenstein-Jensen). Die KUL stellt derzeit die phänotypische und etablierte Methode zum Viabilitätsnachweis von *M. ulcerans* dar, benötigt allerdings unter optimalen Bedingungen Inkubationszeiten von mind. 12 Wochen. Daher eignet sich die KUL nicht für zeitnahe Therapieentscheidungen. Die Sensitivität der KUL betrug in verschiedenen Studien 45-70 %.

4) Die histopathologische Untersuchung von Gewebeproben (HIS) ergab eine Sensitivität und Spezifität von $\geq$ 90 %. Die Methode ist mit der Entnahme von invasiven Proben verbunden, wie z. B. Biopsien, die in Endemiegebieten kaum verfügbar sind. Daher kann diese Untersuchung nur bei speziellen Fragestellungen zur Differentialdiagnose oder bei einem Verdacht auf eine paradoxe Reaktion, einem Rezidiv oder einem Therapieversagen eingesetzt werden.

   [Ross BC, et al. 1997; Stinear T, et al. 1999; Siegmund V, et al. 2005; Fyfe J, et al. 2007; Herbinger KH, et al. 2009[1]; Beissner M, et al. 2010; WHO 2010; WHO 2013; WHO, Ed Portaels F, et al. 2014; Bretzel, et al. 2018].

Für die verschiedenen Untersuchungen stehen je nach der klinischen Erscheinung der BUD unterschiedliche Probematerialien bzw. Abnahmetechniken zur Verfügung. Während der Wundabstrich den Goldstandard zur Laborbestätigung ulzerativer Läsionen mit unterminierten Rändern darstellt, ergab sich zwischen 2007-2010 ein Wandel in der Diagnostik nicht-ulzerativer Läsionen und ulzerativer Läsionen mit vernarbten Wundrändern: Aufgrund von ersten Ergebnissen zweier Pilotstudien aus Ghana und Benin empfahl die „Technical Advisory Group" (TAG) der WHO im April 2007 erstmals die Einführung von Feinnadelaspiraten (FNA) als einer minimal invasiven Abnahmetechnik für die MIK und die PCR Diagnostik aus diesen Läsionsformen. Nach dem Vorliegen umfangreicher Validierungsstudien wurde das Ziel formuliert, Stanzbiopsien langfristig durch FNA zu ersetzen [WHO 2007[1]; Eddyani M, et al. 2009; Phillips RO 2009].

1. Studie: „Efficiency of Fine-Needle Aspiration Compared with Other Sampling Techniques for Laboratory Diagnosis of Buruli Ulcer Disease" [Herbinger KH, et al., 2010].

Vor diesem Hintergrund konnten wir in einer retrospektiven Studie mit 173 klinischen BUD Verdachtsfällen aus Ghana und Togo zeigen, dass 3 mm Stanzbiopsien für die MIK und PCR Bestätigung nicht-ulzerativer Läsionen durch FNAs ohne eine signifikante Abnahme der Sensitivität ersetzt werden können. Als alternative Abnahmetechnik zur Stanzbiopsie wurden FNAs auch bei ulzerativen Läsionen angewandt und ergaben keine signifikant geringere Sensitivität in der MIK und PCR.

Die Erkenntnisse aus 3 weiteren Studien, die in Endemiegebieten Ghanas und Benins durchgeführt wurden, stützen die Ergebnisse unserer Studie in Bezug auf die korrespondierenden Sensitivitäten von MIK und PCR aus FNA Proben und Stanzbiopsien nicht-ulzerativer Läsionen [Eddyani M, et al. 2009; Phillips RO, et al. 2009; Cassisa V, et al. 2010]. Für den Nachweis von *M. ulcerans* aus ulzerativen Läsionen waren die Sensitivitäten für die MIK und PCR in allen Studien signifikant höher bei Wundabstrichen als bei FNA oder Stanzbiopsien; zwischen den letzteren beiden Probenarten ergab sich allerdings kein signifikanter Unterschied in der MIK und PCR. Basierend auf diesen Erkenntnissen empfahl die WHO abschließend die primäre Anwendung von FNA zur MIK und PCR Diagnostik aus nicht-ulzerativen Läsionen [WHO 2010]. Stanzbiopsien und chirurgisch exzidierte Gewebeproben sollten fortan speziellen Fragestellungen im klinischen Management vorbehalten bleiben. Dies gilt z. B. bei einer Laborbestätigung von Ulzera mit vernarbten Wundrändern, wenn Abstriche und FNA Proben trotz dringenden klinischen Verdachtes negative PCR Ergebnisse ergeben. Biopsien sind weiterhin indiziert zur differentialdiagnostischen Abklärung mittels HIS und/oder für

eine kulturelle Viabilitätstestung von *M. ulcerans* bei ausbleibender Heilung oder Rezidiven bzw. vor allem bei Sekundärinfektionen. Die Sensitivität von FNA Proben zum kulturellen Nachweis viabler *M. ulcerans* wurde bislang nur in wenigen Studien untersucht und mit 10-44 % angegeben [Eddyani M, et al. 2009; Phillips RO, et al. 2009; Yeboah-Manu D, et al. 2011; Sarpong-Duah M, et al. 2017].

2. Studie: "Loop-Mediated Isothermal Amplification for Laboratory Confirmation of Buruli Ulcer Disease -Towards a Point-of-Care Test" [Beissner M, et al. 2015[1]].

Da die BUD in West- und Zentralafrika vorwiegend bei Patienten in ländlichen und teils weit entlegenen Regionen vorkommt, stellen die Früherkennung und Laborbestätigung für eine zeitnahe Einleitung der Therapie bei dieser Erkrankung die wichtigsten Herausforderungen dar. Aufgrund von langen Transportwegen der klinischen Proben zu nationalen Forschungseinrichtungen bzw. Referenzlaboren wurde die Entwicklung eines Testverfahrens für eine dezentrale (Point-of-Care [POC]) Labordiagnostik von der WHO als einer der Forschungsschwerpunkte festgelegt.

Im Jahre 2012 wurden drei unterschiedliche *M. ulcerans* „loop-mediated isothermal amplification" (LAMP) Assays publiziert. Die Forschergruppe um de Souza et al. validierte einen LAMP Test mit einer Zielsequenz des Mykolakton Gens lediglich anhand weniger ausgewählter Kulturstämme von *M. ulcerans* und wies eine Spezifität von 100 % nach. Njiru et al. und Ablordey et al. hingegen beschrieben 2 unterschiedliche die IS*2404* von *M. ulcerans* amplifizierende LAMP-Assays. Beide Ansätze wurden anhand von diversen klinischen Isolaten und Umweltproben aus Ghana und Australien ausführlich validiert. Diese beiden LAMP-Assays ergaben 100 % Spezifität für *M. ulcerans* und analytische Sensitivitäten von 0,1 bzw. 1,5 *M. ulcerans* Genomäquivalenten. Nichtsdestotrotz wurden all diese bisherigen *M. ulcerans* LAMP-Assays unter optimalen Bedingungen in Referenzlaboratorien evaluiert und benötigten bis dahin ununterbrochene Kühlketten der verwendeten Reagenzien, die auf Trockeneis in die Endemiegebiete verschickt werden mussten [de Souza DK, et al. 2012; Njiru ZK, et al. 2012; Ablordey A, et al. 2012].

In unserer oben genannten Pilotstudie wurde eine trockenreagenz-basierte (DRB) IS*2404* LAMP zum DNA-Nachweis von *M. ulcerans* etabliert und anhand von 140 klinischen Proben von BUD Verdachtsfällen aus Ghana und Togo validiert. Die IS*2404* DRB LAMP war 100 % *M. ulcerans* spezifisch und wies eine Sensitivität von 91,7 % im Vergleich zur IS*2404* real-time qPCR auf. Die LAMP ergab eine vergleichbare Performance wie die konventionelle Gel-Elektrophorese-basierte IS*2404* PCR. Dieses Format stellt für den Nachweis von *M. ulcerans* in Endemiegebieten den ersten LAMP-Assay dar, für dessen Durchführung die verwendeten Reagenzien bei Raumtemperatur gelagert werden können.

I.4 Therapie und klinisches Management bei neu diagnostizierten Fällen

Die Behandlung der BUD erfolgte bis 2004 vorwiegend chirurgisch und es ergaben sich in Abhängigkeit von der Art der Läsion sowie der Erfahrung des Chirurgen Rezidivraten von bis zu 32 %. Basierend auf Ergebnissen aus Studien im Tiermodell wiesen Etuaful et al. in einer ersten klinischen Studie die Effektivität einer 8-wöchigen Kombinationstherapie bestehend aus täglich Rifampicin (RMP, 10 mg/kg KG/d *per os*) und Streptomycin (SM, 15 mg/kg KG/d intramuskulär) bei

der Behandlung von BUD Patienten nach. Von der WHO wurden 2004 erstmals Empfehlungen zu dieser medikamentösen Kombinationstherapie ergänzt durch chirurgische Interventionen bei großflächigen Ulzera oder Knochenbeteiligung erstellt. Weitere klinische Studien u. a. aus Ghana, Benin und der Demokratischen Republik Kongo bestätigten die Effektivität der Kombinationstherapie in den Folgejahren. Nach der Einführung der antimykobakteriellen Therapie ab 2004 wurden weitaus niedrigere Rezidivraten von < 2 % berichtet. Derzeit können mit dieser Therapie ca. 80 % der Kategorie I Läsionen geheilt werden. In Abwesenheit von primären Präventionsstrategien sind die Früherkennung der BUD und eine umgehende Einleitung einer Therapie essenziell. Bei großflächigen Ulzera wird die Defektheilung oft nur durch eine anschließende Spalthauttransplantation erzielt. Zur Vermeidung von Kontrakturen betroffener bzw. benachbarter Gelenke ist eine frühzeitige Physiotherapie nötig [Etuaful S, et al. 2005; Chauty AM, et al. 2007; Schunk M, et al. 2009; WHO 2004, WHO 2007[2]; WHO 2008[1]; WHO 2008[2]; Sarfo FS, et al. 2010[1]; Nienhuis WA et al. 2010; Kibadi, K et al. 2010; WHO 2012; WHO 2017].

Die Effektivität eines rein oralen Therapieregimes bestehend aus RMP (10 mg/kg KG/d) und Clarithromycin (CLR, 7,5 mg/kg KG 2x/d) wurde von Nienhuis et al. erstmals 2010 für früh diagnostizierte kleine Läsionen (RMP + SM für 4 Wochen gefolgt von RMP + CLR für weitere 4 Wochen) in einer randomisierten und kontrollierten Studie gezeigt. In einer weiteren klinischen Studie konnten Chauty et al. für 30 BUD Patienten aus Benin nachweisen, dass die alleinige Gabe von RMP und CLR über 8 Wochen zu einer Heilungsrate von 100 % führte [Nienhuis WA et al. 2010; Chauty A, et al. 2011; WHO 2017].

I.5 Paradoxe Reaktionen, Rezidive und Therapieversager

Trotz großer Fortschritte in der Therapie der BUD bleibt zu beachten, dass in Einzelfällen paradoxe Reaktionen, Rezidive bzw. Therapieversager auftreten können. Diese gilt es frühzeitig zu erkennen, einer adäquaten Labordiagnostik zuzuführen und das klinische Management für ein optimales Therapieergebnis individuell abzustimmen.

Die nekrotischen Hautläsionen der BUD sind initial durch eine minimale Entzündungsreaktion charakterisiert. Dies wird dem immunsupprimierenden Effekt des Exotoxins Mykolakton – dem Hauptpathogenitätsfaktor von *M. ulcerans* – zugeschrieben, welches sowohl die lokale als auch die systemische Immunantwort auf den Erreger beeinflusst. Paradoxe Reaktionen können unter oder nach dem Abschluss der standardisierten antimykobakteriellen Therapie auftreten. Sie manifestieren sich klinisch als eine Vergrößerung der unter der antibiotischen Therapie zunächst abheilenden Läsion oder als sekundäre Läsion(en) an anderen Lokalisationen. Obgleich die Ätiologie paradoxer Reaktionen noch nicht abschließend geklärt ist, wird ein Immunrekonstitutions-Syndrom diskutiert, welches sich nach dem Absterben der Mykobakterien und einer konsekutiven Abnahme der Mykolakton-Konzentration einstellt. Bakteriologisch lassen sich hierbei keine viablen *M. ulcerans* mehr nachweisen und histopathologisch zeigt sich eine überschießende Entzündungsreaktion. Eine Behandlung kann je nach individuellem Fall durch eine chirurgische Revision (frühestens 6 Wochen nach ausbleibender klinischer Besserung) oder ein konventionelles Wundmanagement unter stringenter klinischer Überwachung erfolgen.

Rezidive an der gleichen bzw. an einer benachbarten Stelle, die nach alleiniger chirurgischer Exzision der Primärläsion auftreten, sind sehr wahrscheinlich auf viable *M. ulcerans* zurückzuführen, die sich im makroskopisch gesund erscheinenden und an die exzidierte Läsion angrenzenden Gewebe befanden und sich weiter replizieren. Hier bedarf es zumeist der Durchführung einer achtwöchigen Kombinationstherapie.

Echten Therapieversagern nach 8-wöchiger antimykobakterieller Standardtherapie hingegen können u. a. persistierende und ggf. antibiotikaresistente *M. ulcerans* zugrunde liegen, die u. a. durch inadäquate Einnahme der antimykobakteriellen Medikamente aufgrund einer mangelnden Compliance der Patienten entstehen können. Zur Laborbestätigung eines echten Therapieversagens wird der Nachweis viabler *M. ulcerans* verlangt und eine Resistenztestung ist erforderlich, um die weitere Therapie individuell so effizient wie möglich gestalten zu können. Betroffene Patienten könnten z. B. von chirurgischen Behandlungen profitieren [WHO 2007; Herbinger KH, et al. 2009[2]; Sarfo FS, et al. 2010[1, 2]; Kibadi K, et al. 2010; Beissner M, et al. 2011; Ruf MT, et al. 2011; Nienhuis WA, et al. 2012; Beissner M, et al. 2012; WHO 2012; O'Brien DP, et al. 2013].

Viabilitätstestung klinischer M. ulcerans Isolate

3. Studie: „Detection of viable *Mycobacterium ulcerans* in clinical samples by a novel combined 16S rRNA reverse transcriptase/IS*2404* real-time qPCR assay" [Beissner M, et al. 2012[1]].

Der Nachweis viabler *M. ulcerans* ist erforderlich, um sekundäre BUD Läsionen und paradoxe Reaktionen zu klassifizieren, den Behandlungserfolg zu überprüfen und neue Antiinfektiva bzw. neue Therapieregime in klinischen Studien zu testen. Da die KUL durch eine niedrige Sensitivität und eine lange Inkubationszeit bestimmt ist, zeigten molekulare Testverfahren das Potential einer spezifischeren, sensitiveren und schnelleren Viabilitätstestung von *M. ulcerans* auf. So wurde in einer von mir betreuten Dissertation ein molekularer und für *M. ulcerans* spezifischer ribosomaler RNA (rRNA) Test in Form eines kombinierten 16S rRNA reverse-Transkriptase (RT)/IS*2404* real-time qPCR Assays etabliert und in einer Pilotstudie in Ghana erstmals validiert. Während die DNA von *M. ulcerans* noch lange Zeit nach der Therapie und dem Absterben der Mykobakterien im Gewebe der Patienten nachweisbar sein kann, hat die mykobakterielle rRNA nur eine Halbwertszeit von wenigen Stunden, weshalb ihr Nachweis als Beweis viabler Erreger gilt. Die analytische Sensitivität, die als untere Nachweisgrenze bestimmt wurde, lag bei sechs (16S rRNA) bzw. bei zwei (IS*2404* DNA) Kopien der Zielsequenz. Die Spezifität betrug 100 % für *M. ulcerans*. Aus Wundabstrichen unbehandelter und durch IS*2404* qPCR bestätigter BUD Patienten (n = 18) wurde in Proben von 15 Patienten *M. ulcerans* rRNA nachgewiesen (83.3 %; 95%iges CI: 66.1–100 %). Wundabstriche, die von 7 BUD Patienten nach abgeschlossener antimykobakterieller Therapie aus noch nicht komplett verheilten Wunden entnommen wurden, ergaben alle negative 16S rRNA und positive IS*2404* (RT) qPCR Ergebnisse.

4. Studie/Case report: "Spontaneous clearance of a secondary Buruli ulcer lesion emerging ten months after completion of chemotherapy – a case report from Togo" [Beissner M, et al. 2012[2]].

Im klinischen Management wurde dieser molekulare Viabilitäts-Test erstmals bei einem Fall aus Togo in einer von mir betreuten Dissertation angewandt. Hier präsentierten wir den Fall eines 9-jährigen Jungen, der zehn Monate nach einer adäquaten RMP und SM Therapie und einer kompletten

Abheilung einer nodulären BUD Läsion am linken Rippenbogen einen sekundären Nodulus an der Rückseite des rechten Oberschenkels entwickelte. Der Nodulus ulzerierte spontan 3 Wochen nach der klinischen Manifestation. Diagnostische Proben von der Sekundärläsion wurden der MIK, der konventionellen IS*2404* PCR, dem kombinierten molekularen Viabilitäts-Assay sowie der KUL zugeführt. Die MIK aus einer FNA ergab den Nachweis von säurefesten Stäbchen. Während die konventionelle IS*2404* PCR negativ war, wurde mittels IS*2404* qPCR *M. ulcerans* DNA aus FNAs nachgewiesen. Die KUL und die 16S rRNA RT qPCR waren negativ. Somit konnte der Beweis geführt werden, dass keine viablen *M. ulcerans* in der Läsion replizierten. Die Hautläsion heilte unter konservativen Maßnahmen der Wundpflege fünf Wochen später spontan ab.

5. Studie: "Clearance of viable *Mycobacterium ulcerans* from Buruli ulcer lesions during antibiotic treatment as determined by combined 16S rRNA reverse transcriptase/IS*2404* qPCR assay" [Sarpong-Duah M, et al. 2017].

Eine weiterführende und prospektive Studie wurde in einer Kollaboration mit dem Kumasi Centre for Collaborative Research in Tropical Medicine, der Kwame Nkrumah University of Science and Technology, Kumasi, Ghana, durchgeführt. Hierbei sollte der kombinierte 16S rRNA RT/IS*2404* qPCR Viabilitäts-Assay die Frage klären, nach welcher Zeit der standardisierten RMP und SM Therapie BUD Patienten keine viablen *M. ulcerans* mehr aufweisen, indem die Ergebnisse der Viabilitätstestung mit dem Heilungsverlauf korreliert wurden. Hierzu wurden FNA und Wundabstriche von 129 klinisch diagnostizierten und durch IS*2404* PCR bestätigten BUD Patienten mittels MIK, KUL und dem RNA Viabilitäts-Assay untersucht. Die Probenentnahme erfolgte vor der Initiierung der antimykobakteriellen Therapie (Woche 0) und in einem 2-wöchigen Intervall im Rahmen der achtwöchigen Kombinationstherapie, sowie bei nicht heilenden Läsionen bis zur Woche 16. Zeitgleich zur Probenentnahme wurden die Heilungsraten und Heilungszeiten dokumentiert und mit den diagnostischen Ergebnissen korreliert. Aus 129 Läsionen der 129 Patienten wurde bei 65 % der Läsionen *M. ulcerans* spezifische rRNA vor der Initiierung der antimykobakteriellen Therapie nachgewiesen. Die *M. ulcerans* rRNA war im weiteren Studienverlauf bei 78 % der Patienten mit ausbleibender Heilung der Läsion nach vier Wochen, bei 52 % dieser Patienten nach acht Wochen, bei 23 % dieser Patienten nach zwölf Wochen und bei 10 % dieser Patienten nach 16 Wochen nachweisbar. Patienten mit einem positiven rRNA Nachweis nach der antibiotischen Therapie hatten initial signifikant höhere Bakterienlasten, längere Heilungszeiten und geringere Heilungsraten in der Woche 4 verglichen mit Patienten, bei denen die rRNA initial nicht nachweisbar war oder nach vier Wochen negativ getestet wurde. Aus den Ergebnissen dieser Studie wurde abgeleitet, dass die antimykobakterielle Therapie mit RMP und SM bei der überwiegenden Mehrzahl der Patienten wirkt. Bei Patienten mit initial hoher *M. ulcerans* Bakterienlast und geringen Heilungsraten bzw. langen Heilungszeiten, die nach acht Wochen noch viable *M. ulcerans* aufweisen, könnte es jedoch nötig sein, die Therapie über acht Wochen hinaus zu verlängern bzw. zu modifizieren.

Resistenztestung klinischer M. ulcerans Isolate

Bisher wurden unter IS*2404* PCR bestätigten BUD Fällen weltweit klinisch keine signifikanten Zahlen von Therapieversagern oder Rezidiven mit einem Verdacht auf das Vorliegen von gegenüber RMP, SM oder CLR resistenten *M. ulcerans* Stämmen beobachtet. Daher ist die Anzahl resistent getesteter und in Studien veröffentlichter klinischer Isolate gering. Die signifikante Bedeutung der

synergistischen und resistenz-vermeidenden Wirkung der Kombinationstherapien wurde in Tiermodellen von Marsollier et al. für RMP und SM bereits 2003 und für RMP und CLR abschließend 2016 von Chauffour et al. verdeutlicht. Ein nur geringer Therapieerfolg bei Monotherapie mit RMP wird auf das Auftreten erworbener Resistenzen gegen das Antibiotikum zurückgeführt. So konnten von Marsollier et al. unter RMP Monotherapie im Tiermodell 3 phänotypisch resistente *M. ulcerans* Stämme mit genotypischem Korrelat nachgewiesen werden. Die Mutationen wurden, wie es von anderen Mykobakterien bekannt ist, in der RMP Resistenz determinierenden Region (RRDR) des *rpoB*-Gens von *M. ulcerans* detektiert. Basierend auf den Sequenzanalysen der RRDR des mykobakteriellen *rpoB*-Gens mit phänotypischer Korrelation – analog zu den Erregern der Tuberkulose und der Lepra – konnten fortan genotypische Resistenztestungen anhand von Gesamt-DNA Extrakten aus klinischen *M. ulcerans* Isolaten durchgeführt werden [Marsollier L, et al. 2003[1, 2]; Beissner M, et al. 2011; Jansson M, et al. 2014; Gupta SK, et al. 2017].

6. Studie: „Comparison of two assays for molecular determination of rifampin resistance in clinical samples from patients with Buruli ulcer disease“ [Jansson M, et al. 2014].

Da ein initial im Rahmen meiner eigenen Dissertation etablierter molekularer Assay zur Sequenzierung der RRDR aus humanen *M. ulcerans* Gesamt-DNA Extrakten aus Ghana lediglich für *Mycobacterium spp.* spezifisch und wenig sensitiv war [Beissner M, et al. 2011], wurde der *rpoB*-Assay optimiert und in einer von mir betreuten Dissertation erneut anhand von klinischen Isolaten aus Ghana und Togo validiert: Der optimierte Assay erwies sich als zu 100 % *M. ulcerans* spezifisch mit einer signifikant niedrigeren unteren Nachweisgrenze von 100-200 Genomäquivalenten verglichen mit 1000-2000 Kopien im ursprünglichen Testverfahren und einer signifikant gesteigerten Rate an analysierbaren *rpoB*-Sequenzen von > 98 % (im Vergleich zu 35 % im ursprünglichen Assay). Das optimale Probenmaterial stellten FNA DNA-Extrakte dar.

I.6 Etablierung eines diagnostischen Netzwerkes und Referenzlabors für BUD in Togo

7. Studie: “Laboratory confirmation of Buruli ulcer disease in Togo, 2007-2010” [Bretzel G, et al. 2011].

Seit den 1990er Jahren wurden mehr als 1.800 BUD Verdachtsfälle in Togo registriert. Jedoch wurden hiervon weniger als 5 % durch Laboruntersuchungen bestätigt. Seit 2007 ermöglichte die finanzielle Unterstützung des togoischen Nationalprogramms zur Bekämpfung der BUD durch die Deutsche Lepra- und Tuberkulose Hilfe (DAHW) die PCR Bestätigung von BUD Verdachtsfällen. In einer Kooperation mit der Abteilung für Infektions- und Tropenmedizin (AITM) des Klinikums der Universität München (LMU) erfolgte dies zunächst über einen Studienzeitraum von 3 Jahren. Die DAHW integrierte hierzu ein aktives „BUD case finding“ in ein schon bestehendes Netzwerk von TB/Lepra Kontrolleuren. Klinische BUD Verdachtsfälle wurden in periphere Gesundheitszentren zur Probenentnahme und Therapie überwiesen. Die MIK wurde lokal durchgeführt und Objektträger zur externen Qualitätssicherung wurden zusammen mit den Proben für die PCR an die AITM geschickt. Auf diese Weise konnten im Studienzeitraum erstmals klinische Proben von 202 BUD Verdachtsfällen aus Togo einer standardisierten Labordiagnostik zugeführt werden. Die Konkordanzrate zwischen den MIK Ergebnissen aus Togo und der AITM betrug 62 %. Insgesamt wurden 109 BUD Verdachtsfälle

(54 %) mittels PCR in der AITM bestätigt. Über die gesamte Studienzeit verbesserte sich die PCR Bestätigungsrate von initial 50 % auf abschließend 70 %, was die damaligen WHO Vorgaben von mindestens 50 % übertraf und die WHO veranlasste, die PCR Bestätigungsrate auf 70 % anzuheben.

8. Studie: "Implementation of a national reference laboratory for Buruli ulcer disease in Togo" [Beissner M, et al. 2013].

Im Januar 2011 hat das „Institut National d'Hygiène" (INH) in Lomé im Rahmen des EU-Projekts „BuruliVac" die Rolle des nationalen Referenzlabors für die MIK und die PCR Bestätigung der BUD in Togo übernommen. Die externe Qualitätssicherung wurde in einem Studienzeitraum von weiteren 3 Jahren von den Laboren der AITM durchgeführt. Die Konkordanzrate der Ergebnisse der MIK betrug 94 % und die der PCR 96 %. Insgesamt wurden 50 % der BUD Patienten mittels MIK und 78 % mittels PCR bestätigt. Im Vergleich zur vorangegangenen Studie stieg die Detektionsrate an frühen und nicht-ulzerativen BUD Läsionen von 37 % auf 50 % und die Rate an spät diagnostizierten und großen Kategorie III Läsionen sank von 30,3 % auf 19,2 % ab. Die mittlere Zeit von der Erkrankungsmanifestation bis zur Diagnose und damit auch bis zum Therapiebeginn verringerte sich signifikant von 182,6 auf 82,1 Tage. Somit konnte der positive Effekt von Qualitätssicherungs- und Trainingsmaßnahmen im Sinne einer früheren Erkennung und einer adäquaten Labordiagnostik von BUD Verdachtsfällen nachgewiesen werden.

9. Studie: „Treatment Outcome of Patients with Buruli Ulcer Disease in Togo" [Beissner M, et al. 2015[2]].

Nach der Einführung der antimykobakteriellen Kombinationstherapie untersuchten einige Studien das Behandlungsergebnis von BUD Patienten in Afrika. Die Analysen erfolgten insbesondere anhand der Heilungszeiten, des Auftretens von Sekundärläsionen und der Funktionseinschränkungen. In Togo wurde das Behandlungsergebnis erstmals in der oben genannten Studie bei 129 laborbestätigten BUD Patienten evaluiert. Die Hautläsionen von 109 Patienten (84,5 %) waren komplikationslos verheilt, fünf Patienten hatten Sekundärläsionen (3,9 %) und 15 Patienten (11,6 %) litten unter Funktionseinschränkungen. Ödeme, Kategorie III Ulzera, Heilungszeiten > 180 Tage und Bewegungseinschränkungen betroffener Gelenke bei der Entlassung stellten signifikante (P-Wert < 0,01) Risikofaktoren für das Auftreten von Funktionseinschränkungen dar. Insgesamt lag die Rate an Funktionseinschränkungen in dieser Studie niedriger als in anderen Studien aus Afrika. Standardisierte Untersuchungs- und Therapiepläne sowie Nachuntersuchungen wurden als Empfehlungen abgeleitet, um diese Rate künftig weiter senken zu können.

I.7 Perspektiven

In Kooperation mit FIND, dem DZIF-Partner-Institut für Mikrobiologie der Bundeswehr, München, der University of Ghana und dem INH Togo wurde die IS*2404* DRB LAMP weiter als POC Test zur Anwendung in einem portablen Fluorimeter optimiert und anhand von 150 klinischen Proben von BUD Verdachtsfällen aus Ghana und Togo validiert. Erste Ergebnisse zeigten eine analytische Sensitivität von 50 Kopien der Zielsequenz, 100 % *M. ulcerans* Spezifität, eine klinische Sensitivität und Spezifität von jeweils 100 % im Vergleich zur real-time PCR und eine geringe Inter- und Intra-

Assay-Variabilität. Der POC Test kann bei Umgebungstemperatur (bis 30 °C) für mind. 24 Monate gelagert werden, ist robust, einfach und schnell durchzuführen. Im weiteren Verlauf soll der Assay kommerziell hergestellt und in einer Feldstudie in Togo und Ghana in Kooperation mit FIND und der WHO getestet werden. Hierzu bedarf es gegenwärtig noch der Optimierung einer feldtauglichen DNA-Extraktionsmethode für die *M. ulcerans* DNA aus klinischen Proben. Frimpong et al. optimierten hierzu die Genolyse® (Hein-lifescience, Nehren, Germany) DNA Extraktion für die Anwendung eines der LAMP ähnelnden isothermalen Amplifikationsverfahrens zum Nachweis von *M. ulcerans* DNA aus klinischen Proben. Die Ergebnisse sind von hoher Relevanz für eine künftige POC Testung von BUD Verdachtsfällen mittels IS*2404* LAMP [Frimpong et al., 2019]. Im Rahmen des 2019 gegründeten WHO BUD LabNet, in dem die AITM der LMU durch Frau Prof. Dr. Bretzel und mich als Berater vertreten wird, soll diese Extraktionsmethode künftig in allen endemischen Ländern Afrikas Anwendung finden. Eine erste Validierung gegen eine der bisherigen Referenzmethoden (Puregene Extraktion) ergab keinen signifikanten Nachteil bei der Amplifikation durch die IS*2404* qPCR [persönliche Kommunikation, Dr. Sarah Eyangoh, Institut Pasteur, Yaounde, Kamerun]. Des Weiteren wird die Etablierung einer feldtauglichen Viabilitätstestung von *M. ulcerans* mittels 16S rRNA LAMP in einem künftigen Projekt gegenwärtig geplant.

Ergebnisse aus diagnostischen Studien zur BUD erwiesen sich auch als anwendbar auf die Diagnostik der Lepra nach für das Pathogen spezifischen Optimierungen. So konnten von unserer Arbeitsgruppe sowohl der Viabilitäts-Assay als auch die LAMP für *M. leprae* etabliert und validiert werden.

Im Jahr 2020 kam es zum Abschluss einer von mir mitbetreuten Dissertation zur Laborbestätigung von Lepra Patienten aus Togo mittels Nasenabstrich und Testung durch eine 100 % *M. leprae* spezifische und kombinierte 16S rRNA RT/RLEP qPCR (Viabilitäts-Assay, analog zum etablierten und validierten Testsystem für *M. ulcerans*) [Beissner et al., 2019]. Dieser Assay wurde in der AITM der LMU bereits angewandt, um den klinisch diagnostizierten Rückfall eines multibazillären Lepra Patienten 5 Jahre nach der Ersttherapie labordiagnostisch zu bestätigen und zu verfolgen [Saar et al. 2019].

In einer noch laufenden Dissertation wurde diese Methode zur Früherkennung von *M. leprae* infizierten Kontaktpersonen aus Togo an der AITM der LMU untersucht. Zudem wurde eine *M. leprae* spezifische RLEP DRB LAMP zur Anwendung unter Feldbedingungen etabliert und unter optimalen Laborbedingungen unter meiner Aufsicht validiert. In einem Folgeprojekt ist die Etablierung feldtauglicher *M. leprae* spezifischer RNA/DNA Extraktionsverfahren und (RT) LAMP basierter Nachweiseverfahren zur POC Testung von Lepra Patienten und Kontaktpersonen in Endemiegebieten mit den bisherigen und neuen Kooperationspartnern unter der Leitung der AITM der LMU und dem Institut für Tropenmedizin Antwerpen, Belgien in Planung.

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III) Publikationen der kumulativen Habilitationsschrift

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IV) Publikationen der kumulativen Habilitationsschrift im Ausdruck

Erste Originalarbeit

Herbinger KH, Beissner M, Huber K, Awua-Boateng NY, Nitschke J, et al. **2010**. Efficiency of fine-needle aspiration compared with other sampling techniques for laboratory diagnosis of Buruli ulcer disease. J Clin Microbiol 48(10):3732-4.

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Efficiency of Fine-Needle Aspiration Compared with Other Sampling Techniques for Laboratory Diagnosis of Buruli Ulcer Disease▽

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In accordance with recent WHO recommendations, this study evaluates the sensitivities of PCR and microscopy for fine-needle aspiration (FNA) versus techniques involving swabs and punch biopsy specimens and suggests that FNA can replace punch biopsies for nonulcerative lesions and may serve as an alternative for ulcerative lesions in cases where scarred edges prevent the collection of swabs.

Buruli ulcer disease (BUD), caused by *Mycobacterium ulcerans*, is an emerging disease predominantly affecting West and Central Africa. BUD initially presents as a painless nodule, papule, and plaque (nonulcerative lesions), evolving into a painless ulcer with characteristically undermined edges (ulcerative lesions). Scarring and contractures may cause severe functional disability (9, 11, 12). Among the currently available diagnostic laboratory methods (microscopy, culture, PCR, and histopathology), PCR provides the highest sensitivity and is therefore regarded as the method of choice for laboratory confirmation. The WHO encourages all countries where BUD is endemic to ensure PCR confirmation of at least 50% of all cases (1, 12, 13). With the introduction of antimycobacterial treatment, laboratory confirmation of suspect cases became

TABLE 1. Sensitivities of dry-reagent-based IS*2404* PCR and microscopic examination[a]

Type of lesion and diagnostic test	Sensitivity (%) (no. of positive results/no. of tested specimens)[b]				*P*[c]			
	Swab	FNA	PB	TS	FNA vs swab	FNA vs PB	FNA vs TS	Swab vs PB
Nonulcerative (n = 37)								
PCR	NA	88.9 (32/36)	87.5 (28/32)	NC (3/3)		0.86		
Microscopic examination	NA	58.3 (21/36)	55.6 (15/27)	NC (1/2)		0.83		
Ulcerative (n = 73)								
PCR	75.0 (51/68)	55.6 (40/72)	66.2 (43/65)	30.0 (3/10)	0.02*	0.20	0.13	0.26
Microscopic examination	46.4 (32/69)	22.2 (16/72)	37.5 (18/48)	20.0 (1/5)	<.01*	0.07	0.91	0.34

[a] The diagnostic results for swabs, fine-needle aspirates (FNA), punch biopsy specimens (PB), and surgically excised tissue (TS) from 110 laboratory-confirmed BUD cases presenting with nonulcerative (n = 37) and ulcerative (n = 73) lesions from Ghana (Agogo Presbyterian Hospital, Agroyesum Hospital, Apromase Hospital, and Dunkwa Governmental Hospital) and Togo (Tsévié Regional Hospital) were analyzed. For subgroups with sample sizes of <5, the sensitivity was not calculated (NC). NA, not applicable.

[b] The *P* values comparing the sensitivities of 2 tests for laboratory diagnosis of BUD (PCR and microscopic examination), stratified into 4 techniques of specimen collection (involving swabs, fine-needle aspirates [FNA], punch biopsy specimens [PB], and surgically excised tissue [TS]), were as follows: for swabs, <0.01 for ulcerative lesions; for FNA, <0.01 for nonulcerative and ulcerative lesions; for PB, <0.01 for nonulcerative and ulcerative lesions; and for TS, 0.68 for ulcerative lesions. *P* values of <0.05 were considered significant.

[c] The *P* values comparing the sensitivities of 2 out of 4 techniques of specimen collection (involving swabs, fine-needle aspirates [FNA], punch biopsy specimens [PB], and surgically excised tissue [TS]), stratified into 2 tests for laboratory diagnosis of BUD (PCR and microscopic examination), are given. *P* values of <0.05 were considered significant (*).

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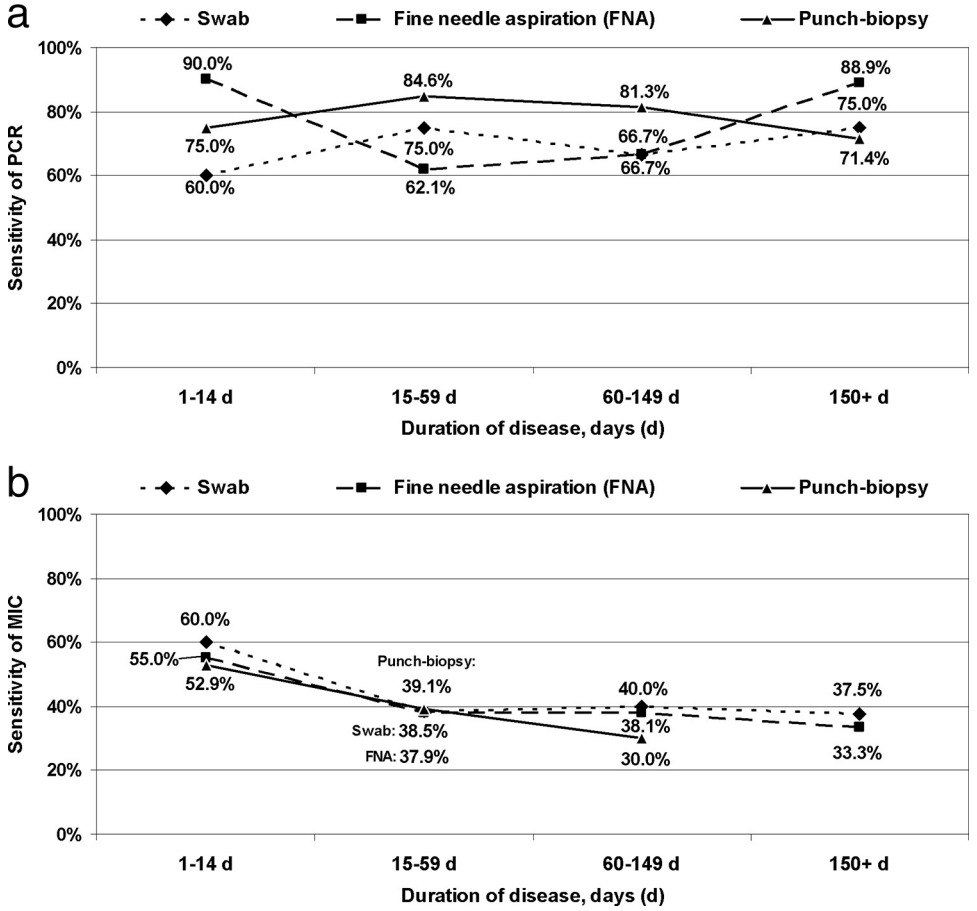

FIG. 1. Correlation between sensitivity of IS*2404* PCR (a) and microscopy (MIC) (b) and duration of disease among 79 laboratory-confirmed BUD patients (35 nonulcerative lesions and 44 ulcerative lesions) without previous antimycobacterial treatment. The analysis includes swabs (from ulcerative lesions), FNA samples, and punch biopsy specimens (from ulcerative and nonulcerative lesions). The patients were divided into four groups according to duration of disease (1 to 14 days, 15 to 59 days, 60 to 149 days, and ≥150 days). Due to a sample size of <5, calculation of the sensitivity of microscopy for punch biopsy specimens was not possible (b). Linear trends in proportions are shown as coefficient of determination (R^2) for PCR (R^2_{swab}, 0.42; R^2_{FNA}, <0.01; R^2_{punch}, 0.09) and for microscopy (R^2_{swab}, 0.63; R^2_{FNA}, 0.77; R^2_{punch}, 0.99).

crucial for clinical management of the disease (6, 12–15). Swabs, punch biopsy specimens, and surgically excised tissue are suitable diagnostic samples (3, 5–7). Recently, the WHO recommended fine-needle aspiration (FNA) as a minimally invasive method for nonulcerative lesions as well as for ulcerative lesions where scarring of edges prevents collection of swab samples (15).

The present study retrospectively compares the sensitivities of PCR and microscopy for FNA samples, swabs, punch biopsy specimens, and surgically excised tissue.

From February 2008 until December 2008, 173 clinically suspected BUD cases from Ghana (n = 112) and Togo (n = 61) were included in the study. FNA was performed with 21-gauge needles by transdermal aspiration. The needle was inserted into the center of the nonulcerative lesions or the subcutaneous tissue of the ulcer (the maximal distance from the margins was 1 to 2 cm) and was moved back and forth about three times in different directions under suction without withdrawal of the needle. Swabs, 3-mm punch biopsy specimens, and surgically excised tissue were taken according to standardized procedures, and all samples were stored in transport media as previously described and forwarded to the laboratories (5). In Ghana, 68 swabs, 112 FNA samples, 108 punch biopsy specimens, and 14 surgically excised tissue samples were subjected to microscopy and dry-reagent-based IS*2404* PCR at the Kumasi Centre for Collaborative Research in Tropical Medicine, Kumasi (5, 10). The samples from Togo (43 swabs, 61 FNA samples, 45 punch biopsy specimens, and 7 surgically excised tissue samples) were analyzed at the Centre National de Référence et de Traitement d'Ulcère de Buruli, Tsévié (microscopy), and the Department of Infectious Diseases and Tropical Medicine, University of Munich, Germany (IS*2404* standard PCR), in accordance with standardized procedures (5, 10).

One hundred ten suspects (63.6%) with 37 (33.6%) nonulcerative lesions and 73 (66.4%) ulcerative lesions were confirmed by at least one positive test result. The categories of the lesions according to the WHO definitions were known for 107 of these cases (12) (category I, 49 cases [45.8%]; category II, 44 cases [41.1%]; and category III, 14 cases [13.1%]).

Among the 37 nonulcerative cases, the sensitivities of PCR, defined as the number of positive test results divided by the

number of laboratory-confirmed cases (5), were 88.9% (32/36) for FNA samples and 87.5% (28/32) for punch biopsy specimens, and the sensitivities of microscopy were 58.3% (21/36) for FNA samples and 55.6% (15/27) for punch biopsy specimens. For both tests, there was no significant difference in sensitivity between the two types of samples (the *P* value for PCR [P_{PCR}] was 0.86, and the *P* value for microscopy [$P_{microscopy}$] was 0.83) (Table 1).

Among the 73 ulcerative cases, the sensitivities of PCR were 75.0% (51/68) for swabs, 55.6% (40/72) for FNA samples, 66.2% (43/65) for punch biopsy specimens, and 30.0% (3/10) for surgically excised tissue. The sensitivities of microscopy were 46.4% (32/69) for swabs, 22.2% (16/72) for FNA samples, 37.5% (18/48) for punch biopsy specimens, and 20.0% (1/5) for surgically excised tissue. For both tests, the sensitivities for FNA samples were significantly lower than the sensitivities for swabs ($P_{PCR} = 0.02$; $P_{microscopy} < 0.01$) but not significantly different from the sensitivities for punch biopsy specimens (P_{PCR}, 0.20, and $P_{microscopy}$, 0.07) or surgically excised tissue (P_{PCR}, 0.13, and $P_{microscopy}$, 0.91) (Table 1).

There was no significant correlation between category of lesion and sensitivity of PCR and microscopy for swabs, FNA samples, and punch biopsy specimens ($P_{PCR\text{-}swab}$, 0.25; $P_{PCR\text{-}FNA}$, 0.48; $P_{PCR\text{-}punch}$, 0.15; $P_{microscopy\text{-}swab}$, 0.23; $P_{microscopy\text{-}FNA}$, 0.55; and $P_{microscopy\text{-}punch}$, 0.06). As shown in Fig. 1a and b, for 79 cases without previous antimycobacterial treatment, sensitivity of PCR was not associated with duration of disease ($R^2_{PCR\text{-}swab}$ [coefficient of determination], 0.42; $R^2_{PCR\text{-}FNA}$, <0.01; and $R^2_{PCR\text{-}punch}$, 0.09); however, a negative trend was noted for microscopy ($R^2_{microscopy\text{-}swab}$, 0.63; $R^2_{microscopy\text{-}FNA}$, 0.77; and $R^2_{microscopy\text{-}punch}$, 0.99). Also, after stratification into nonulcerative and ulcerative lesions, no correlation between test sensitivity and the independent variables involving category of lesion and duration of disease was found (data not shown). For 69 ulcerative lesions (44 untreated and 25 treated for ≥28 days), sensitivity of PCR and microscopy for swabs, FNA samples, and punch biopsy specimens were not significantly correlated with duration of treatment ($P_{PCR\text{-}swab}$, 0.42; $P_{PCR\text{-}FNA}$, 0.16; $P_{PCR\text{-}punch}$, 0.40; $P_{microscopy\text{-}swab}$, 0.28; $P_{microscopy\text{-}FNA}$, 0.29; $P_{microscopy\text{-}punch}$, 0.07).

In the past, punch biopsy specimens were considered suitable diagnostic samples for laboratory confirmation of BUD (5, 7). However, due to the invasive character of the sample collection method, a consensus has been reached that in the interest of the patient, the method should be restricted to special diagnostic questions (e.g., differential diagnosis or identification of failure and recurrent cases) and that other methods should be applied for routine laboratory confirmation. Our findings regarding PCR assessment of FNA from nonulcerative lesions are in line with three recent studies from Ghana (8) and Benin (2, 4). All groups report sensitivities around 90%, which equals the sensitivity for punch biopsy specimens according to our data and the findings of Eddyani et al. (4). We also determined equal sensitivities for microscopy of FNA samples (corresponding to 65%, as reported by Eddyani et al. [4]) and punch biopsy specimens from nonulcerative lesions. For ulcerative lesions, available data, including our own, suggest that for both diagnostic tests, swabs are clearly superior to tissue samples (2) and that there are no significant differences in sensitivity between the use of FNA samples and that of punch biopsy specimens (4, 8).

In conclusion, FNA can replace punch biopsies for nonulcerative lesions and may serve as an alternative for ulcerative lesions in cases where scarred edges prevent the collection of swabs.

So far, only two studies report data on the sensitivity of FNA cultures, with values ranging from 10 to 44% (4, 8). As certain diagnostic questions still require assessment of cultures, further studies are needed to resolve the issue if FNA can be considered adequate diagnostic samples for culturing as well.

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PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Loop-Mediated Isothermal Amplification for Laboratory Confirmation of Buruli Ulcer Disease—Towards a Point-of-Care Test

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Abstract

Background

As the major burden of Buruli ulcer disease (BUD) occurs in remote rural areas, development of point-of-care (POC) tests is considered a research priority to bring diagnostic services closer to the patients. Loop-mediated isothermal amplification (LAMP), a simple, robust and cost-effective technology, has been selected as a promising POC test candidate. Three BUD-specific LAMP assays are available to date, but various technical challenges still hamper decentralized application. To overcome the requirement of cold-chains for transport and storage of reagents, the aim of this study was to establish a dry-reagent-based LAMP assay (DRB-LAMP) employing lyophilized reagents.

Methodology/Principal Findings

Following the design of an IS*2404* based conventional LAMP (cLAMP) assay suitable to apply lyophilized reagents, a lyophylization protocol for the DRB-LAMP format was developed. Clinical performance of cLAMP was validated through testing of 140 clinical samples from 91 suspected BUD cases by routine assays, i.e. IS*2404* dry-reagent-based (DRB) PCR, conventional IS*2404* PCR (cPCR), IS*2404* qPCR, compared to cLAMP. Whereas qPCR rendered an additional 10% of confirmed cases and samples respectively, case confirmation and positivity rates of DRB-PCR or cPCR (64.84% and 56.43%; 100% concordant results in both assays) and cLAMP (62.64% and 52.86%) were comparable and there was no significant difference between the sensitivity of the assays (DRB PCR and cPCR, 86.76%; cLAMP,

83.82%). Likewise, sensitivity of cLAMP (95.83%) and DRB-LAMP (91.67%) were comparable as determined on a set of 24 samples tested positive in all routine assays.

Conclusions/Significance

Both LAMP formats constitute equivalent alternatives to conventional PCR techniques. Provided the envisaged availability of field friendly DNA extraction formats, both assays are suitable for decentralized laboratory confirmation of BUD, whereby DRB-LAMP scores with the additional advantage of not requiring cold-chains. As validation of the assays was conducted in a third-level laboratory environment, field based evaluation trials are necessary to determine the clinical performance at peripheral health care level.

Author Summary

Buruli ulcer disease (BUD) mainly occurs in remote rural areas of Sub-Saharan Africa, affects skin and soft tissue, and may lead to severe disabilities. Therefore, early diagnosis and treatment with antimycobacterial therapy are essential whereby the WHO recommends laboratory confirmation of 70% of the cases. As the current diagnostic gold standard (polymerase chain reaction [PCR]) is restricted to third-level laboratories, development of confirmatory point-of-care (POC) tests for BUD applicable at primary health care level has become a research priority to bring diagnosis closer to where the patients are. Loop-mediated isothermal amplification (LAMP) has been selected by the WHO as one of the promising candidate technologies for POC tests. The aim of this study was to establish and validate a LAMP assay applying lyophilized reagents which are stable at ambient temperature, thus avoiding the need for cold-chains. The results from this study suggest that the assay provides a valuable alternative to other PCR tests as currently used for laboratory confirmation of BUD.

Introduction

Buruli ulcer disease (BUD), caused by *Mycobacterium ulcerans*, is an infectious disease affecting skin, soft tissues and sometimes the bones. The major endemic foci occur in rural areas of Sub-Saharan Africa where BUD mainly affects children below the age of 15 years.

Antimycobacterial therapy can cure up to 80% of patients diagnosed in early stages of the disease. If treated in advanced stages or left untreated, extensive destruction of tissue followed by fibrous scarring and contractures may lead to severe sequelae such as functional limitation of affected joints, which occur in up to 25% of cases. In the absence of proven preventive strategies, early diagnosis and treatment are therefore crucial to avoid disease related disabilities [1–2].

The WHO recommends laboratory confirmation of at least 70% of clinically suspected BUD cases per country [3]. Application of the 100% *M. ulcerans* specific diagnostic reference standard for clinical samples, i.e. amplification of the multicopy insertion sequence (IS) *2404* by dry-reagent-based (DRB) PCR, conventional gel-based PCR (cPCR), or quantitative real-time PCR (qPCR) requires fully equipped molecular biology units with highly-skilled personnel and is thus mostly restricted to tertiary (reference) level laboratories or national research centres [4–9]. However, as the major burden of BUD exists in (remote) rural areas of endemic countries and up to one-third of BUD cases are diagnosed in advanced category III stages [10–

12], molecular IS*2404* detection formats applicable as point-of-care (POC) tests are urgently needed to bring diagnosis closer to where the patients live [13].

Behind this background, an expert group convened by the Foundation for New Innovative Diagnostics (FIND) and the WHO in November 2013 selected loop-mediated isothermal amplification (LAMP) as promising nucleic acid based candidate POC technology applicable for decentralized diagnosis at primary health care level [14].

The salient features of LAMP technology are attributable to the *Bacillus stearothermophilus*-derived *Bst* polymerase, which is characterized by strand-displacement activity (without 5'-3' exonuclease activity), enzyme activity at constant temperature (~ 65 +/- 3°C) without the need of steps for denaturation of double-stranded DNA or primer annealing at different temperatures, high amplification efficiency (up to 10^{10} copies in 60 minutes) and low susceptibility to classical PCR inhibitors (e.g. melanin, collagen, humic acids). Furthermore, the ability to specifically amplify target sequences by the use of four distinct primers recognizing 6 distinct regions in a single step without the need for sophisticated laboratory equipment made this nucleic acid detection method promising as POC test. LAMP applications were thus established and validated for the diagnosis of various human pathogens such as (protozoan) parasites (e.g. *Plasmodium falciparum*, *Leishmania spp*., *Trypanosoma brucei*, *Giardia duodenalis*, *Schistosoma mansoni/haematobium*, *Taenia solium*), bacteria (e.g. *Listeria monocytogenes*, *Staphylococcus aureus*, *Mycobacterium tuberculosis*) as well as viruses and fungi in settings with limited resources [15–25].

To date, three different LAMP assays for laboratory confirmation of BUD were published. The assay described by de Souza et al. targets the enoyl reductase gene of the *M*. *ulcerans* virulence plasmid, technical validation of the assay however was conducted only on a limited number of samples [26]. Njiru et al. and Ablordey et al. reported two LAMP assays amplifying different regions of the IS*2404* of *M*. *ulcerans*. Both assays underwent validation on various clinical and environmental samples of BUD patients and infected animals from Ghana and Australia, were 100% *M*. *ulcerans* specific (without any false positive result) and revealed analytical sensitivities of 20 [27] as well as 30–300 [28] copies of the respective IS*2404* target sequence, which equals 0.1 to 1.5 genome equivalents of *M*. *ulcerans*, respectively. These analytical sensitivities approach that of cPCR [6, 27–28], but not that of qPCR [7, 29]. However, both assays were evaluated under optimal laboratory conditions applying high-standard DNA extraction and purification procedures in third level laboratories or national research centers, which may not be practicable at primary health care level. To simulate technical feasibility under field conditions, crude (i.e. boiled) DNA extracts were used without further purification for LAMP testing of clinical samples and led to a significant decrease in sensitivity [28]. Moreover, all LAMP assays described so far require unlimited cold-chains as well as shipment of reagents on dry-ice, which is a major cost factor for endemic settings and not always feasible at decentralised facilities. Therefore, technical advancement of LAMP technology and DNA extraction into utterly field friendly formats is unanimously recommended [27–28].

Against this background, the aim of this study was to establish an IS*2404* detection based LAMP assay employing lyophilized reagents (dry-reagent-based [DRB] LAMP) which provides significant benefit for application under tropical climate conditions, to validate the assay on clinical samples including fine needle aspirates (FNA) which were largely omitted in previous studies, and to provide a prototype assay for future large-scale field testing.

Materials and Methods

Ethical statement

The study was approved by the Ghanaian KNUST (CHRPE/91/10) and the national Togolese (14/2010/CRBS) ethics committees. All samples analyzed in this study were collected for

diagnostic purposes. Written informed consent was obtained from all study participants and/or their legal representative, if aged below 18 years.

Study participants, clinical samples and data collection

Clinically suspected BUD patients were recruited from two study sites in Ghana (Agogo Presbyterian Hospital, Asante Akim North District, n = 12; Tepa Government Hospital, Ahafo Ano North District, n = 20) and one study site in Togo ("Centre Hospitalier Régional de Tsévié", region "Maritime", n = 59) and 140 diagnostic samples (FNA, n = 66; swab samples, n = 32; punch biopsy samples, n = 42) were collected according to standardized procedures. Briefly, swabs were taken by circling the undermined edges of ulcerative lesions, and FNA or 3mm punch biopsies were obtained from the center of non-ulcerative lesions. Samples were transported to the Kumasi Center for Collaborative Research in Tropical Medicine (KCCR, Kumasi, Ghana) or the "Institut National d'Hygiène" (INH, Lomé, Togo) in 2 ml screw cap tubes containing 700 μl (swab and punch biopsy samples) or 300 μl (FNA samples) cell lysis solution (CLS; Qiagen, Hilden, Germany) within one day at ambient temperature [10, 30–33].

Clinical, epidemiological and routine laboratory data were collected by means of WHO BU 01.N forms [34] and standardized project specific laboratory data entry forms, and were entered in a web-based database as previously described [10].

Laboratory confirmation by PCR

Whole genome DNA was extracted from clinical samples in CLS at KCCR or INH using the Gentra Puregene DNA extraction kit (Qiagen) with minor modifications of the manufacturer's instructions as described in S1 Protocol [6]. DNA extracts were stored at 4–8°C (up to one week) or -18°C (long-term storage).

For routine on-site laboratory confirmation DNA extracts were subjected to IS*2404* DRB PCR at KCCR and INH as previously described [6, 10, 35]. For comparative testing in the context of external quality assurance programs with conventional, gel-based IS*2404* PCR (cPCR) [4–5, 33, 35, 36] and a recently described modified IS*2404* qPCR based on the assay published by Fyfe et al. [7, 29] aliquots of DNA extracts were shipped to the Department of Infectious Diseases and Tropical Medicine (DITM), Munich, Germany by courier service at ambient temperature [10].

Development of an IS*2404* detection-based LAMP assay

The development and validation of the LAMP assay was conducted in the laboratories of DITM.

DNA extracts. For establishment and technical validation of an IS*2404* LAMP assay, sequencing confirmed DNA extracts of five *M. ulcerans* strains from cultures (i.e. "must detect" samples), twelve mycobacterial species (i.e. "must not detect" samples: *M. avium*, *M. chelonae*, *M. fortuitum*, *M. gordonae*, *M. intracellulare*, *M. kansasii*, *M. marinum*, *M. smegmatis*, *M. szulgai*, *M. tuberculosis*, *M. xenopi* and *M. lentiflavum*) were available at DITM [29, 37–38].

IS*2404* plasmid standard. To generate a plasmid standard applicable as positive control and calibration template with known copy numbers for regions amplified by gel-based IS*2404* (DRB) PCR, IS*2404* qPCR as well as the novel IS*2404* LAMP assays, respectively, the complete IS*2404* sequence was amplified by conventional PCR from a sequencing confirmed *M. ulcerans* culture extract. The primers were IS*2404*-fwd (5`-3`: ATG GCT TTG TTG GCG ATC GC) and IS*2404*-rev (5`-3`: TTA GCA GGC TTG TGA GCT GG). The reaction mixture contained 13.5 μl molecular grade H_2O (Carl Roth, Karlsruhe, Germany), 2.5 μl 10-fold PCR buffer for *Thermococcus kodakaraenis* (KOD) derived DNA polymerase (Merck, Darmstadt, Germany),

2.0 μl $MgSO_4$, 2.5 μl dNTP mix (2 mM each), 10 pmol of each primer, 2 μl DNA template and 0.5 μl KOD Hot-Start Polymerase (Merck). The amplification was performed at 95°C for 2 min., followed by 35 cycles of 95°C for 20 sec., 57°C for 15 sec., 70°C for 20 sec. and a final incubation at 70°C for 2 min. The PCR product was purified from a 1.2% agarose TAE gel by means of the Double Pure Kit (Bio&SELL, Feucht, Germany) according to the manufacturer's instructions. Then, a 3'A-overhang was added to the purified PCR product by incubating the following reaction mixture for 20 minutes at 72°C: 38 μl purified PCR product, 5 μl PCR-reaction buffer (10-fold), 5 μl $MgCl_2$ (25 mM), 1 μl dATP (10 mM), 1 μl Taq polymerase (Bio&SELL, each). The 'A'-tailed PCR product was again purified with the Double Pure Kit and then ligated into a pGEM-T-vector (Promega, Mannheim, Germany) using the following reaction mixture incubated at 4°C overnight: 5 μl 2-fold Rapid Ligation Buffer, 3 μl DNA (with 3'A-overhangs), 1 μl pGEM-T-vector and 1 μl T4 DNA Ligase. For cloning the plasmid to bacteria, 2 μl of the ligation reaction were mixed with 50 μl *E. coli* JM109 z-competent cells (Zymo, Freiburg, Germany), inoculated onto LB agar plates with 100 mg/L ampicillin (Carl Roth) and incubated at 37°C overnight. The selected *E. coli* clone was cultivated in 5 ml LB medium with 100 mg/L ampicillin (Carl Roth), incubated at 37°C overnight and then subjected to plasmid preparation using the matrix-based HiYield Plasmid Mini kit (Süd-Laborbedarf, Gauting, Germany) according to the manufacturer's specification. The cloned plasmid sequence was confirmed by direct DNA sequencing as previously described [37]. Purity of extracted IS*2404* plasmids was assessed by photometry on a BioPhotometer plus (Eppendorf, Wesseling-Berzdorf, Germany) and agarose gel-electrophoresis on a 1% TAE gel. Quantification of plasmid DNA extracts was done using the fluorescence quantification kit "Quant-It dsDNA Broad Range" on a Qubit (Life Technologies, Karlsruhe, Germany) according to the manufacturer's instruction and plasmid standards were adjusted to 10^6 copies per μl.

Analytical sensitivity was determined as lower limit of detection (LOD), i.e. lowest template concentration rendering positive amplification of 95% of samples. The LOD of each assay (including DRB PCR, cPCR and qPCR) was determined at DITM using the plasmid standard in 10-fold serial dilutions [39].

IS*2404* LAMP primers. A set of four primers was designed for amplification of the *M. ulcerans* specific IS*2404* by manually analyzing the target sequence and designing the primers according to the needs for LAMP amplification. Specificity of the primers for *M. ulcerans* was confirmed in silico by means of the basic local alignment search tool (BLAST, GenBank, NCBI) [40]. The primer set consisted of two smaller oligonucleotides with forward (MU2-F3) and reverse complementary (MU2-B3) sequences and two larger oligonucleotides (MU2-FIP and MU2-BIP) with a complex sequence construction. Primer sequences are provided in Table 1 and binding sites within the IS*2404* are displayed in Fig 1. Primers MU2-B3 and MU2-BIP were first published by Ablordey et al. [28].

Conventional IS*2404* LAMP (cLAMP) protocol. Each cLAMP reaction mix consisted of 1 μl *Bst* DNA polymerase (large fragment, 8 U/μl; New England Biolabs [NEB], Frankfurt am Main, Germany), 1.0 μl dNTP mix (2 mM each, Merck), 1.0 μl of primers MU2-F3 and MU2-B3 (5 pmol/μl) and 2.0 μl of primers MU2-FIP and MU2-BIP (10 pmol/μl), respectively (TibMolBiol, Berlin, Germany), 2.0 μl betaine (5 M; Sigma-Aldrich, Taufkirchen, Germany), 2.5 μl 10-fold Thermopol buffer for *Bst* DNA polymerase (NEB) and 11.5 μl molecular grade H_2O (Carl Roth). Following the addition of 1 μl DNA extract (template), cLAMP reactions (final volume: 25 μl) were carried out in 1.5 ml SafeSeal reaction tubes (Sarstedt, Nümbrecht, Germany) at 65°C for 60 minutes in a conventional thermoblock (HLC Thermomixer MKR 13, HLC BioTech, Bovenden, Germany) and a final step at 80°C for 10 minutes terminated the amplification.

Each run included negative extraction and no template (H_2O) as well as positive controls.

Table 1. Primer sequences of the IS*2404* LAMP assay.

Primer	Sequence (forward)	Sequence (reverse complementary)	Complete sequence[a]
MU2-F3	ACT GCG GAA TCG AGA ACA G	N/A	ACT GCG GAA TCG AGA ACA G
MU2-B3[b]	N/A	CGG TTG GCG GTC AAA GC	*CGG TTG GCG GTC AAA GC*
MU2-FIP		GTG CGC CGT GTC TGG TAT GTG G	*GTG CGC CGT GTC TGG TAT GTG G*CT GCA CTG GAT ACG CGA CG
	CTG CAC TGG ATA CGC GAC G		
MU2-BIP[c]	AGG TCC TAG CAA CGC TAC GCA		AGG TCC TAG CAA CGC TAC GCA *AAT CCG GCA GGC TTC GG*
		AAT CCG GCA GGC TTC GG	

Table 1 shows the sequences of a set of four *M. ulcerans* specific LAMP primers targeting the IS*2404*.

N/A, not applicable.

[a] The reverse complementary sequence of nucleotides is displayed in italics.

[b] MU2-B3, the same primer sequence was originally published by Ablordey et al. as primer "Buruli-B3" [28].

[c] MU2-BIP, the same primer sequence was originally published by Ablordey et al. as primer "Buruli-BIP" [28].

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ATGGCTTTGTTGGCGATCGCGGTTCTGGCCACTGCCGCGGGGATGCGCGGCTATGCTGGTTTTGCCACATGGGCGGCCACCGCTTCC

GATGATGTGTTGGCCCAAGTAGGGGTCCGGTTCCGGCGGCCCAGTGAGAAGACCTTCCGCGCTGTTTTGTCTCGGCTAGACCCCGCC

GACCTCAACGCCAGGATGGGCAGTTACTTCACTGCACACGTGGCCAGCAGCGACCCCAGTGGATTGGTGCCGATCGCGTTGGACGG

CAAGATGCTGCGTGGTGCTTTACGCGCCAAAGCGACAGCCACGCATCTCGTGTCGGTGTTCGCCCACCGTGCCCGATTGGTGCTCGG

TCAACTCGCTGTCGCCGAGAAAAGCAATGAAATTCCCTGCGTATGTGCCCTGCTCACGCTGCTGCCGGATAACTTGCGGTGGCTGGT

CACCGTGGATGCGATGCATACCCAGGTCGTCATCGCGAAGTTGATCTGCGCCACCTTGAAGTCGCACTACCTGATGATCGTCAAGTC

CAACCAAGCCAAAATACTTGCCCGTATCACCGCGCTGCCCTGGGCCGAGGTGCCCGCAGCCGCTACCGACGACTCCCGCGGCCACG

GCCGTGTCGAGACCCGCACCCTGCAAATCATCACCGCTGCACGAGGAATCGGCTTCCCCTACGCAAAACAAATCATCCGGATCACTC

GTGAACGCTTGATCACCGCCACCGACCAGCGCAGCGTGGAGGTGGTCTATGCCATCTGCAGCCTGCCGTTCGAGCACGCCCGCCCTA

CCGCGATCATGACCTGGATGCGTCAACACTGCGGAATCGAGAACAGCCTGCACTGGATACGCGACGTCACCTTCGACGAAGACCGT

CAAAGGCCACATACCAGACACGGCGCACAGGTCCTAGCAACGCTACGCAACACCGCGATCAGTCTGCACCGCCTCAACGGCGCCGA

CAACATCGCCGAAGCCTGCCGGATCACCGCTTTGACCGCCAACCGCCGCCTAGACCTCCTCAATCCACAATTCCCCAGCTCACAAGC

CTGCTAA

Fig 1. Binding sites of LAMP primers within the IS*2404*. Fig 1 shows binding sites of LAMP primers within the IS*2404* of *M. ulcerans* strain Agy 99 (Genbank accession number: CP000325.1). Primer MU2-FIP consists of a first reverse complementary region "F1" and a second forward region "F2". Primer MU2-BIP (first described by Ablordey et al. [28]) consists of a first forward region "B1" and a second reverse complementary region "B2". Primers and corresponding regions are highlighted in colors; red: primer MU2-F3 (region "F3"); yellow: primer MU2-FIP (region "F2"); light blue: primer MU2-FIP (region "F1"); dark blue: primer MU2-BIP (region "B1"); green: primer MU2-BIP (region "B2") and pink: primer MU2-B3 (region "B3"; first described by Ablordey et al. [28]).

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IS*2404* dry-reagent-based LAMP (DRB LAMP) protocol. The DRB LAMP reaction mix contained the same concentrations of reagents and primers as described for the conventional LAMP assay. However, as the wildtype *Bst* polymerase large fragment contained glycerol it was not possible to lyophilize. Therefore, a customized *Bst* polymerase dissolved in H_2O and reaction buffer (NEB) were applied for the DRB LAMP assay. The DRB LAMP reaction mix was prepared for each reaction in 1.5 ml reaction tubes and subjected to lyophilization by means of a RVC 2–25 CD plus Vacuum Concentrator (Christ, Osterode, Germany) at 1.0 mbar and a safety pressure of 1.000 mbar according to the manufacturer's specifications. During the process of validation, DRB LAMP reaction tubes were stored at ambient temperature in the dark and reactions were carried out within one week after lyophilization as described for cLAMP following the addition of 1 μl template DNA and adjustment with 24 μl molecular grade H_2O (Carl Roth) to a final reaction volume of 25 μl.

LAMP product visualization. LAMP products were detected by two different methods: i) gel-electrophoresis on a 0.5x TBE gel containing 0.01% GelRed (Biotium, Cologne, Germany) and ii) SYBR Green I (Life Technologies) staining (0.5 μl of 1:5 diluted SYBR Green I staining solution and 12.5 μl of LAMP product) followed by UV-transillumination.

Comparative testing of clinical samples

Confirmation rates of routine assays (PCR [DRB PCR, cPCR] and qPCR) compared with cLAMP. The confirmation rate was defined as the number of patients with a positive PCR, qPCR or cLAMP test result divided by the number of all suspected BUD cases.

Sensitivity rates of PCR (DRB PCR, cPCR) and cLAMP among confirmed BUD patients. The sensitivity rate was defined as the number of patients with a positive PCR or cLAMP test result divided by the number of all qPCR confirmed patients.

Positivity rates of routine assays (PCR [DRB PCR, cPCR] and qPCR) and cLAMP among clinical samples from suspected BUD cases. The positivity rate was defined as the number of clinical samples from suspected BUD cases with a positive PCR, qPCR or cLAMP test result divided by the number of all samples tested.

Sensitivity rates of routine assays (PCR [DRB PCR, cPCR]) compared with cLAMP among clinical samples from confirmed BUD cases. The sensitivity rate was defined as the number of clinical samples from confirmed BUD patients with a positive PCR or cLAMP test result divided by the number of all samples with a positive qPCR result.

Positivity rates of DRB LAMP compared with routine assays (PCR [DRB PCR, cPCR] and qPCR) and cLAMP among clinical samples from suspected BUD cases. The positivity rate was defined as the number of clinical samples with a positive PCR, qPCR or cLAMP test result divided by the number of all samples tested.

Sensitivity rates of cLAMP and DRB LAMP among clinical samples from confirmed BUD cases. The sensitivity rate was defined as the number of clinical samples from confirmed BUD patients with a positive cLAMP or DRB LAMP test result divided by the number of all samples with a positive result in DRB PCR, cPCR and qPCR each.

Statistical analysis

The study was observational and transversal (cross-sectional study design). An approximative test (McNemar chi-square test for matched pairs of samples with categorical test results) and estimation of standard error of proportion (to calculate 95 percent confidence intervals [95%-CI] of categorical test results) were conducted. Significant differences were defined as *P*-values below 0.05 or as not overlapping of 95%-CI of proportions.

Results

Performance characteristics of routine assays (DRB PCR, cPCR and qPCR) compared with cLAMP

In silico analysis of the novel IS*2404* LAMP primers and testing of 17 DNA extracts from mycobacterial cultures (*M*. *ulcerans*, n = 5; other mycobacteria, n = 12) by cLAMP and DRB LAMP revealed 100% specificity of both assays for *M*. *ulcerans*. The LODs were 50 (DRB PCR and cPCR), 3 (qPCR) and 100 (cLAMP) copies of the target sequence IS*2404*, corresponding to 0.2, 0.01, and 0.5 *M*. *ulcerans* genome equivalents, respectively.

Laboratory confirmation of suspected BUD cases

Out of the 91 patients with suspected BUD, 68 were laboratory confirmed as BUD patients (74.73%) by routine methods. Among 68 confirmed BUD patients, 40 patients (58.82%) were in age group 5–14 years (age range 5–56 years, mean 14 years, median 11 years), 33 patients (48.53%) were male, and 36 patients (52.94%) presented with non-ulcerative lesions.

Confirmation rates of routine assays (DRB PCR, cPCR and qPCR) compared with cLAMP among suspected BUD cases

DRB PCR (on-site at KCCR or INH) and cPCR (DITM, 100% concordance between DRB and cPCR results) confirmed 59/91 (64.84%; 95%-CI: 55.02%-74.65%) of the suspected BUD cases, the qPCR confirmed 68/91 (74.73%; 95%-CI: 65.80%-83.65%), thus added an additional diagnostic yield of 9.89%. The confirmation rate for cLAMP was 62.64% (95%-CI: 52.70%-72.58%; n = 57). Neither DRB PCR nor cPCR or cLAMP had false positive results compared with qPCR, and confirmation rates were not significantly different. According to McNemar test, there was no significant difference between DRB PCR or cPCR (100% concordant results) compared with qPCR (OR_{crude} = 1.60; 95%-CI: 0.81–3.20; *P*-value = 0.15), between cLAMP compared with qPCR (OR_{crude} = 1.76; 95%-CI: 0.89–3.50; *P*-value = 0.08) and between DRB PCR or cPCR compared with cLAMP (OR_{crude} = 1.10; 95%-CI: 0.57–2.11; *P*-value = 0.76).

Sensitivity rates of DRB PCR, cPCR and cLAMP among confirmed BUD cases

Among the 68 BUD cases confirmed by qPCR, the sensitivity was 86.76% (95%-CI: 78.71%-94.82%; n = 59) for DRB PCR and cPCR and 83.82% (95%-CI: 75.07%-92.58%; n = 57) for cLAMP. According to McNemar test, there was no significant difference between DRB PCR and cPCR compared with cLAMP (OR_{crude} = 1.27; 95%-CI: 0.44–3.63; *P*-value = 0.63).

Positivity rates of routine assays (DRB PCR, cPCR and qPCR) and cLAMP among clinical samples from suspected BUD cases

Among the 140 samples from 91 clinically suspected BUD cases, the positivity rate was 56.43% (95%-CI: 48.21%-64.64%; n = 79) for DRB PCR and cPCR, 67.14% (95%-CI: 59.36%-74.92%; n = 94) for qPCR, and 52.86% (95%-CI: 44.59%-61.13%; n = 74) for cLAMP. Neither DRB PCR nor cPCR or cLAMP revealed false positive results compared with qPCR. According to McNemar test, there was no significant difference between DRB PCR or cPCR compared with qPCR (OR_{crude} = 1.58; 95%-CI: 0.94–2.64; *P*-value = 0.07) and between DRB PCR or cPCR compared with cLAMP (OR_{crude} = 1.16; 95%-CI: 0.70–1.90; *P*-value = 0.55), whereas the difference between cLAMP compared with qPCR was significant (OR_{crude} = 1.82; 95%-CI: 1.09–3.05; *P*-value = 0.02).

Stratification into sample types did not reveal significant differences in positivity rates of DRB PCR, cPCR, qPCR and cLAMP among FNA, swab or punch biopsy samples.

Sensitivity rates of routine assays (DRB PCR and cPCR) compared with cLAMP among clinical samples from confirmed BUD cases

Among the 94 samples from 68 BUD cases confirmed by qPCR, the sensitivity was 84.04% (95%-CI: 76.64%-91.45%; n = 79) for DRB PCR and cPCR, and 78.72% (95%-CI: 44.59%-61.13%; n = 74) for cLAMP. According to McNemar test, there was no significant difference between DRB PCR or cPCR compared with cLAMP (OR_{crude} = 1.42; 95%-CI: 0.64–3.18); *P*-value = 0.35).

Performance characteristics of DRB LAMP

DRB LAMP revealed the same performance characteristics as determined for cLAMP (i.e. 100% *M. ulcerans* specificity and a LOD of 0.5 *M. ulcerans* genome equivalents).

Positivity rates of DRB LAMP compared with routine assays (DRB PCR, cPCR and qPCR) and cLAMP among clinical samples from suspected BUD cases

To compare DRB LAMP with DRB PCR, cPCR, qPCR and cLAMP, 32 samples (FNA and swab samples, n = 16, respectively) from 32 suspected BUD cases were subjected to the assays. The positivity rate was 75.0% (95%-CI: 62.41%-87.59%; n = 24) for DRB PCR, cPCR and qPCR, 71.88% (95%-CI: 87.84%-100%; n = 23) for cLAMP, and 68.75% (95%-CI: 80.61%-100%; n = 22) for DRB LAMP. Neither cLAMP nor DRB LAMP revealed false positive results compared with DRB PCR, cPCR and qPCR.

According to McNemar test, there was no significant difference neither between DRB PCR, cPCR or qPCR compared with cLAMP (OR_{crude} = 1.17; 95%-CI: 0.34–4.10; *P*-value = 0.78), nor between DRB PCR, cPCR or qPCR compared with DRB LAMP (OR_{crude} = 1.36; 95%-CI: 0.40–4.49; *P*-value = 0.58), nor between cLAMP compared with DRB LAMP (OR_{crude} = 0.86; 95%-CI: 0.26–2.87; *P*-value = 0.79).

Sensitivity rates of cLAMP and DRB LAMP among clinical samples from confirmed BUD cases

Among the 24 samples from 24 BUD patients confirmed by DRB PCR, cPCR and qPCR, the sensitivity was 95.83% (95%-CI: 87.84%-100%; n = 23) for cLAMP and 91.67% (95%-CI: 80.61%-100%; n = 22) for DRB LAMP. According to McNemar test, there was no significant difference between cLAMP compared with DRB LAMP (OR_{crude} = 0.48; 95%-CI: 0.02–7.54); *P*-value = 0.56).

IS*2404* LAMP detection methods

Out of 74 amplicons derived from cLAMP reactions, 74/74 (100%) were judged positive by gel-electrophoresis and 73/74 (98.65%) by SYBR Green I staining. All products derived from DRB LAMP were likewise analyzed and the concordance rate was 22/22 (100%) between both detection methods.

Table 2 shows confirmation rates, sensitivity, specificity and significance of the applied molecular tests.

Table 2. Confirmation rates, sensitivity, specificity and significance of the applied molecular tests.

Number of BUD cases	Number of samples[a]	Statistical parameter	DRB PCR and cPCR[b]	qPCR	cLAMP	DRB LAMP
91 suspected	140	Positive results	59	68	57	N.A.
		Confirmation rate [%]	64.84	74.73	62.64	N.A.
		95%-CI[c] [%]	55.02–74.65	65.80–83.65	52.70–72.58	N.A.
		Specificity[d] [%]	100	N.A.	100	N.A.
68 confirmed[e]	140	Positive results	59	68	57	N.A.
		Sensitivity [%]	86.76	N.A.	83.82	N.A.
		95%-CI[c] [%]	78.71–94.82	N.A.	75.07–92.58	N.A.
91 suspected	140[#]	Positive results	79	94	74	N.A.
		Positivity rate [%]	56.43	67.14*	52.86*	N.A.
		95%-CI[c] [%]	48.21–64.64	59.36–74.92	44.59–61.13	N.A.
		Specificity[d] [%]	100	N.A.	100	N.A.
68 confirmed[e]	94[#]	Positive results	79	94	74	N.A.
		Sensitivity [%]	84.04	100	78.72	N.A.
		95%-CI[c] [%]	76.64–91.45	N.A.	44.59–61.13	N.A.
32 suspected	32	Positive results	24	24	23	22
		Positivity rate [%]	75.00	75.00	71.88	68.75
		95%-CI[c] [%]	62.41–87.59	62.41–87.59	87.84–100	80.61–100
		Specificity[d] [%]	100	N.A.	100	100
24 confirmed[e]	24	Positive results	N.A.	N.A.	23	22
		Sensitivity [%]	N.A.	N.A.	95.83	91.67
		95%-CI[c] [%]	N.A.	N.A.	87.84–100	80.61–100
		Specificity[d] [%]	N.A.	N.A.	100	100

Table 2 shows the results of DRB PCR, cPCR, qPCR, cLAMP, and DRB LAMP from clinical samples of clinically suspected and confirmed BUD cases recruited at Agogo Presbyterian Hospital, Ghana, Tepa Government Hospital, Ghana, and at Centre Hospitalier Régional de Tsévié, Togo.

N.A., not applicable.

[a] Number of clinical samples tested.

[#] indicates if the presented results refer to the number of samples–all other results refer to the number of patients or [¶]the number of patients and samples was equal.

[b] Results of the DRB PCR and cPCR were 100% concordant for all samples tested.

[c] 95 percent confidence interval.

[d] Specificity was calculated as proportion of truly positive test results out of all positive results of the same test, based on the results of qPCR as reference test.

[e] Laboratory confirmation was defined as positive IS*2404* qPCR test result of any sample tested per patient.

* Significantly different proportions of positive results among all clinical samples tested by two different tests, calculated by McNemar test.

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Discussion

BUD belongs to the currently five neglected diseases in line for the IDM (innovative and intensified disease management) approach demanding a major scaling up of active detection, treatment, monitoring and surveillance. Development of diagnostic tests that bring health services closer to where NTDs are is considered a research priority. LAMP, a technology that features cost effectiveness, robustness and modest needs in terms of equipment, has recently been selected by the WHO as one of the promising tools for decentralized diagnostics [13–14, 41].

Several investigators recently succeeded in developing *M. ulcerans* specific LAMP assays which showed performance characteristics comparable to conventional PCR formats [26–28]. Based on longstanding experience with a DRB PCR format for laboratory confirmation of BUD in Ghana and Togo [6, 10, 30, 33, 36, 42], the development of a DRB LAMP assay

applicable under tropical climate conditions at primary health care level was envisaged in this study. Thermodynamic reasons (i.e. leaving out an initial denaturation step for annealing of primers) required the design of modified primers, therefore as a first step a new cLAMP assay was established that constituted the basis for the DRB format. During development of the DRB assay lyophilization of the reaction mix initially constituted a major challenge. Due to the glycerol content of *Bst* polymerase and reaction buffer as employed in previous cLAMP formats (including our own), customized glycerol-free reagents had to be obtained and adequate lyophilization protocols had to be established.

The comparable performance of cLAMP, DRB LAMP and DRB PCR as well as cPCR suggests that both LAMP formats constitute a reliable alternative to conventional routine assays. Our data also show that the LAMP assays and the DRB PCR as well as cPCR have equal sensitivity for FNA samples. Both LAMP formats are applicable at primary health care level, the DRB format however provides significant advantages such as a simplified test layout and the possibility of storage of reagents at ambient temperature. Decentralized utilization of LAMP technology furthermore would lead to cost saving due to reduced expenditures for transportation of samples to a reference center as well as reduced test costs, i.e. US$ 1–2 per LAMP reaction as compared to US$ 8–10 per DRB PCR or cPCR reaction.

In this study it was not possible to assess long-term storage of DRB-LAMP reaction tubes under tropical conditions. Long-term storability of DRB PCR reaction tubes was however previously proven [6, 36] which allows the conclusion that maximum storage periods of up to 12 months also apply for LAMP reagents.

Although in our study routine PCR and LAMP assays for the most part did not perform significantly different from qPCR, it must be assumed that qPCR renders an additional diagnostic yield of approximately 10% [10]. Therefore, regardless of the method used, confirmation of negative samples by qPCR e.g. through the global network of laboratories for confirming *M. ulcerans* infection [43] should be attempted. Likewise, participation of laboratories in external quality assurance programs as implemented by Eddyani et al. in collaboration with the WHO is strongly recommended [44].

While the amplification procedure of LAMP technology especially in the DRB format can be considered field friendly without restriction, current DNA extraction procedures are not yet entirely appropriate for POC testing and need optimization. As shown by Ablordey et al. the use of boiled crude DNA extracts led to a significant decrease in sensitivity [28]. Other options such as one-tube silica-membrane based extraction protocols [45] or one-tube enzyme-based lyophilized reactions are yet to be evaluated. A field friendly approach to storage of DNA extracts for purposes of quality assurance could be the filter paper technology as successfully applied for TBC [46].

In conclusion, the cLAMP and DRB LAMP formats evaluated in this study are equivalent alternatives to conventional PCR techniques and, provided the availability of field friendly DNA extraction formats, constitute valuable tools for decentralized laboratory confirmation of BUD. As in the case of other investigators who previously developed BUD specific LAMP assays, the validation of the LAMP assays presented in this study was conducted in a third-level laboratory environment, therefore field based evaluation trials are necessary to determine the clinical performance at peripheral health care level.

Supporting Information

S1 Protocol. Extraction of mycobacterial DNA from clinical specimens.
(PDF)

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Author Contributions

Conceived and designed the experiments: MBe ROP FB GB. Performed the experiments: MBe MBa MF IM AR KLH DS MJ. Analyzed the data: MBe ROP FB MBa KB FSS FXW ABK KHH TL GB. Contributed reagents/materials/analysis tools: ROP FB KB FXW ABK KHH TL GB. Wrote the paper: MBe ROP FB MBa TL GB. Patient management: ROP FSS EP. Study logistics: FXW.

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PLOS | NEGLECTED TROPICAL DISEASES

From Innovation to Application

Detection of Viable *Mycobacterium ulcerans* in Clinical Samples by a Novel Combined 16S rRNA Reverse Transcriptase/IS*2404* Real-Time qPCR Assay

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Introduction

Buruli ulcer disease (BUD) caused by *Mycobacterium ulcerans* involves the skin and soft tissue. If left untreated, extensive destruction of tissue followed by scarring and contractures may lead to severe functional limitations. Following the introduction of standardized antimycobacterial chemotherapy with rifampicin and streptomycin, recurrence rates of less than 2% were reported. However, treatment failures occur and a variety of secondary lesions necessitating customized clinical management strategies have been reported. True recurrences by definition occur more than three months after completion of antibiotic treatment, are characterised by the presence of viable bacilli, and require a second course of antibiotics. "Non-healers" may harbour viable, possibly drug-resistant *M. ulcerans* strains and may benefit from surgical intervention. Early-onset immune-mediated paradoxical reactions emerging during or shortly after treatment do not contain viable bacilli and may heal under conventional wound care and/or minor surgery; late-onset secondary lesions presumably attributable to secondary infection foci may clear spontaneously through enhanced immune responses primed by initial treatment. None of the current diagnostic techniques is applicable to rapidly address the pivotal question of the presence of viable bacilli in non-healers and patients with secondary BUD lesions, and optimal time points for collection of follow-up samples have not yet been investigated. Therefore, to date treatment monitoring is mainly based on clinical observation [1–5]. Reverse transcriptase assays targeting 16S rRNA and mRNA were successfully applied for the rapid detection of viable mycobacteria in clinical samples from patients with tuberculosis and leprosy [6,7]. To employ this technique for classification of BUD lesions and monitoring of treatment success we developed a *M. ulcerans*–specific RNA-based viability assay combining a 16S rRNA reverse transcriptase real-time PCR (RT-qPCR) to determine bacterial viability with an IS*2404* quantitative real-time PCR (qPCR) for increased specificity and simultaneous quantification of bacilli.

Development and Validation

Ethical Approval

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/28/09). Written informed consent was obtained from all study participants, or their legal representatives.

Bacterial Strains, DNA Extracts, and Clinical Samples

Technical validation of the assay was performed with 29 *M. ulcerans* strains originating from Cameroon [8] and Ghana (Table 1), as well as DNA extracts from 18 closely related human pathogenic mycobacterial species and five bacterial species frequently colonizing human skin (Table 2).

Clinical validation was conducted on pre-treatment swab samples in PANTA (BD, Heidelberg, Germany) from 24 suspected BUD cases from Agogo Presbyterian Hospital ($n = 14$) and Tepa Government Hospital ($n = 10$), Ghana (Protocol S1). In addition, post-treatment swab samples from seven IS*2404* PCR confirmed BUD patients with incomplete wound healing were collected at week nine (Figures 1 and 2).

All clinical samples were subjected to routine diagnostics (microscopy and IS*2404* dry-reagent-based [DRB] PCR) at the Kumasi Centre for Collaborative Research (KCCR) [3].

Primers and Probes

Primers and a hydrolysis probe (TibMolBiol, Berlin, Germany) for specific amplification of *M. ulcerans* 16S rRNA were designed using DNAsis Max (MiraiBio, San Francisco, USA) by alignment of 16S rRNA gene sequences (GenBank, National Center for Biotechnology Information [NCBI]) from closely related mycobacteria and other bacteria potentially contaminating the human skin (Table 2).

For simultaneous quantification by IS*2404* qPCR, the primers described by Fyfe et al. [9] were used in combination

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Table 1. *M. ulcerans* cultures subjected to the 16S rRNA RT/IS*2404* qPCR assay.

M. ulcerans Strain	Source	Origin[a]	16S rRNA RT-qPCR[b]	IS*2404* qPCR[c]	IS*2404* qPCR – Wipeout Control[d]
K4s-C1	DITM	Human isolate – Kamerun	Positive	Positive	Negative
K4s-C2	DITM	Human isolate – Kamerun	Positive	Positive	Negative
K4s-C3	DITM	Human isolate – Kamerun	Positive	Positive	Negative
K5d-C1	DITM	Human isolate – Kamerun	Positive	Positive	Negative
K5d-C2	DITM	Human isolate – Kamerun	Positive	Positive	Negative
K5d-C1	DITM	Human isolate – Kamerun	Positive	Positive	Negative
K5d-C2	DITM	Human isolate – Kamerun	Positive	Positive	Negative
K5d-C3	DITM	Human isolate – Kamerun	Positive	Positive	Negative
K5d-C4	DITM	Human isolate – Kamerun	Positive	Positive	Negative
K5s-C1	DITM	Human isolate – Kamerun	Positive	Positive	Negative
K5s-C2	DITM	Human isolate – Kamerun	Positive	Positive	Negative
K5s-C3	DITM	Human isolate – Kamerun	Positive	Positive	Negative
K5s-C4	DITM	Human isolate – Kamerun	Positive	Positive	Negative
K5s-C5	DITM	Human isolate – Kamerun	Positive	Positive	Negative
K7b-C1	DITM	Human isolate – Kamerun	Positive	Positive	Negative
K7b-C2	DITM	Human isolate – Kamerun	Positive	Positive	Negative
K7b-C3	DITM	Human isolate – Kamerun	Positive	Positive	Negative
K7b-C4	DITM	Human isolate – Kamerun	Positive	Positive	Negative
K7s-C1	DITM	Human isolate – Kamerun	Positive	Positive	Negative
K7s-C2	DITM	Human isolate – Kamerun	Positive	Positive	Negative
K12S-C1	DITM	Human isolate – Kamerun	Positive	Positive	Negative
941328-C1	DITM	Human isolate – Ghana	Positive	Positive	Negative
07-C1	DITM	Human isolate – Ghana	Positive	Positive	Negative
DS1-C1	DITM	Human isolate – Ghana	Positive	Positive	Negative
97680-C1	DITM	Human isolate – Ghana	Positive	Positive	Negative
G.A.P.001-C1	KCCR	Human isolate – Ghana	Positive	Positive	Negative
G.A.P.033-C1	KCCR	Human isolate – Ghana	Positive	Positive	Negative
G.A.P.071-C1	KCCR	Human isolate – Ghana	Positive	Positive	Negative
G.A.P.078-C1	KCCR	Human isolate – Ghana	Positive	Positive	Negative

Table 1 shows 29 *M. ulcerans* cultures that were available at the Department of Infectious Diseases and Tropical Medicine (DITM) and the Kumasi Centre for Collaborative Research (KCCR) for development and technical validation of the 16S rRNA RT/IS*2404* qPCR viability assay and the corresponding test results. Sequence analysis of 16S rRNA genes from the listed strains revealed 100% nucleotide concordance of the corresponding genomic regions amplified by the 16S rRNA RT-qPCR; no SNPs or mutations were detected, suggesting a high selectivity of the assay. Sequencing primers are described in Table 3 [11].

[a]*M. ulcerans* cultures were available from previous studies from Kamerun ($n = 21$) and Ghana ($n = 4$) at DITM [8] or were available at KCCR ($n = 4$) from the present study. All strains were of human origin (BUD patients) and confirmed by conventional IS*2404* PCR and sequencing of *rpoB*- and *rpsL*-genes that revealed the *M. ulcerans* Agy99 wild-type sequences (GenBank accession no. CP000325.1) [11,12].

[b]Results of the 16S rRNA RT-qPCR of mycobacterial RNA extracts.

[c]Results of the IS*2404* qPCR of mycobacterial DNA extracts.

[d]Results of the IS*2404* qPCR of genomic DNA (gDNA) wipeout controls (see Protocols S2 and S3); a positive result indicates gDNA contamination of RNA extracts following DNAse digestions, and a negative result indicates RNA extracts free of gDNA.

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with a hydrolysis probe (Table 3) that was re-designed by DNAsis Max for thermodynamic reasons.

Combined RNA/DNA Extraction, Reverse Transcription, and Real-Time qPCR

Culture suspensions and swab samples were stabilized by RNA protect (Qiagen, Hilden, Germany) and subjected to All-Prep DNA/RNA extraction kit (Qiagen) (Protocols S1 and S2).

M. ulcerans whole transcriptome RNA from cultures and swab samples was transcribed to cDNA by QuantiTect Reverse Transcription Kit (Qiagen) including genomic DNA (gDNA) wipeout (Protocol S2). DNA and cDNA were subjected to IS*2404* qPCR and 16S rRNA RT-qPCR, respectively, with corresponding controls (Table 4, Protocols S3 and S4).

Intra- and Inter-Assay Variability

Intra- and inter-assay variability was assessed by testing of each sample in quadruplicate within one 96-well plate, repeated on three different days (Table 5).

Sensitivity

The analytical sensitivity was determined as lower limit of detection (LOD, lowest template concentration rendering amplification of 95% of samples) [10] for

Table 2. Specificity of 16S rRNA and IS*2404* qPCR assays.

Bacterial Species	Source[a]	Origin[b]	16S rRNA[d]	IS*2404*[e]
M. abscessus	NRZ	Human isolate[P]	–	–
M. africanum	NRZ	Human isolate[P]	–	–
M. avium	NRZ	Human isolate[P]	–	–
M. bovis	NRZ	Cattle isolate[P]	–	–
M. chelonae	NRZ	Human isolate[P]	–	–
M. fortuitum	NRZ	Human isolate[c]	–	–
M. gordonae	NRZ	Human isolate[c]	–	–
M. gordonae	DITM	Human isolate[c]	–	–
M. kansasii	NRZ	Human isolate[P]	–	–
M. leprae	DITM	Human isolate[P]	–	–
M. malmoense	NRZ	Human isolate[c]	–	–
M. marinum	NRZ	Human isolate[P]	+	–
M. microti	NRZ	Mouse isolate[P]	–	–
M. scrofulaceum	NRZ	Human isolate[P]	–	–
M. smegmatis	NRZ	Human isolate[P]	–	–
M. szulgai	NRZ	Human isolate[P]	–	–
M. tuberculosis	NRZ	Human isolate[P]	–	–
M. ulcerans	DITM	Human isolate[P]	+	+
M. xenopi	NRZ	Human isolate[c]	–	–
E. coli	MVP	Human isolate[c]	–	–
P. acnes	MVP	Human isolate[P]	–	–
Staph. aureus	MVP	Human isolate[c]	–	–
Staph. epidermidis	MVP	Human isolate[c]	–	–
Str. pyogenes	MVP	Human isolate[P]	–	–

Table 2 shows DNA extracts from closely related mycobacterial species and bacteria potentially contaminating the human skin subjected to the combined 16S rRNA RT/IS*2404* qPCR viability assay and the corresponding test results. Mycobacterial species were selected according to their respective genetic contiguousness to *M. ulcerans* Agy99 (GenBank accession no. CP000325.1) within the 16S rRNA gene sequences as determined by BLASTN analysis (GenBank, NCBI) [13]. *M.*, *Mycobacterium*; *E.*, *Escherichia*; *P.*, *Propionibacterium*; *Staph.*, *Staphylococcus*; *Str.*, *Streptococcus*. While in-silico analysis revealed that the combined 16S rRNA RT/IS*2404* assay will also amplify mycolactone-producing mycobacteria (MPM) other than *M. ulcerans* (e.g., *M. pseudoshottsii*, *M. liflandii*, and the environmental *M. marinum* [GenBank accession No. NR_042988.1, AY500838.1, and AF456241.1, respectively]), these MPM species were not included in specificity testing.

[a]DNA extracts that were not available at the DITM were provided by the National Reference Center (NRZ) for Mycobacteria, Borstel, Germany, and the Max von Pettenkofer-Institute (MVP), Ludwig-Maximilians University, Munich, Germany.

[b]The respective primary patient isolates were considered as [P]pathogenic bacteria or as [c]commensals/contaminants of clinical samples.

[d]Results of the 16S rRNA RT-qPCR of DNA extracts; "+" indicates a positive and "–" a negative test result.

[e]Results of the IS*2404* qPCR of DNA extracts; "+" indicates a positive and "–" a negative test result.

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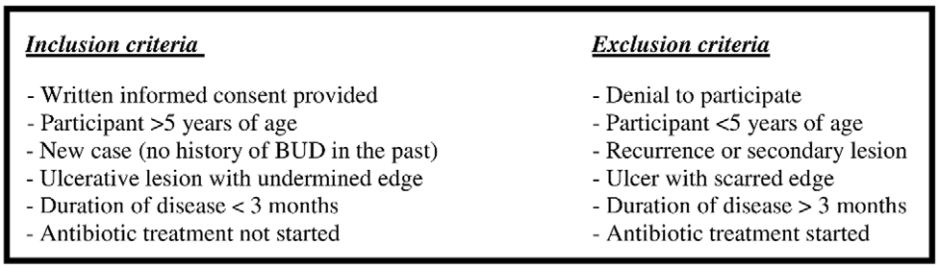

Figure 1. Enrolment criteria for the pre-treatment study population. Figure 1 describes enrolment criteria for clinically suspected BUD patients presenting at Agogo Presbyterian Hospital ($n = 14$) and Tepa Governmental Hospital ($n = 10$), Ghana, respectively. None of the eligible study participants was excluded.

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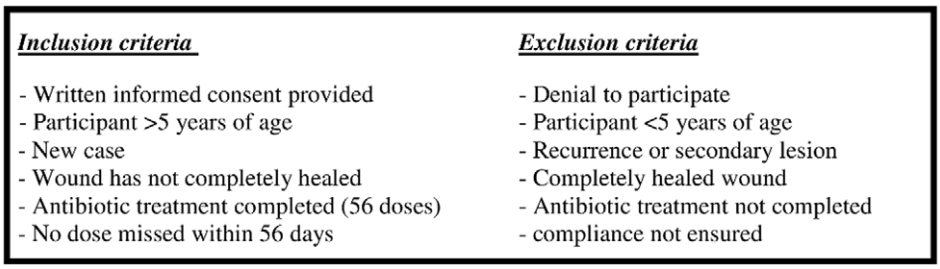

Figure 2. Enrolment criteria for the post-treatment study population. Figure 2 describes enrolment criteria for IS*2404* PCR confirmed BUD patients with incomplete wound healing (collection of swab samples feasible) who presented at Agogo Presbyterian Hospital, Ghana ($n = 7$), following completion of 56 doses of rifampicin and streptomycin administered within eight weeks. None of the eligible study participants was excluded.
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both qPCR components using 10-fold serial dilutions of cloned IS*2404* templates (GenExpress, Berlin, Germany) with known copy numbers (IS*2404* qPCR) and exactly quantified *M. ulcerans* whole genome DNA extracts from cultures (16S rRNA RT-qPCR). The LOD was two (IS*2404*) and six templates (16S rRNA gene), respectively (Figures 3 and 4).

M. ulcerans DNA and rRNA was detected in all culture extracts. Out of 24 pre-treatment swab samples, 18 (75.0%; 95%-CI: 57.7%–92.3%) had a positive IS*2404* qPCR result, 12 out of those were also positive in routine DRB PCR, and rRNA was detected in 15 out of these 18 samples (83.3%; 95%-CI: 66.1%–100%); quantification of the three negative samples revealed a bacillary load below the LOD of the 16S rRNA RT-qPCR (Table 6).

All seven post-treatment swab samples were IS*2404* qPCR positive and 16S rRNA negative.

Specificity

Analysis of DNA extracts revealed 100% specificity for the combined assay. *M. marinum* (human isolate) was amplified by 16S rRNA RT-qPCR; however, simultaneous IS*2404* qPCR was negative (Table 2).

Bacillary Survival Times

To investigate the effect of sample transport on bacillary survival, mycobacteriological transport media (PANTA and LTM) [3] were spiked with viable *M. ulcerans* and stored at 4°C and 31°C. RNA was detectable in both media for >4 weeks (4°C and 31°C).

After heat-inactivation of *M. ulcerans*–spiked PANTA-samples, RNA positivity decreased significantly within 12 h, whereas DNA was still detectable after seven days.

Future Application

The assay will support clinicians in classification of secondary lesions and selection of adequate clinical management strategies and provides a powerful tool for clinical research evaluating novel treatment regimens (Box 1).

Through analysis of sequential samples collected during antimycobacterial treatment, the assay will be employed to determine the proportional decrease of bacterial viability over time and to establish laboratory-based evidence for optimal time-points to collect follow-up samples for treatment monitoring.

Whereas the current format of the assay is restricted to reference laboratories,

Table 3. Primers and probes.

Primer/Probe[a]	Sequence (5′–3′)	Target Gene[b]	Nucleotide Position[c]	Amplicon Size[d]
MU16S TF	CGA TCT GCC CTG CAC TTC	16S rRNA	4414800–4414817	100 bp
MU16S TR	CCA CAC CGC AAA AGC TT		4414718–4414734	
MU16S TP	6 FAM-CAC AGG ACA TGA ATC CCG TGG TC-BBQ[e]		4414740–4414762	
IS2404 TF	AAA GCA CCA CGC AGC ATC T	IS*2404*	96685–96667	59 bp
IS2404 TR	AGC GAC CCC AGT GGA TTG		96627–96644	
IS2404 TP2	6 FAM-CCG TCC AAC GCG ATC GGC A-BBQ[e]		96664–96646	
T13[f]	TGC ACA CAG GCC ACA AGG GA	16S rRNA	4413906–4413925	935 bp
T39[f]	CG AAC GGG TGA GTA ACA CG		4414822–4414840	

Table 3 indicates primers and probes designed for the 16S rRNA RT-qPCR, the primers described by Fyfe et al., and a re-designed hydrolysis probe used for the amplification, detection, and quantification of IS*2404* [9].
[a]TF, forward primer; TR, reverse primer; TP2, hydrolysis probe (TibMolBiol, Berlin, Germany).
[b]16S rRNA, gene for the ribosomal 16S RNA detected as 16S cDNA; IS*2404*, insertion sequence *2404*.
[c]Nucleotide positions are provided for the first (IS*2404*) or single (16S rRNA) copy of the respective amplicon in *M. ulcerans* Agy99 (GenBank accession no. CP000325.1) as determined by BLASTN analysis within GenBank (NCBI) [13].
[d]bp, base pairs.
[e]6 FAM, 6-Carboxyfluorescein fluorescent dye; BBQ, BlackBerry Quencher.
[f]Primers T13 (forward) and T39 (reverse) were used for the amplification of a 935-bp region of the *M. ulcerans* 16S rRNA gene, encompassing the region amplified by qPCR primers MU16S TF and MU16S TR, to generate single copy replicates. Furthermore, these primers were used for sequencing of the *M. ulcerans* 16S rRNA gene (Table 1).
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Table 4. Controls applied in 16S rRNA RT/IS*2404* qPCR.

Control	Purpose	Material	
		16S rRNA RT-qPCR[a]	**IS*2404* qPCR[b]**
gDNA wipeout control[c]	To exclude DNA contamination of RNA extracts	Aliquot of each RNA extract following gDNA wipeout before reverse transcription	NA
Internal positive control	To exclude false negative results due to inhibition	TaqMan exogenous internal positive control (IPC)[d]	TaqMan exogenous internal positive control (IPC)[d]
Positive run control	To ensure adequate performance of PCR	*M. ulcerans* cDNA[e]	Cloned IS*2404* standard
Negative no template control	To exclude contamination during PCR set up	H_2O	H_2O
Negative extraction control	To exclude contamination during extraction procedure	NA	Extract treated in the same way as samples

Table 4 indicates controls applied in 16S rRNA RT/IS*2404* qPCR. NA, not applicable.
[a]16S rRNA RT PCR, reverse transcriptase real-time PCR targeting the 16S ribosomal RNA of *M. ulcerans*.
[b]IS*2404* qPCR, quantitative real-time PCR targeting the insertion sequence (IS) *2404* of *M. ulcerans*.
[c]gDNA, genomic DNA wipeout was conducted using DNAses provided in the QuantiTect Reverse Transcription Kit (Qiagen).
[d]TaqMan exogenous internal positive control (Applied Biosystems, Carlsbad, CA).
[e]cDNA, complementary DNA obtained through reverse transcription of *M. ulcerans* RNA by QuantiTect Reverse Transcription Kit (Qiagen).
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Table 5. Intra- and inter-assay variability of the 16S rRNA RT/IS*2404* qPCR assay.

qPCR Target[a]	Standard No.	Run No. 1		Run No. 2		Run No. 3		Intra-Assay Variability		Inter-Assay Variability			
		Ct-range[b]	CV[c]	Ct-range[b]	CV[c]	Ct-range[b]	CV[c]	ΔCt max.[d]	CV max.[e]	Ct-range[f]	CV[g]	ΔCt max.[h]	CV max.[i]
16S rRNA	1	0.23	0.50	0.12	0.48	0.17	0.42	0.23	0.49	0.55	1.33	0.75	1.33
	2	0.09	0.19	0.16	0.30	0.19	0.35			0.24	0.53		
	3	0.12	0.18	0.06	0.20	0.20	0.32			0.31	0.55		
	4	0.15	0.22	0.17	0.25	0.12	0.22			0.75	1.15		
	5	0.07	0.10	0.15	0.20	0.16	0.20			0.71	0.92		
IS*2404*	1	0.12	0.53	0.13	0.54	0.10	0.42	0.35	0.65	0.61	2.67	0.80	2.66
	2	0.18	0.65	0.15	0.48	0.18	0.57			0.71	2.35		
	3	0.02	0.07	0.23	0.60	0.11	0.28			0.80	2.13		
	4	0.18	0.39	0.14	0.28	0.10	0.22			0.80	1.76		
	5	0.31	0.58	0.25	0.42	0.22	0.38			0.58	1.09		
	6	0.15	0.23	0.31	0.47	0.20	0.32			0.31	0.58		
	7	0.35	0.48	0.15	0.33	0.08	0.29			0.74	1.10		

Table 5 shows intra- and inter-assay variability of the 16S rRNA RT/IS*2404* qPCR assay.
16S rRNA RT-qPCR: 16S rRNA gene standards (935 bp) were generated by conventional PCR according to Talaat et al. [12]. Quantification of PCR products was conducted by Picogreen fluorometry (Invitrogen) and copy numbers were calculated based on the known mass of one amplicon. Serial standards were prepared from PCR products in 5 Log dilutions ranging from 3E+6 (standard no. 1) to 3E+2 copies (standard no. 5) of the 16S rRNA amplicon (PCR template: 2 µl) and were subjected to the 16S rRNA RT-qPCR in quadruplicate on one 96-well plate to assess intra-assay variability. The runs were repeated on three days to determine the inter-assay variability between runs 1 through 3. The intra- and inter-assay variability of the 16S rRNA RT-qPCR was low with maximum coefficients of variation (CV) of 0.49 (intra-assay) and 1.33 (inter-assay).
IS*2404* qPCR: Cloned IS*2404* replicates (1,047 bp, complete sequence; *M. ulcerans* Agy99) were used as standards. Quantification of IS*2404* templates was conducted by Picogreen fluorometry (Invitrogen) and copy numbers were calculated based on the known mass of one template. Serial standards were prepared in 7 Log dilutions ranging from 2E+8 to 2E+2 copies of the IS*2404* (PCR template: 2 µl) and were subjected to the IS*2404* qPCR in quadruplicate on one 96-well plate to assess intra-assay variability. The runs were repeated on three days to determine the inter-assay variability between runs 1 through 3. The intra- and inter-assay variability of the IS*2404* qPCR was low with maximum CV of 0.65 (intra-assay) and 2.66 (inter-assay).
[a]16S rRNA, target of the 16S rRNA RT-qPCR; IS*2404*, target of the IS*2404* qPCR.
[b]Ct-range, range of Ct-values of samples tested in the same dilution.
[c]CV, coefficient of variation of copy numbers from samples tested in quadruplicate of the same dilution.
[d]ΔCt max., maximum Ct-variation of all samples tested within one run.
[e]CV max., maximum CV of all samples tested within one run.
[f]Ct-range, range of Ct-values of samples tested in the same dilution within three runs.
[g]CV of samples in the same dilution tested within three runs.
[h]ΔCt max. of all samples tested within three runs.
[i]CV max. of all samples tested within three runs.
doi:10.1371/journal.pntd.0001756.t005

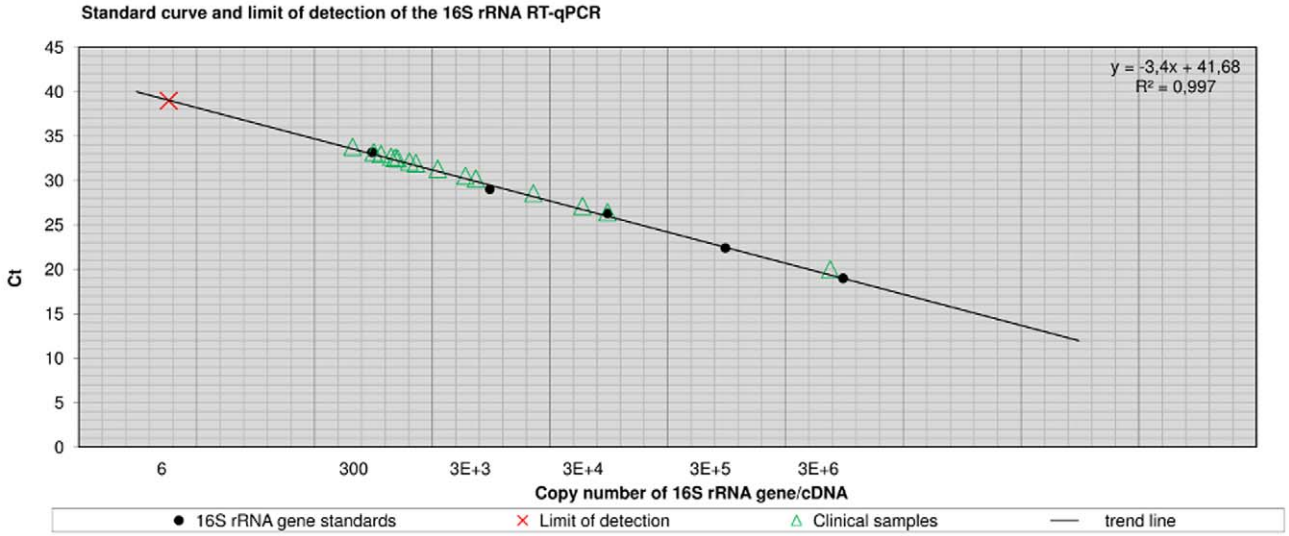

Figure 3. Standard curve and limit of detection of the 16S rRNA RT-qPCR. Figure 3 shows Ct-values of clinical samples plotted versus quantified 16S rRNA copy numbers. Standards for the 16S rRNA RT-qPCR were generated by conventional PCR amplification (Table 5). Log 10 fold serial dilutions ($n = 5$) were prepared ranging from 3E+6 to 300 copies of the 16S rRNA gene (PCR template: 2 µl) and were subjected to the assay in quadruplicate to generate a calibration curve. The regression line was $y = -3.4x+41.68$ with a coefficient of correlation >0.99 and the efficiency was $E = 0.97$. *M. ulcerans* whole genome extracts were quantified by means of IS*2404* qPCR and the analytical sensitivity was determined as limit of detection (LOD) by subjecting 10 aliquots of a dilution series containing 30, 15, 10, 8, 6, 3, or 2 copies of the 16S rRNA gene to the assay. The LOD was 6 copies of the target sequence. The copy number ($n = 1$) of the 16S rRNA gene per *M. ulcerans* genome was determined by copy number variation assay (unpublished data).
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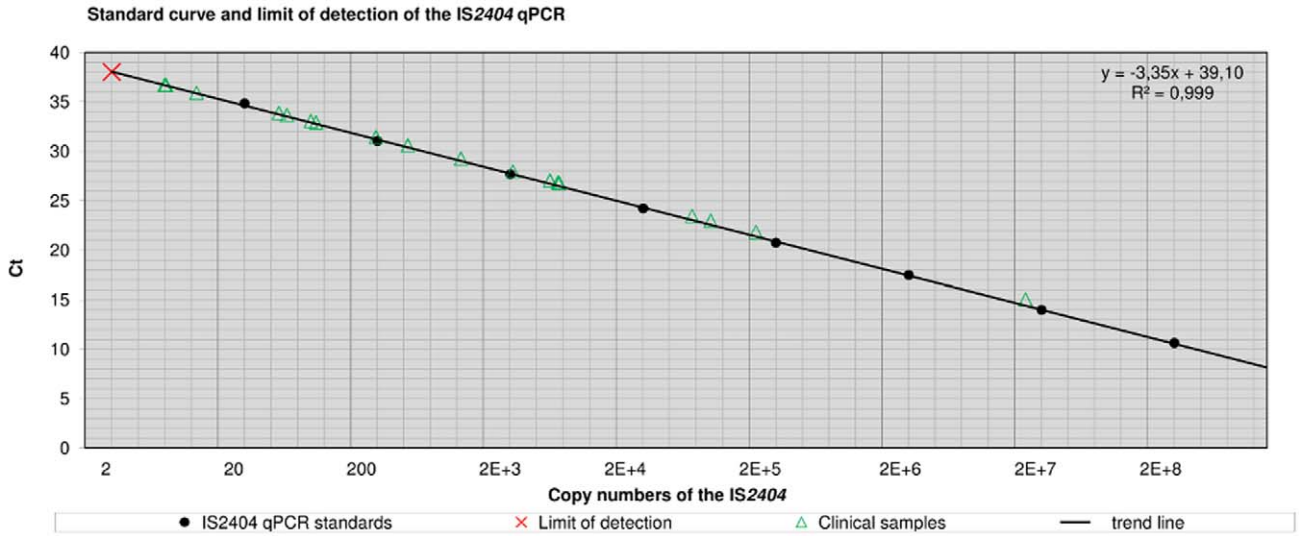

Figure 4. Standard curve and limit of detection of the IS*2404* qPCR. Figure 4 shows mean Ct-values of calibration standards and clinical samples plotted versus the quantified copy number of IS*2404*. Cloned IS*2404* templates were used as standards (Table 5). Log 10 fold serial dilutions ($n = 8$) were prepared ranging from 2E+8 to 20 copies of the IS*2404* (PCR template: 2 µl) and were subjected to the IS*2404* qPCR in quadruplicate to generate a calibration curve. The regression line was $y = -3.35x+39.10$ with a coefficient of correlation >0.99 and the efficiency was $E = 0.97$. The analytical sensitivity was determined as limit of detection (LOD) by subjecting 10 aliquots of a dilution series containing 10, 5, 4, 3, 2, or 1 copy of the IS*2404* to the assay. The LOD was 2 copies of the target sequence.
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Table 6. Study participants, clinical information, and diagnostic results.

Clinical Data				Molecular Viability Assay[a]			Routine Diagnostics[b]	
No.[c]	BUD Patient[d]	Duration (Weeks)[e]	Category of Lesion[f]	IS*2404* [Ct][g]	Bacillary Load[h]	16S rRNA[i]	MIC[k]	PCR[l]
1	No	NA	NA	Neg [NA]	NA	Neg	0	Neg
2	Yes	6	III	Pos [15,04]	>1000	Pos	+1	Pos
3	Yes	4	III	Pos [26,80]	584	Pos	+1	Pos
4	Yes	9	III	Pos [32,93]	6–10	Pos	0	Neg
5	Yes	4	I	Pos [35,94]	1–5	Neg	0	Neg
6	Yes	8	II	Pos [36,72]	1–5	Neg	0	Neg
7	Yes	2	I	Pos [36,74]	1–5	Neg	0	Neg
8	Yes	10	I	Pos [27,05]	497	Pos	+1	Pos
9	No	NA	NA	Neg [NA]	NA	Neg	0	Neg
10	Yes	3	I	Pos [30,61]	42	Pos	+1	Pos
11	Yes	8	II	Pos [33,89]	6–10	Pos	0	Neg
12	Yes	9	I	Pos [33,68]	6–10	Pos	0	Neg
13	Yes	3	III	Pos [29,27]	106	Pos	+1	Pos
14	Yes	3	I	Pos [27,98]	261	Pos	+1	Pos
15	Yes	1	I	Pos [26,85]	571	Pos	+1	Pos
16	Yes	2	I	Pos [33,07]	6–10	Pos	0	Pos
17	Yes	2	II	Pos [31,44]	24	Pos	+1	Pos
18	Yes	3	II	Pos [21,85]	>1000	Pos	+2	Pos
19	Yes	4	III	Pos [22,98]	>1000	Pos	+1	Pos
20	Yes	3	I	Pos [23,47]	>1000	Pos	+2	Pos
21	No	NA	NA	Neg [NA]	NA	Neg	0	Neg
22	No	NA	NA	Neg [NA]	NA	Neg	0	Neg
23	No	NA	NA	Neg [NA]	NA	Neg	0	Neg
24	No	NA	NA	Neg [NA]	NA	Neg	0	Neg

Table 6 shows suspected BUD cases with ulcerative lesions enrolled in the pre-treatment cohort (Figure 1), clinical information, and diagnostic results. Swab samples from 24 suspected BUD cases were subjected to 16S rRNA RT/IS*2404* qPCR viability assay (swab 1 in PANTA), microscopic examination and enumeration of acid fast bacilli (AFB) following Ziehl-Neelsen staining (swab 2, direct smear), and conventional IS*2404* dry-reagent-based (DRB) PCR (swab 3 in Cell Lysis Solution [Qiagen]). 18 patients were laboratory confirmed by IS*2404* qPCR and 15 out of those were RNA positive; the quantification by IS*2404* qPCR revealed a bacillary load (1–2 bacilli per sample) below the lower limit of detection of the RNA assay for samples from three RNA negative patients. All samples from six IS*2404* qPCR negative study participants were also RNA negative. Direct correlation of AFB enumeration with IS*2404* qPCR quantification is not feasible due to inhomogeneous distribution of *M. ulcerans* in different clinical samples. NA, not applicable; Neg, negative test result; Pos, positive test result.

[a]Results of the 16S rRNA RT/IS*2404* qPCR viability assay. Clinical swab samples in PANTA were directly processed at KCCR, and *M. ulcerans* DNA and cDNA were transported to DITM and subjected to qPCR.

[b]Routine diagnostics were conducted following standardized procedures at KCCR [3].

[c]No., consecutive number of study participants.

[d]Yes, IS*2404* qPCR confirmed BUD patients; No, IS*2404* negative study participants.

[e]Duration of disease before presentation of study participants in weeks.

[f]Category of lesion according to the World Health Organization's clinical criteria [1].

[g]Results of the IS*2404* qPCR with corresponding cycle threshold (Ct)-values.

[h]The bacillary load in the respective swab samples (No. 2) was estimated on the basis of IS*2404* quantification given an IS*2404* copy number of 209 copies per *M. ulcerans* genome [9]. For bacterial numbers <10 ranges were estimated.

[i]Results of the 16S rRNA RT-qPCR.

[k]MIC, microscopic detection and enumeration of AFB was conducted at KCCR including external quality assurance by DITM. The following scale was applied: 0 = negative, +1 = 10–99 AFB/100 fields, +2 = 1–10 AFB/1 field, +3 = more than 10 AFB/1 field.

[l]PCR, conventional, single target gel-based IS*2404* DRB PCR.

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Box 1. Advantages and Disadvantages of the Molecular Viability Assay

Advantages

- Provides a rapid, sensitive, and specific tool to detect viable bacilli in clinical samples of BUD patients, thus offering an alternative to cultures.
- Supports classification of secondary BUD lesions and monitoring of treatment success.

Disadvantages

- Current test format requires well equipped laboratory with real-time PCR facilities.
- Costs per test (approximately 14 €) may limit the applicability.

sample collection on FTA cards in combination with isothermal dry-reagent-based reverse transcription and amplification formats would facilitate processing of samples also at a peripheral level and at lower costs.

Conclusions

The novel combined 16S rRNA RT/IS*2404* qPCR assay proved to be highly sensitive, specific, and efficient in detecting viable *M. ulcerans* in clinical samples under field conditions. The assay is applicable for classification of secondary lesions and monitoring of treatment success and provides a powerful tool for clinical research.

GenBank Accession Numbers

Genes or DNA sequences of mycobacterial strains used in this study were retrieved from GenBank (NCBI) [13]. The respective sequences and accession numbers are summarized in Table S1.

Supporting Information

Protocol S1 Preparation of PANTA transport medium and stabilisation of *M. ulcerans* RNA/DNA in swab samples and culture suspensions.
(PDF)

Protocol S2 Simultaneous RNA/DNA extraction from swab samples and reverse transcription of whole transcriptome RNA from *M. ulcerans*.
(PDF)

Protocol S3 Combined 16S rRNA RT/IS*2404* qPCR assay.
(PDF)

Protocol S4 16S rRNA RT/IS*2404* qPCR run protocol.
(XLS)

Table S1 GenBank accession numbers.
(DOC)

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Vierte Originalarbeit (Case report)

Beissner M, Piten E, Maman I, Symank D, Jansson M, et al. **2012**. Spontaneous clearance of a secondary Buruli ulcer lesion emerging ten months after completion of chemotherapy - a case report from Togo. PLoS Negl Trop Dis 6(7):e1747.

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PLoS NEGLECTED TROPICAL DISEASES

Symposium

Spontaneous Clearance of a Secondary Buruli Ulcer Lesion Emerging Ten Months after Completion of Chemotherapy—A Case Report from Togo

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Presentation of Case

An eight-year-old boy from Togo presented with a nodule of 30 mm in diameter at the left costal arch (Figure 1) clinically compatible with Buruli ulcer disease (BUD) at the "Centre Hospitalier Régional Maritime" (CHR Maritime), Tsévié, in July 2010. His hometown, a village located close to the river "Haho" in the central district "Yoto" of the "Région Maritime", constitutes one of the BUD-endemic foci in Togo [1]. The patient's BCG vaccination status was positive and no other family member was diagnosed with BUD before. The lesion was laboratory confirmed by conventional IS*2404* PCR from a 3-mm punch biopsy sample (Table 1) at the Department of Infectious Diseases and Tropical Medicine (DITM), Munich, and a full course of rifampicin (300 mg/d) and streptomycin (0.5 g/d) was administered for eight weeks at the peripheral health post ("Unité de Soins Périphérique", USP). The patient was fully compliant throughout the entire period of treatment and no complications were reported. In September 2010, the lesion was completely healed under scarification (Figure 2) and the patient was considered cured. Weekly follow-up was conducted by the BUD nurse of the village for three months following healing accompanied by monthly follow-up by the BUD nurse of CHR Maritime. No pathological findings were observed until June 2011. In July 2011, the boy (in the meantime nine-year-old) presented again with a secondary nodule (diameter: 30 mm) at the back of the right thigh (Figure 3) at CHR Maritime one week after its emergence. The lesion was clinically compatible with BUD and clinical samples were collected and forwarded to the newly established BUD Reference Laboratory at the "Institut National d'Hygiène" (INH), Lomé, as well as DITM (Table 1). Microscopy of a Ziehl-Neelsen stained FNA smear was (scanty) positive and the presence of *Mycobacterium ulcerans* DNA was confirmed by IS*2404* real-time qPCR at DITM while conventional IS*2404* PCR remained negative for all samples tested. The secondary lesion ulcerated three weeks after emergence and further samples were collected from the ulcer (diameter: 25×30 mm) and forwarded to DITM for analysis. Whereas IS*2404* qPCR reconfirmed the presence of *M. ulcerans* DNA, viability testing of *M. ulcerans* by analysis of mycobacterial ribosomal 16S RNA through a newly established 16S rRNA RT qPCR (specificity: 100%, positivity rate for pre-treatment swab samples: 83.3% [95%-CI: 66.1%–100%], limit of detection: six copies of the target sequence) (unpublished data) and culture were negative (Table 1). Under stringent clinical observation, conventional wound care by daily cleaning with normal saline, disinfection with povidone-iodine, and sterile dressing of the ulcerated lesion was performed at the USP for two weeks. The lesion healed completely (Figure 4) five weeks after onset of disease. The patient's parents gave written informed consent for publication.

Case Discussion

BUD caused by infection with *M. ulcerans* may lead to extensive destruction of the skin, soft tissue, and bone with severe fibrous scarring and formation of contractures if left untreated. Pathogenesis of BUD is mediated by the cytotoxic and immunosuppressive exotoxin mycolactone [2]. During the last decade, significant advances in the treatment of BUD have been made and the introduction of standardized antimycobacterial chemotherapy with rifampicin and streptomycin resulted in recurrence rates below 2% [3].

While recurrences after surgical excision alone presumably are attributable to the persistence of mycobacteria in macroscopically healthy tissue bordering surgical excision [4], little is known about the pathogenesis and immunological mechanisms of secondary BUD lesions evolving after completion of standardized antimycobacterial treatment [5].

Development of new skin lesions during antimycobacterial treatment are currently assumed to be caused by immune-mediated, paradoxical reactions (i.e., deteriorating responses to treatment of an infection after initial improvement) which are likely

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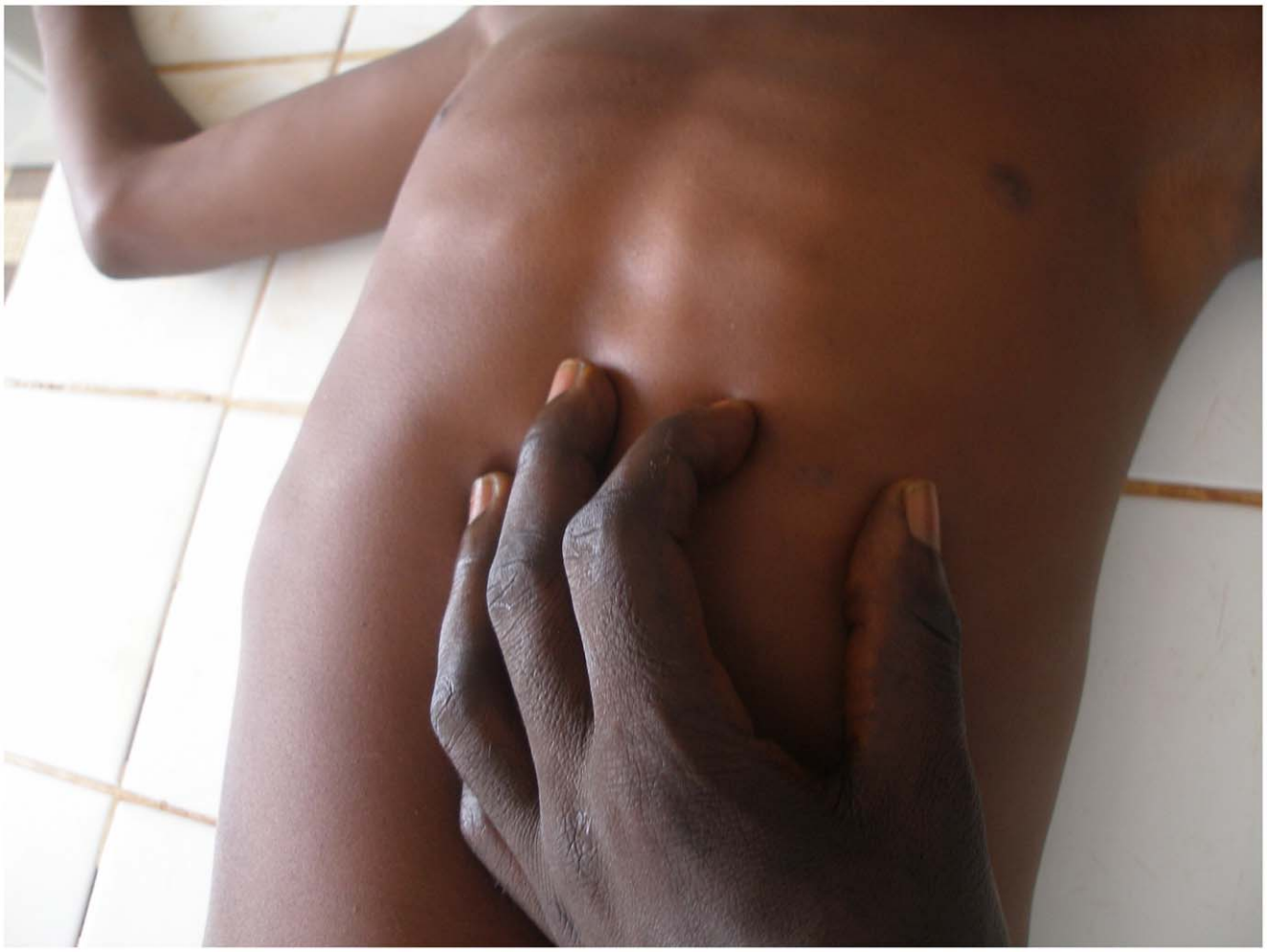

Figure 1. Primary nodule at the left costal arch, June 2010.
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to be triggered by mycobacterial antigens and immune-stimulators released from killed mycobacteria [6,7].

Ruf et al. recently reported two BUD patients from Benin who developed a series of secondary BUD lesions after completion of chemotherapy [5]. These lesions may partly represent secondary infection foci that were already present during treatment and appeared as a consequence of delayed paradoxical reactions. However, in particular, lesions occurring more than one year after completion of treatment may have been associated with new *M. ulcerans* infection or mycobacteria surviving antimycobacterial treatment and may have been resolved by immune responses triggered by successful treatment of primary lesions.

In accordance with the other cases published so far, in the present case a secondary *M. ulcerans* lesion was laboratory confirmed by microscopic detection of acid fast bacilli and IS*2404* real-time qPCR, whereas cultures remained negative. Furthermore, analysis of mycobacterial ribosomal 16S RNA did not provide evidence for the presence of viable bacilli.

As shown by Ruf et al. histopathological analysis of surgically excised late-onset secondary lesions revealed characteristical features of BUD as well as massive leukocyte infiltration of necrotic areas characteristic for successfully treated lesions. As there was no surgical intervention for the secondary lesion of the Togolese patient, clinical samples for histopathological analysis were not available.

Pathogenesis of the secondary BUD lesion in the present case might either be attributable to a second unrecognized focus of killed *M. ulcerans* during antibiotic chemotherapy ten months earlier which became clinically apparent due to a late inflammatory response to residual mycobacterial antigens (i.e., late paradoxical reaction), or to re-inoculation of *M. ulcerans* that was cleared by an elevated immune response primed by the successful initial treatment. However, available laboratory methods did not allow distinguishing between late paradoxical reaction and spontaneous host clearance during a second exposure.

While mycolactone plays a major role in the pathogenesis of primary BUD lesions, the question whether and to which extent the toxin is involved in the pathogenesis of secondary BUD lesions remains unresolved. Sarfo et al. recently demonstrated the detection of mycolactone in human tissue, suggesting its usefulness as a biomarker for monitoring the clinical response to treatment [8]. Detection of mycolactone in secondary lesions may support the hypothesis that new infection foci are associated with secondary lesions. However, to our knowledge, data on mycolactone in secondary lesions are still lacking.

Beside previous anecdotal observations on spontaneous clearance of lesions in

Table 1. Clinical samples and laboratory results of primary and secondary BUD lesions.

Date of Sample Collection	Clinical Presentation and Diameter of Lesion	Sample Type	Transport Medium	Laboratory Results				
				MIC[a]	IS*2404* PCR[b]	IS*2404* qPCR[c]	16S RT qPCR[d]	CUL[e]
July 20, 2010	Primary nodule, 30 mm	FNA[f]	CLS[g]	neg	neg	ND[h]	ND	ND
		Punch biopsy	CLS	ND	pos	ND	ND	ND
July 13, 2011	Secondary nodule, 30 mm	FNA(1)	CLS	pos (1AFB[i])	neg	pos	ND	ND
		FNA(2)	PANTA[j]	neg	pos	neg	ND	ND
July 21, 2011	Ulcerated secondary nodule, 25×30 mm	Punch biopsy	PANTA	neg	ND	ND	ND	No growth[k]
		Swab (1)	PANTA	neg	ND	ND	ND	No growth
		Swab (2)	PANTA	ND	neg	pos	neg	ND
		Swab (3)	PANTA	ND	neg	pos	neg	ND

Table 1 shows samples collected from the primary and secondary lesions in July 2010 and 2011 and the corresponding laboratory results. ''Neg'' indicates a negative test result, ''pos'' indicates a positive test result.
[a]MIC, microscopic examination of acid fast bacilli (AFB) following Ziehl-Neelsen staining conducted at CHR, DITM, and INH (samples from secondary lesion only).
[b]IS*2404* PCR, conventional, single-step, gel-based IS*2404* polymerase-chain-reaction conducted at DITM.
[c]IS*2404* qPCR, real-time quantitative IS*2404* polymerase-chain-reaction conducted at DITM.
[d]16S RT qPCR, *Mycobacterium ulcerans*–specific reverse-transcription real-time quantitative polymerase-chain-reaction targeting the ribosomal 16S RNA of *M. ulcerans* conducted at DITM.
[e]CUL, mycobacterial culture on Löwenstein-Jensen medium conducted at IML red, synlab, Asklepios Gauting, Germany.
[f]FNA, fine-needle aspiration.
[g]CLS, Puregene cell lysis solution, Qiagen, Germany.
[h]ND, not done.
[i]AFB, acid fast bacilli.
[j]PANTA, transport medium for viable mycobacteria containing Polymyxin B, Amphotericin, Nalidixic acid, Trimethoprim, and Azlocillin.
[k]No growth, culture result negative, no growth of acid fast bacilli.
doi:10.1371/journal.pntd.0001747.t001

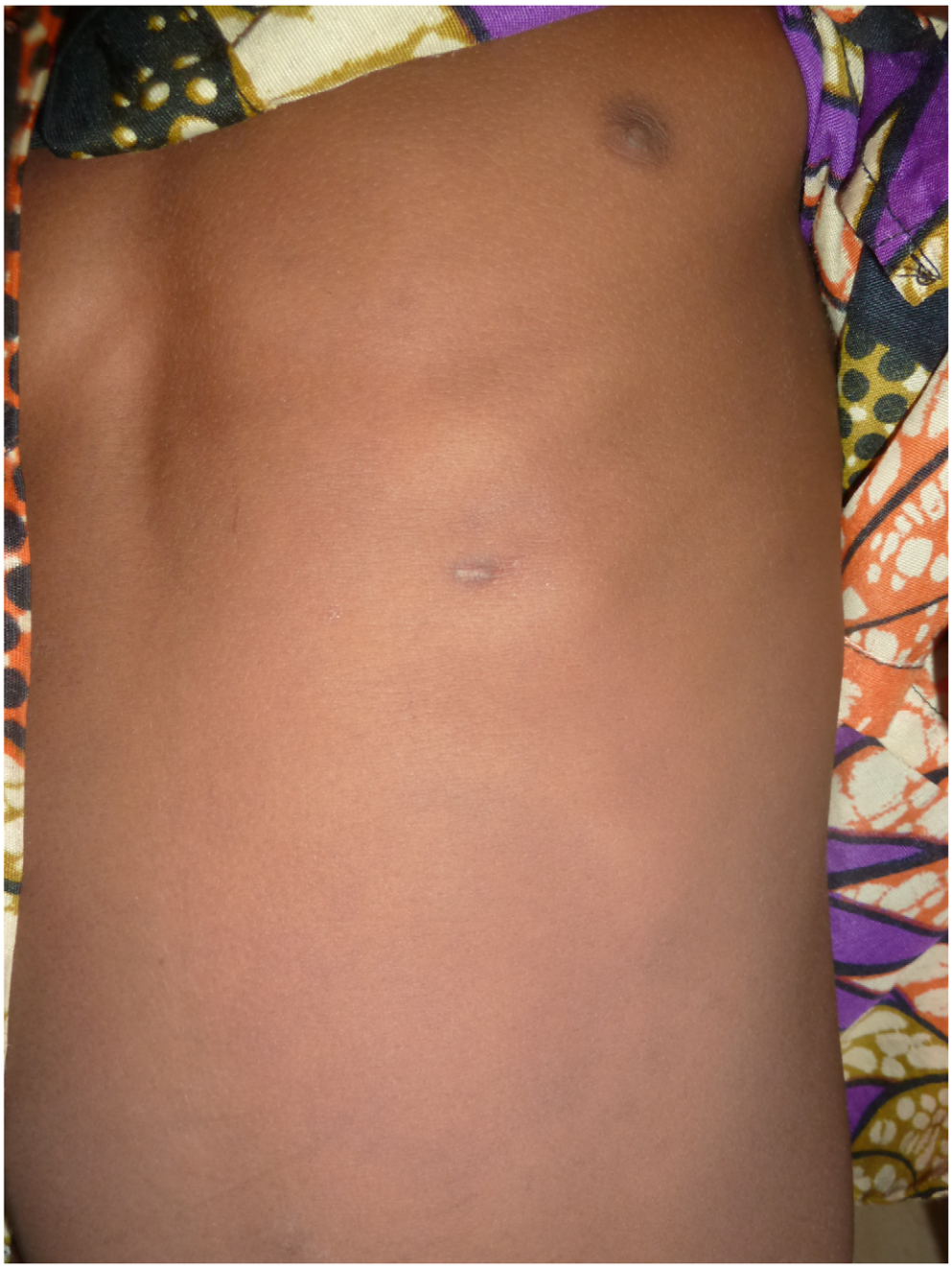

Figure 2. Scar of the primary nodule at the left costal arch, September 2010.
doi:10.1371/journal.pntd.0001747.g002

clinically suspected BUD cases, Gordon et al. recently reported the first case of spontaneous resolution of a laboratory confirmed BUD ulcer in a patient from Australia [9]. Whereas the secondary lesions of the two BUD patients from Benin were surgically excised, the ulcerated lesion of the Togolese case also healed under conventional wound care.

In the absence of evidence-based guidelines for reliable identification of late-onset secondary immune-mediated lesions and

Learning Points

- Secondary BUD lesions may occur as paradoxical reaction (i.e., deteriorating responses to treatment of an infection after initial improvement) during or shortly after treatment; late-onset secondary lesions may occur up to more than one year after completion of treatment.
- Characteristic diagnostic results for secondary BUD lesions are positive microscopy and PCR results without evidence for viable bacilli.
- The case of the Togolese patient shows that complete healing of secondary lesions without antibiotic or surgical treatment occurs. Therefore, conventional wound care can be considered as a treatment option if continuous clinical observation is possible.

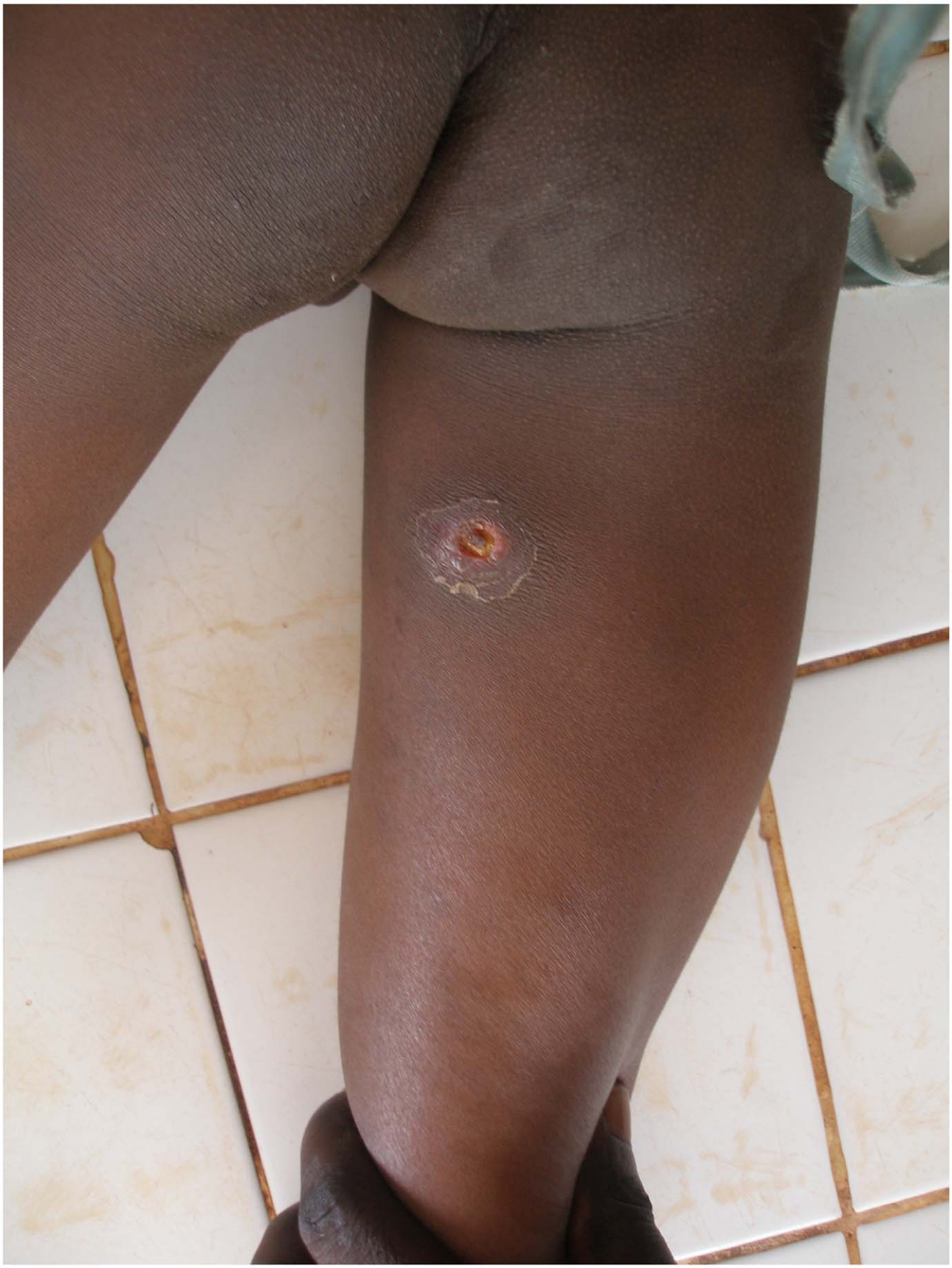

Figure 3. Secondary ulcerated nodule at the back of the right thigh, July 2011.
doi:10.1371/journal.pntd.0001747.g003

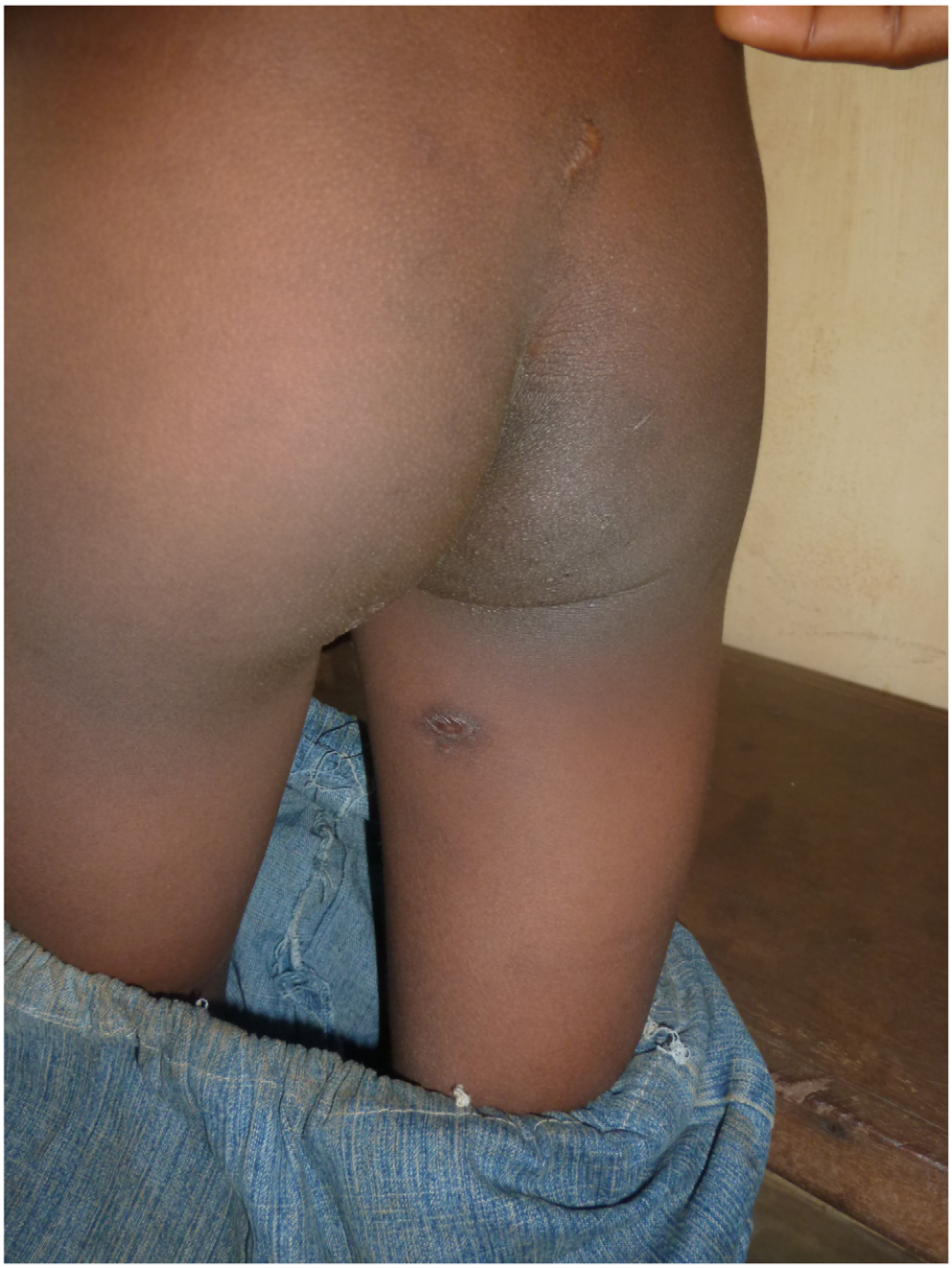

Figure 4. Scar of secondary nodule at the back of the right thigh, September 2011.
doi:10.1371/journal.pntd.0001747.g004

their clinical management, it may be advisable to consider the possibility of spontaneous healing under stringent clinical observation and regular wound care.

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Fünfte Originalarbeit

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PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Clearance of viable *Mycobacterium ulcerans* from Buruli ulcer lesions during antibiotic treatment as determined by combined 16S rRNA reverse transcriptase /IS 2404 qPCR assay

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Abstract

Introduction

Buruli ulcer (BU) caused by *Mycobacterium ulcerans* is effectively treated with rifampicin and streptomycin for 8 weeks but some lesions take several months to heal. We have shown previously that some slowly healing lesions contain mycolactone suggesting continuing infection after antibiotic therapy. Now we have determined how rapidly combined *M. ulcerans* 16S rRNA reverse transcriptase / IS*2404* qPCR assay (16S rRNA) became negative during antibiotic treatment and investigated its influence on healing.

Methods

Fine needle aspirates and swab samples were obtained for culture, acid fast bacilli (AFB) and detection of *M. ulcerans* 16S rRNA and IS*2404* by qPCR (16S rRNA) from patients with IS*2404* PCR confirmed BU at baseline, during antibiotic and after treatment. Patients were followed up at 2 weekly intervals to determine the rate of healing. The Kaplan-Meier survival analysis was used to analyse the time to clearance of *M. ulcerans* 16S rRNA and the influence of persistent *M ulcerans* 16S rRNA on time to healing. The Mann Whitney test was used to compare the bacillary load at baseline in patients with or without viable organisms at week 4, and to analyse rate of healing at week 4 in relation to detection of viable organisms.

Results

Out of 129 patients, 16S rRNA was detected in 65% of lesions at baseline. The *M. ulcerans* 16S rRNA remained positive in 78% of patients with unhealed lesions at 4 weeks, 52% at 8

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weeks, 23% at 12 weeks and 10% at week 16. The median time to clearance of *M. ulcerans* 16S rRNA was 12 weeks. BU lesions with positive 16S rRNA after antibiotic treatment had significantly higher bacterial load at baseline, longer healing time and lower healing rate at week 4 compared with those in which 16S rRNA was not detected at baseline or had become undetectable by week 4.

Conclusions

Current antibiotic therapy for BU is highly successful in most patients but it may be possible to abbreviate treatment to 4 weeks in patients with a low initial bacterial load. On the other hand persistent infection contributes to slow healing in patients with a high bacterial load at baseline, some of whom may need antibiotic treatment extended beyond 8 weeks. Bacterial load was estimated from a single sample taken at baseline. A better estimate could be made by taking multiple samples or biopsies but this was not ethically acceptable.

Author summary

Buruli ulcer (BU) caused by *Mycobacterium ulcerans* is effectively treated with rifampicin and streptomycin for 8 weeks but some lesions take several months to heal. We have shown previously that some slowly healing lesions contain the *M. ulcerans* toxin, mycolactone, suggesting continuing infection after completion of antibiotic therapy. In the present study we have determined how soon *M. ulcerans* was killed during antibiotic treatment using the *M. ulcerans* 16S rRNA assay combined with qPCR for IS*2404* to detect live bacilli in clinical samples and investigated its influence on healing. This assay is more sensitive than culture for the organism. Using samples collected from one hundred and twenty-nine BU patients prior to antibiotic treatment, viable organisms were detected by culture in 34% but the 16S rRNA assay was positive in 65%. The 16S rRNA remained positive in 78% of patients with unhealed lesions at 4 weeks, 52% at 8 weeks, 23% at 12 weeks, and 10% at week 16. Lesions with positive 16S rRNA after antibiotic treatment also contained a higher number of bacteria at baseline, had a lower rate of healing at week 4 and took a longer time to heal compared with those in which the organism was undetectable at baseline or by week 4. Positive 16S rRNA was less likely in ulcerative compared with nodular forms of disease 4 weeks after antibiotic treatment. It may be possible to shorten the treatment to 4 weeks in patients with low numbers of bacteria at baseline. Since persistent infection appears to contribute to slow healing, some patients with a high bacterial load at baseline may need antibiotic treatment for longer than 8 weeks.

Introduction

Buruli ulcer is a neglected tropical disease caused by infection with *Mycobacterium ulcerans* (Mu) which is common in rural parts of West African countries including Ghana [1]. It causes large, disfiguring skin ulcers mainly in children aged 5 to 15 years although any age can be affected [2]. The initial lesion is a subcutaneous painless nodule tethered to the skin or an intradermal plaque sometimes associated with oedema. These enlarge over a period of days to weeks and ulcerate in the centre. Ulcers are painless and have a necrotic base and irregular, undermined edges. There is surrounding oedema in about 10% of cases. Ulcers enlarge

progressively and may cover the whole of a limb or the trunk if left untreated but the patient remains systemically well unless secondary bacterial infection occurs [3] [4] [5]. The mode of transmission remains unknown[5, 6]but there have been major advances in understanding the mechanism of disease since the establishment of the WHO Buruli ulcer initiative in 1998 together with improved diagnosis and clinical management.

Treatment of Buruli ulcer has changed considerably since 2004 with the introduction of antibiotics as an alternative to surgery. It has now been established that the combination of rifampicin and streptomycin administered daily for 8 weeks is effective in healing all forms of lesion caused by Mu disease and this has reduced the recurrence rate from 6–47% after surgery to 0–2% after antibiotic treatment [6, 7]. This treatment can be administered by community health nurses and admission to hospital is rarely necessary except when skin grafting is needed. The current duration of antibiotic therapy (8 weeks) was based on observations in patients with early Mu lesions which were excised after treatment for 2, 4, 8 or 12 weeks. All lesions remained culture positive after 2 weeks but thereafter all were culture negative [3]. Thus it is likely that a shorter course of treatment may be successful in some patients which would be highly desirable, not least because streptomycin has to be injected intramuscularly. This is supported by recent experience of treating *M ulcerans* disease in Australia with antibiotic durations of less than 8 weeks suggesting that successful outcomes may be achieved in selected patients [8]. In spite of the success of rifampicin and streptomycin treatment for 8 weeks some lesions take much longer than others to heal despite having appeared identical before treatment. Available data from various studies suggest that healing of up to two thirds of patients occurs within 25 weeks from the start of treatment [9–11].

One reason for slow healing may be that active infection persists despite antibiotic treatment for 8 weeks. In our recent study of BU treated with rifampicin and streptomycin for 8 weeks, persistent infection with *M. ulcerans* was shown by positive cultures in some lesions 4 weeks after completion of antibiotic treatment despite full adherence to therapy. Furthermore mycolactone, the toxin produced by *M. ulcerans*, was detected in lesions which were culture negative as well as in culture positive samples, suggesting that it is a more sensitive marker for the presence of viable organisms [12]. However it is not known how long mycolactone can remain in human BU lesions after *M. ulcerans* is killed and it is vital to establish how often infection persists after a standard course of antibiotic treatment.

Reverse transcriptase assays targeting ribosomal or messenger RNA have been applied successfully for the rapid detection of viable mycobacteria in clinical samples from patients with tuberculosis, leprosy and recently Buruli ulcer [13] [14] [15] and as a surrogate for response to chemotherapy in tuberculosis [13]. With respect to Buruli ulcer, the assay is fast, 100% specific for *M. ulcerans* and highly sensitive with an analytical sensitivity of 6 templates of the targeted 16S rRNA. The excellent performance on clinical samples makes this tool highly promising for monitoring the therapeutic response with the goal of optimizing the duration of antimycobacterial treatment [15]. The aim of the present study was to determine how rapidly combined *M. ulcerans* 16S rRNA reverse transcriptase / IS*2404* qPCR assay (hereafter referred to as 16S rRNA) became negative during antibiotic treatment and to relate this to the rate of healing.

Materials and methods

Patients

In the period from June 2013 to June 2015, patients more than 5 years of age with suspected Buruli ulcer and subsequent confirmation by *M. ulcerans* IS*2404* dry reagent based (DRB) PCR presenting to treatment clinics at the Tepa Government Hospital, Nkawie-Toase Government Hospital, Dunkwa Government Hospital and Agogo Presbyterian Hospital were

screened for inclusion. Patients who had already been under antimycobacterial treatment at the time of study initiation were excluded.

Study procedures

Demographic data were collected using standard BU01 forms from the WHO together with a careful history to establish when lesions were first observed and their type. The dimensions of lesions were documented with Silhouette (ARANZ Medical, Christchurch, New Zealand) a 3-dimensional imaging and documentation system together with digital photographs. The Silhouette camera captures an image of the wound, a tracing of the wound boundary is generated and the wound dimensions including the area, depth and volume are automatically calculated. For oedematous lesions, only digital photographs were obtained. Patients were reviewed at 2 weekly intervals during standard antibiotic treatment and monthly thereafter with further recordings of clinical data as routinely conducted for all BU patients until complete healing. These measurements enabled calculation of healing rate at week 4 and predicted healing time in relation to lesion size and type. Rate of healing in mm per week was calculated by subtracting the mean diameter of the lesion in millimeters determined at week 4 from that determined at week 0 and dividing this result by 4. Mean diameter was the mean of the maximum diameter and the largest diameter at right angles to that [16]. Two fine needle aspirates (FNA) or swabs samples were collected from skin lesions to confirm the diagnosis of Buruli ulcer by microscopy and conventional IS*2404* DRB PCR. An additional sample for culture and another for the 16S rRNA reverse transcriptase/IS*2404* qPCR assay (16S rRNA) were collected at baseline and during (week 4 and 8) or after treatment (week 12 and 16) from unhealed lesions, immediately placed in either 500μl PANTA media or 500μl RNA protect respectively on site. Human GAPDH mRNA assay was performed on the samples in the RNA protect to assess the stability of the RNA in the solution (Qiagen, UK).

All routine laboratory tests were conducted at Kumasi Centre for Collaborative Research in Tropical Medicine (KCCR) immediately upon arrival of samples. Prior to the study a human GAPDH mRNA reverse transcriptase qPCR was established and validated at the Department for Infectious Diseases and Tropical Medicine (DITM) of the University Hospital of the Ludwig-Maximilians-University (LMU) in Munich, Germany. During the study all molecular assays were conducted at the KCCR by trained laboratory staff supervised by Kwame Nkrumah University of Science and Technology (KNUST) staff.

Whole genome DNA and whole transcriptome RNA were extracted at the KCCR immediately on arrival of samples in RNA protect and subjected to the *M. ulcerans* 16S rRNA assay [15].

Routine laboratory confirmation

For laboratory confirmation of Buruli ulcer disease, AFB microscopy, IS*2404* dry reagent based (DRB)-PCR and cultures were performed. IS*2404* qPCR were performed by well established methods as previously described [17][18] [15]. IS*2404* qPCR was also performed on all samples. A final diagnosis of Buruli ulcer was based on IS*2404* DRB-PCR and qPCR results which were the most sensitive tests.

Combined 16S rRNA reverse transcriptase / IS*2404* qPCR assay

FNA and swab samples were transported from study site to the KCCR stabilized in 500 μl RNA protect (Qiagen, UK). Whole transcriptome RNA and whole genome DNA were extracted from the same clinical sample. The RNA and DNA isolation was carried out within 5 hours of sample collection using the AllPrep DNA/RNA Micro kit (Qiagen, UK) as previously

described with minor modification[15]. Here, homogenizing was carried out with the QiaShredder (Qiagen, UK) according to the manufacturers instruction in a biosafety cabinet. 12 μl RNA extracts were immediately reverse transcribed whilst 50 μl DNA extracts obtained were stored at 4–8˚C (short-term) or -20˚C (long-term).

To remove potentially contaminating genomic DNA (gDNA) from the *M. ulcerans* whole transcriptome RNA extracted, 2 μl DNA wipe out buffer (Qiagen, UK) was added to 12 μl of the total RNA extracts, incubated for 5 min at 42˚C and the reaction was terminated by incubating at 95˚C for 3 min. 2 μl gDNA free *M. ulcerans* whole transcriptome RNA extracted was included as a wipe out control. The remaining *M. ulcerans* whole transcriptome RNA was then reverse transcribed into cDNA using QuantiTect Reverse transcription kit (Qiagen, UK) according to the manufacturer's instructions as described elsewhere[15]. The cDNA samples were stored at -20˚C until further processing.

"To exclude false negative 16S rRNA RT qPCR results (e.g. due to RNA degradation during sample transport or RNA extraction procedures), the cDNA prepared as described above was subjected to qPCR for detection of the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (S1 Protocol)[18]. The performance of the GAPDH mRNA reverse transcriptase qPCR is provided as supplementary material (S2 Protocol). All whole transcriptome RNA extracts from Buruli ulcer patients tested positive when subjected to GAPDH mRNA RT qPCR at baseline.

The cDNA was then subjected to 16S rRNA qPCR and DNA to IS*2404* qPCR to increase the specificity for *M. ulcerans* and quantification of the bacterial load as previously described [15]. Quantitative PCR of IS*2404* (DNA), and 16S rRNA (cDNA) targets were carried out at 95˚C for 15 min, and then 40 cycles of 95˚C for 15 sec and 60˚C for 60 sec in a BioRad CFX 96 real time PCR detection system (BIORAD, Singapore). Each run included negative extraction controls, negative "no template" controls, negative gDNA wipe-out controls (16S rRNA qPCR only), inhibition controls (exogenous IPC) and positive controls. Ten fold serial dilutions of known amounts of a plasmid standard of IS*2404* (99 bp) and 16S rRNA (147 bp) (Eurofins MWG Operon, Ebersberg, Germany) were included with PCR amplification for preparation of a standard curve. *M. ulcerans* bacillary loads in original clinical samples were calculated based on threshold cycle values per template of IS*2404* qPCR (standard curve method) adjusted to the whole amount of DNA extract and the known copy number of 207 IS*2404* copies per *M. ulcerans* genome on average.

Statistical analysis

The raw data generated from the study was entered in Microsoft Excel (Microsoft Corporation, Redmond, WA) and analyzed using Graphpad Prism version 5.0 (GraphPad Software, Inc., La Jolla, CA) and Microsoft Excel (Microsoft Corporation). The Kaplan-Meier survival analysis (log rank test) was used to determine the time to clearance of *M. ulcerans* 16S rRNA, as well as to determine the influence of persistent *M ulcerans* 16S rRNA on time to healing. This approach was used to offset bias due to patient censoring for not showing up at study time points. Mann Whitney test was used to compare the bacillary load at baseline in patients with presence or absence of viable organisms at week 4, and also to analyse rate of healing at week 4 in relation to detection of viable organisms. Mann Whitney test were used due to variable distribution of data. Fisher's exact test was used to compare positive results of 16S rRNA assay with culture due to small sample size. P value < 0.05 was considered statistically significant in all the analyses. All statistical tests were two-tailed.

Ethics statement

Verbal and written informed consent was obtained from all eligible participants, and from parents or legal representatives of participants aged 18 years or younger. Ethical approval was obtained from the Committee of Human Research Publication and Ethics, School of Medical Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana (CHRPE/AP/229/12).

Results

Characteristics and diagnosis of study participants

Of 150 patients presenting to treatment centers with clinically suspected Buruli ulcer, *M. ulcerans* infection was confirmed by IS*2404* PCR in 129 cases (Table 1): in 104 out of these by gel-

Table 1. Demographic data and diagnostic test results for Buruli ulcer patients.

	No. of Participants: N (%) n = 129
Age(years)	
Median(IQR [e])	14(10–30)
Sex	
Male Female	61 (47.3) 68 (52.7)
Lesion Form [a]	
Nodule	29 (22.4))
Plaque	24 (18.6))
Oedema	4 (3.1))
Ulcer	68(52.7)
Ulcer with oedema	4 (3.1)
Category of lesion	
I (< = 5cm)	57 (44.2)
II(5-15cm)	56 (43.4)
III(>15cm)	16(12.4)
Sample type	
FNA	63(48.8)
Swab	66(51.2)
Diagnostic confirmation	
Microscopy[b]	50/125(40.0)
Culture[d]	44/129(34.1)
IS *2404* DRB-PCR[c]	104/127(81.9)
IS *2404* qPCR	129/129(100)

[a]FNA samples were taken from 3 patients presenting with ulcers because they did not have undermined edges and from 1 patient presenting with ulcerated oedema.

Diagnostic tests used in the study were smear microscopy for AFB, culture for *M. ulcerans*, dry-reagent-based (DRB) IS*2404* PCR and IS*2404* real time PCR (qPCR).

[b] Microscopy was not done for 4 participants

[c] IS*2404* DRB-PCR was not done for 2 participants

[d]6 of these patients had a positive *M. ulcerans* culture result from a sample taken after 4 weeks of antibiotics treatment

[e] IQR interquartile range

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Table 2. Sensitivity of *M. ulcerans* 16S rRNA assay compared with culture for Buruli ulcer patients at baseline.

	Mu Culture (No. of patients[a]) N = 124		
Mu 16S rRNA N = 124	**Positive (n = 38)**	**Negative (n = 86)**	**Sensitivity (95% CI)**
Positive (n = 84)	36	48	95(82–99)
Negative (n = 40)	2	38	

[a]All Patients were *M. ulcerans* IS*2404 qPCR* positive.

https://doi.org/10.1371/journal.pntd.0005695.t002

based DRB PCR and qPCR, and for the remaining 25 cases by IS*2404* qPCR only. Fifty seven (44%) had pre-ulcerative lesions and 16 (12%) had lesions larger than 15 cm in maximum diameter (category III). There were 8 lesions with oedema, 4 of which were pre-ulcerative. Out of 129 IS*2404* PCR positive patients, direct smears for the detection of AFB were available for 125 patients (96.9%) and 50 (40%) tested positive. Samples were taken for culture from 129 patients of which 44 (34%) were positive.

Detection of *M. ulcerans* 16S rRNA and response to antibiotic treatment

Positive results for *M. ulcerans* 16S rRNA were obtained in 84 out of 129 patients (65%) at baseline (Table 2). Although the sensitivity of 16S rRNA was substantially higher than that for culture (34%), 2 of 38 samples yielding a positive culture had negative 16SrRNA, presumably as a result of sampling error.

After initiation of antibiotic therapy, *M. ulcerans* 16S rRNA was detected in 78% of patients with unhealed lesions at 4 weeks, 52% at 8 weeks, 23% at 12 weeks, and 10% at week 16 (Fig 1). Of 15 patients censored at week 16 when sampling ended, 3 had positive *M. ulcerans* 16S rRNA but in 12 patients a sample could not be obtained. Thus despite antibiotic treatment for 8 weeks, positive 16S rRNA was still detected in 52% lesions sampled at week 8 and the median for detection of *M. ulcerans* by Kaplan-Meier curve analysis was 12 weeks (95% CI 8–16). The number of patients whose lesions yielded a positive *M. ulcerans* culture decreased to 24% at week 4, 5% at week 8 and none by week 16. *M. ulcerans* was detected by culture for a median time of 4 weeks (95% CI 4–6) (S1 Table).

Relationship of bacterial load before treatment to clearance of *M. ulcerans* 16S rRNA

Before antibiotic treatment, 28 patient lesions in which *M. ulcerans* 16S rRNA was negative and 27 patients with detectable *M. ulcerans* 16S rRNA at baseline but subsequently undetectable after 4 weeks of antibiotic treatment had a significantly lower bacterial load based on qPCR for IS*2404* ($p = 0.003$; Mann Whitney) (Fig 2), than those of 74 patients with detectable 16S rRNA at week 4 or later.

Detection of *M. ulcerans* 16S rRNA and healing outcome

Patients with positive 16S rRNA at week 4 had a 3.7-fold increase (95% CI 2.43–5.04) in the time to complete healing of Buruli ulcer lesions compared to those with negative 16S rRNA result at week 4 (Fig 3). This was not attributable to lesion size at baseline because there was no significant difference in initial size of patient lesions with or without detectable 16S rRNA at week 4 ($p = 0.0798$, Mann Whitney). Fig 4 shows that the rate of wound healing (ROH) determined at week 4 was higher for patients with undetectable 16S rRNA at week 4 [2.4 (0.8 to 6.2)

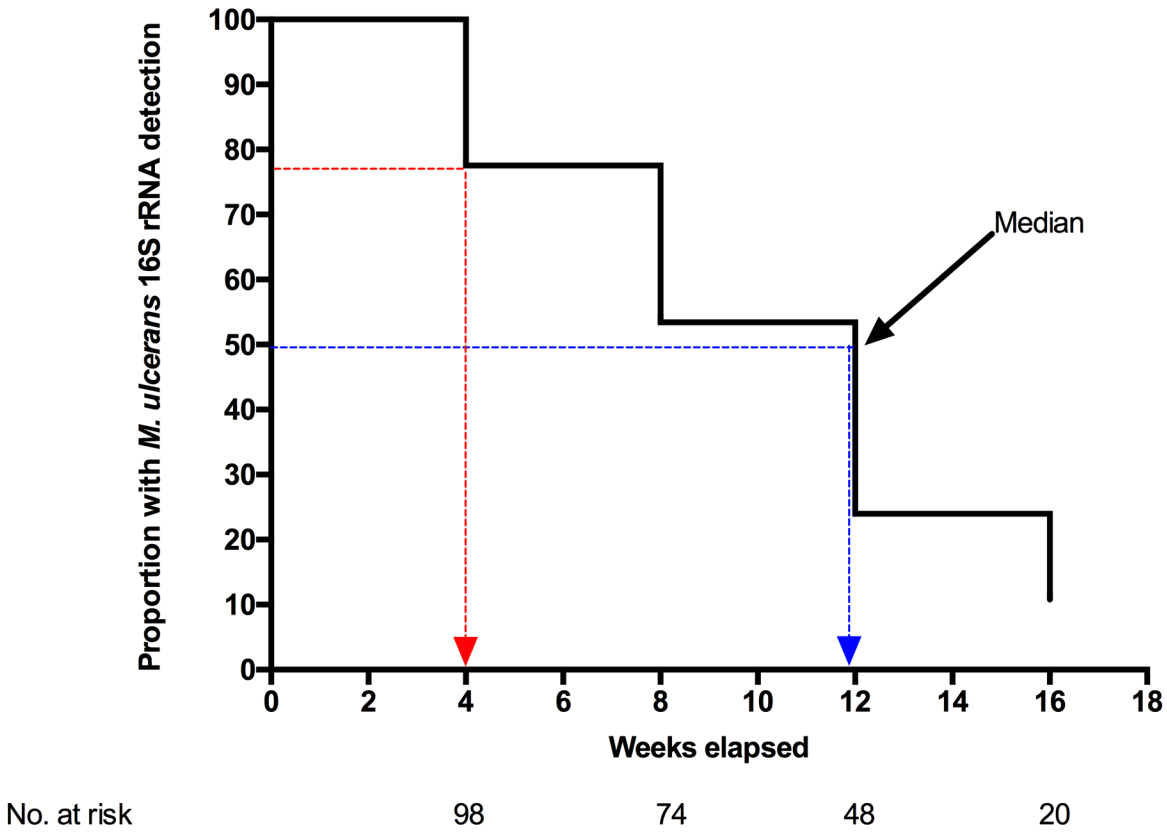

Fig 1. Kaplan-Meier analysis of *M. ulcerans* 16S rRNA in Buruli patients on antibiotic treatment. Blue line: Median time (weeks) for detection of *M. ulcerans* 16S rRNA. Red line: Proportion of patients with positive *M. ulcerans* 16S rRNA at week 4.

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mm/week; median (interquartile range)] compared to those with positive 16S rRNA at week 4 [0.3 (-2.0 to 3.3) mm/week] (p = 0.0003, Mann Whitney).

Discussion

Simultaneous detection of 16S rRNA and IS*2404* by qPCR has been shown to be a specific marker for the presence of viable *M. ulcerans* in human tissue [15]. In this study, we have investigated the time taken for the 16S rRNA assay to become negative during antibiotic treatment for 8 weeks. The assay detected viable bacteria in 65% of samples taken from patients proven to have Buruli ulcer by PCR for IS*2404*. Since these samples were from untreated patients, they should all have been *M. ulcerans* 16S rRNA positive. One possible explanation for false negatives would be loss of mRNA during transport to the laboratory so we measured concurrent detection of human GAPDH mRNA. This was positive showing that mRNA was present in the 16S rRNA negative samples. Sampling error is the most likely explanation for the false negatives which is not surprising since the volume of FNA samples is less than 50 μl and *M. ulcerans* is not evenly distributed within lesions [19]. We found that there was a relationship between bacterial load measured by qPCR for IS*2404* and the result of the 16S rRNA assay; bacterial load was significantly lower in samples with negative 16S rRNA. Thus the combination of low bacterial load and a less sensitive 16S rRNA assay may also account for false negatives. The 16S rRNA assay was more sensitive than culture for *M. ulcerans* as shown in Table 2; negative 16S rRNA with positive culture was detected in only 2 patient lesions whereas negative culture with positive 16S rRNA was found in 48 lesions.

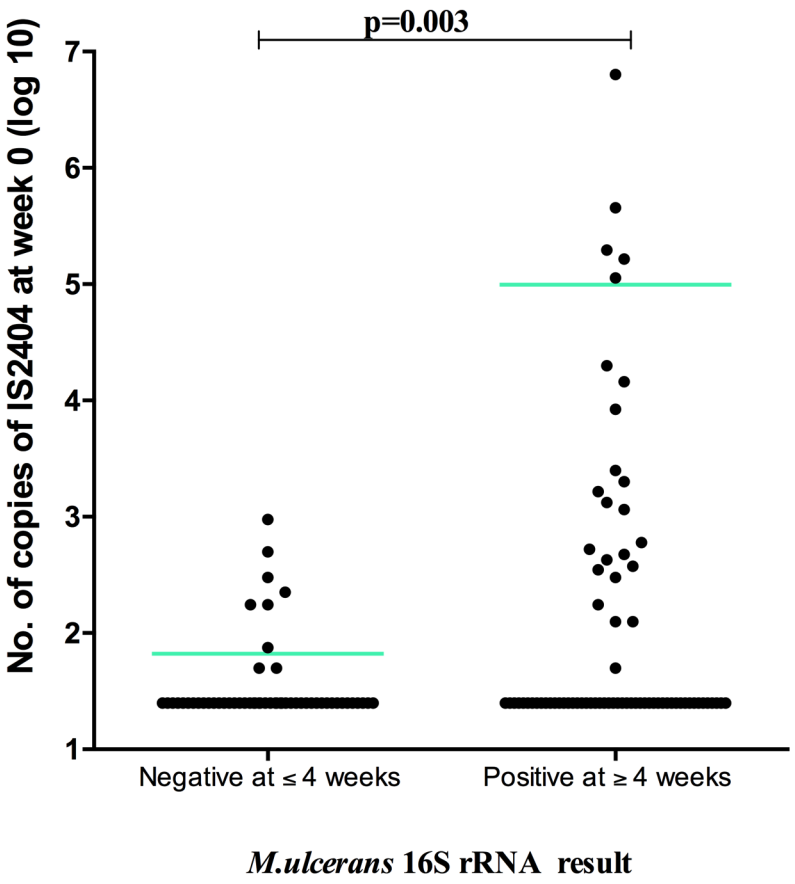

Fig 2. Comparison of baseline *M. ulcerans* IS*2404* in Buruli ulcer patients with a positive or negative 16S rRNA result at week 4.

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At week 4, 20 of 129 (16%) lesions had healed and 22% of unhealed lesions had no detectable viable *M. ulcerans* (16S rRNA) in the lesion (Fig 1). If these patients could be identified before or during the early stages of treatment it is possible that the course of antibiotics could be shortened substantially with considerable benefit to patients as well as a reduction in the cost of management. The recommendation that patients receive treatment for 8 weeks was derived from the finding that early lesions excised after 2 weeks antibiotic treatment were still culture positive but those excised after 4 weeks were all negative [3]. The 16S rRNA assay is more sensitive than culture as shown in the present study and if lesions could be shown to be 16S rRNA negative at 4 weeks it would be justified to abbreviate the course of antibiotics. This would need to be assessed by a clinical trial, using the currently recommended combination of clarithromycin and rifampicin. Evidence for shorter treatment for selected patients is supported by recent data from Australia where complete healing was achieved after 14 to 28 days of antibiotics in selected patients but most of the patients had received early surgical treatment in addition to antibiotics and the study was retrospective [8]. The cost and skill requirement for the 16S rRNA assay limits its routine use in most countries where Buruli ulcer is endemic but it may be possible to predict rapid responders in other ways. This is the subject of ongoing studies.

The healing rate was faster over the first 4 weeks in patients who had cleared active infection by that time (Fig 3). Also the time to complete healing was significantly longer in patients with

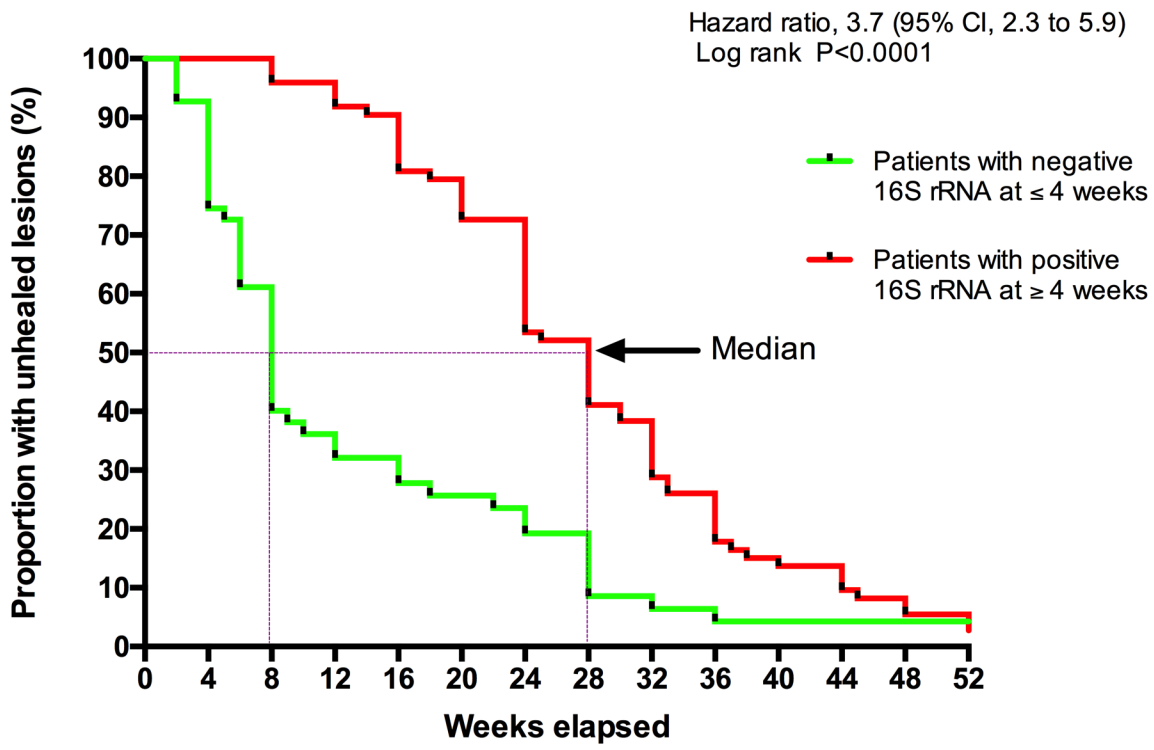

Fig 3. Survival curve for time to healing in Buruli patients with a negative or positive *M. ulcerans* 16S rRNA at week 4. Purple lines: Median time to healing.

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persistent infection independently of the initial lesion size. There has been speculation about why some lesions heal slower than others despite appearing clinically comparable before treatment and the findings from this study suggest that persistent infection is an important contributing factor. Furthermore several observations imply that the initial bacterial load may determine the time to total clearance of viable bacteria from BU lesions. A crude estimate of bacterial load was made by quantifying the number of copies of IS*2404* using qPCR. A better estimate could be made by taking multiple samples or biopsies but this was not considered ethically acceptable. Given the limitations of the data it is not surprising that there was not a significant correlation between initial bacterial load and the time for which viable bacteria remained detectable but Fig 1 illustrates that they are probably related since the bacterial load in lesions with negative *M. ulcerans* 16S rRNA at week 0 was significantly lower than that in all other groups.

At the end of the standard 8 week period of antibiotic treatment 52% of lesions were 16S rRNA positive (Fig 1) raising the question whether antibiotic treatment should be prolonged for a selected subgroup of patients. We have found positive *M. ulcerans* culture in 2 patients who had fully complied with treatment for 8 weeks in an earlier study[12]. The finding that healing was delayed in this group compared with those with negative 16S rRNA supports the idea of continuing antibiotics, perhaps for a further 4 weeks but against this is the fact that all the lesions healed eventually without further antibiotic treatment. There is also the difficulty of identifying such lesions except within the context of a research study since this assay is

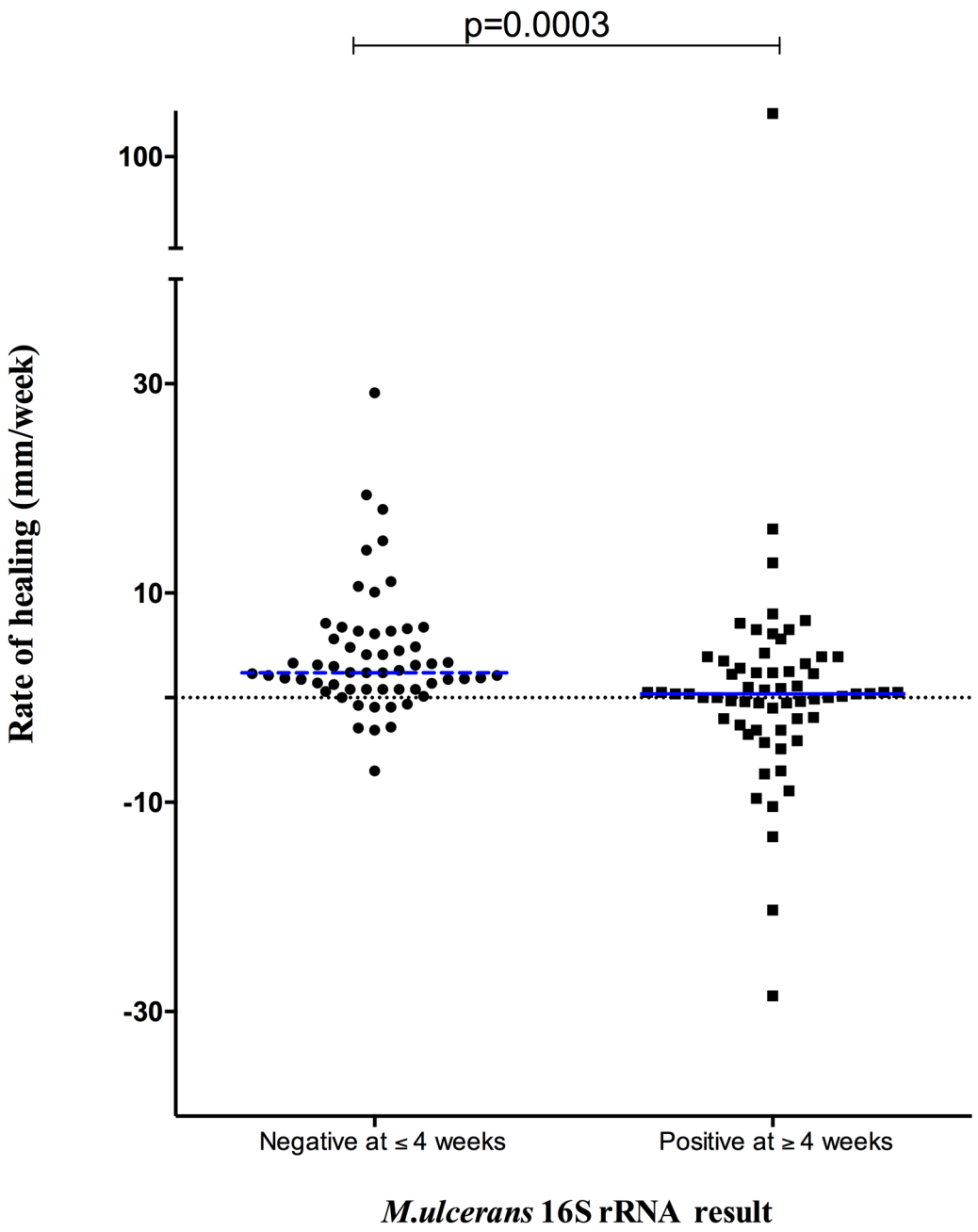

Fig 4. Rate of wound healing at week 4 in Buruli patients with a negative or positive *M. ulcerans* 16S rRNA. Rate of healing was highest in patients where *M. ulcerans* 16S rRNA was negative at baseline or 4 weeks after starting antibiotic treatment. The rate of healing at week 4 (ROH) was computed in millimeters per week by subtracting the mean diameter of the lesion at week 4 from that at week 0 and dividing this result by 4.

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relatively expensive and labor intensive for routine use. At present a judgment would have to be made on purely clinical grounds.

The presence of detectable *M. ulcerans* 16S rRNA after chemotherapy with rifampicin and streptomycin may be indicative sometimes of a persistent altered physiological state of *M. ulcerans* such that it can reactivate to cause recurrent disease later. An analogous situation

arises when *M*. *tuberculosis* is treated with rifampicin or pyrazinamide. Subpopulations consisting of dormant or semi-dormant, antibiotic tolerant persisters survive longest during chemotherapy and are difficult to kill with any new antibacterial drug. They are thought to be responsible for the prolonged period required for effective chemotherapy in tuberculosis [20–22]. In human *M*. *ulcerans* disease, lesions with persistent viable organisms still go on to heal, albeit slowly, presumably due to immune clearance of the organism whereas in tuberculosis, residual viable organisms invariably cause disease. In BU, as mycolactone concentration decreases in lesions during antibiotic therapy [12], IFN-gamma levels [23] increase possibly due to *M*. *ulcerans* antigens interacting normally with the immune system. The slow clearance of these organisms may however explain the slow healing of some of these wounds due to the inhibition of vital wound healing factors by mycolactone.

It is not known whether antibiotic tolerant persisters cause relapse in *M*. *ulcerans* disease but current evidence does not support this. Recurrent *M*. *ulcerans* disease was fairly common before the antibiotic era when 6–47% of patients experienced relapse after surgical treatment alone, [24] [25] probably because there were residual *M*. *ulcerans* in apparently healthy tissue at resection margins [26]. However, since observed antibiotic therapy was introduced, reported series have shown relapse rates below 2% [7, 9]. Individuals with a deeply compromised immune system such as those co-infected with HIV are at risk of relapse or overwhelming disseminated disease but this is more likely due to the need for a competent immune response to clear infection [27] [28]. That the presence of *M*. *ulcerans* 16S rRNA indicates persistence of viable organisms in the tissue is supported by our previous findings that mycolactone can be detected in some patients after they finish antibiotics as can positive cultures for *M*. *ulcerans* [12]. The presence of mycolactone, the toxin secreted by *M*. *ulcerans*, probably indicates that viable organisms are still extant but the pharmacokinetics of mycolactone are not known and it could persist after killing of the organism. Mycolactone is a powerful inhibitor of many growth factors and if it persists in a Buruli ulcer it is likely to retard healing [29]. Further investigations are ongoing to identify lesions containing the toxin after the end of treatment in the present study. However further work is also needed to determine if there is an association between *M*. *ulcerans* 16S rRNA and mRNA detection suggestive of transcriptional activity which would indicate that the organisms are in a replicative state.

In conclusion this study has demonstrated that current antibiotic therapy for BU disease is highly successful in most patients but it may be possible to abbreviate the treatment to 4 weeks in patients with a low initial bacterial load. On the other hand evidence has been presented that persistent infection contributes to slow healing in other patients, probably those with a high bacterial load, who may need antibiotics for longer than 8 weeks.

Supporting information

S1 Protocol. Human GAPDH mRNA RT qPCR.
(DOCX)

S2 Protocol. Validation and performance of human GAPDH mRNA RT qPCR.
(DOCX)

S1 Table. Patient information.
(XLSX)

S1 Checklist. STROBE checklist.
(DOCX)

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Sechste Originalarbeit

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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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PLoS NEGLECTED TROPICAL DISEASES

Laboratory Confirmation of Buruli Ulcer Disease in Togo, 2007–2010

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Abstract

Background: Since the early 1990s more than 1,800 patients with lesions suspicious for Buruli ulcer disease (BUD) have been reported from Togo. However, less than five percent of these were laboratory confirmed. Since 2007, the Togolese National Buruli Ulcer Control Program has been supported by the German Leprosy and Tuberculosis Relief Association (DAHW). Collaboration with the Department for Infectious Diseases and Tropical Medicine (DITM), University Hospital, Munich, Germany, allowed IS*2404* PCR analysis of diagnostic samples from patients with suspected BUD during a study period of three years.

Methodology/Principal Findings: The DAHW integrated active BUD case finding in the existing network of TB/Leprosy Controllers and organized regular training and outreach activities to identify BUD cases at community level. Clinically suspected cases were referred to health facilities for diagnosis and treatment. Microscopy was carried out locally, external quality assurance (EQA) at DITM. Diagnostic samples from 202 patients with suspected BUD were shipped to DITM, 109 BUD patients (54%) were confirmed by PCR, 43 (29.9%) by microscopy. All patients originated from Maritime Region. EQA for microscopy resulted in 62% concordant results.

Conclusions/Significance: This study presents a retrospective analysis of the first cohort of clinically suspected BUD cases from Togo subjected to systematic laboratory analysis over a period of three years and confirms the prevalence of BUD in Maritime Region. Intensified training in the field of case finding and sample collection increased the PCR case confirmation rate from initially less than 50% to 70%. With a PCR case confirmation rate of 54% for the entire study period the WHO standards (case confirmation rate $\geq$50%) have been met. EQA for microscopy suggests the need for intensified supervision and training. In January 2011 the National Hygiene Institute, Lomé, has assumed the role of a National Reference Laboratory for PCR confirmation and microscopy.

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Introduction

Buruli ulcer disease (BUD), caused by *Mycobacterium ulcerans*, has become the third most common mycobacterial disease after tuberculosis and leprosy. Cases have been reported from more than 30 countries worldwide with a focus on West Africa. The disease mainly affects impoverished inhabitants of remote rural areas, particularly children under the age of 15 years [1].

BUD involves the skin and the subcutaneous adipose tissue. The disease starts as painless papule, plaque or nodule that evolves into large painless ulcerations with characteristically undermined edges and may be accompanied by edema of the surrounding skin. Large ulcers may affect the subjacent bones resulting in osteomyelitis [1]. Self-healing processes may lead to scarring and contractures. Though mortality is low, morbidity and subsequent functional disability are severe [2–5].

Since 2004, antimycobacterial treatment (if necessary followed by surgical intervention) has been considered the treatment of choice [1,6–9]. With the introduction of antimycobacterial treatment, the laboratory confirmation of clinically suspected BUD cases became crucial for the clinical management of BUD. Therefore, WHO strongly recommends collection of diagnostic samples from all clinically suspected BUD cases [1,10,11].

Currently available diagnostic laboratory tests include microscopic examination, culture, IS*2404* polymerase chain reaction (PCR) and histopathological analysis. Swab samples, fine needle

Author Summary

Buruli ulcer disease (BUD) is an emerging disease particularly affecting children under the age of 15 years. Due to scarring and contractures BUD may lead to severe functional disability. Introduction of antimycobacterial treatment necessitated the laboratory confirmation of BUD, and WHO recommends confirmation of at least 50% of patients with suspected BUD by polymerase chain reaction (PCR). In Togo, cases have been reported since the early 1990s. However, less than five percent were laboratory confirmed. Since 2007, the German Leprosy and Tuberculosis Relief Organization (DAHW) has supported the Togolese National Buruli Ulcer Control Program in the area of training, treatment and laboratory confirmation of BUD. In close collaboration of DAHW and the Department for Infectious Diseases and Tropical Medicine, University Hospital, Munich (DITM), diagnostic samples from Togolese patients with suspected BUD were subjected to PCR. Out of 202 suspected BUD cases 109 BUD patients (54%) were PCR confirmed over a period of three years. Whereas the PCR case confirmation rate initially was below 50%, intensified training measures for health staff in the field of clinical diagnosis and collection of diagnostic samples ultimately resulted in 69% PCR confirmed cases. Our findings confirm the prevalence of BUD in Maritime Region.

aspirates (FNA), punch biopsies and surgical biopsies have been used as diagnostic specimens. Microscopy is considered a suitable first line diagnostic test to be applied in field settings. PCR provides the highest sensitivity, therefore is regarded the method of choice for laboratory confirmation as well as sufficient evidence to commence antimycobacterial treatment. WHO encourages all endemic countries to ensure that at least 50% of all cases are confirmed by PCR [1,11–14].

Since the early 1990s patients with lesions clinically suspicious for BUD have been treated in Togolese hospitals. The first two laboratory-confirmed and well documented BUD patients from Togo were described in 1996 by Meyers and colleagues [15]. A case series of 21 clinically diagnosed BUD patients hospitalized from 1994 through 1996 was reported by Songné [16]. A hospital based study conducted from 2000 through 2001 identified 180 patients with suspected BUD, 23 out of those were laboratory confirmed [17]. According to data available at the Togolese Ministry of Health, from 1996 through 2004 more than 100 suspected BUD cases were notified, and approximately 20% of these were PCR confirmed at the Institute for Tropical Medicine, Antwerp, Belgium. In 2004, a nationwide survey detected 1505 suspected cases of BUD [“Politique Nationale de Lutte contre L’Ulcère de Buruli.” Ministère de la Santé, République Togolaise, Lomé 2007; 18].

In 1999, Togo established its National Buruli Ulcer Control Program (Programme National de Lutte contre L’Ulcère de Buruli [PNLUB], since 2010: Programme National de Lutte contre L’Ulcère de Buruli – Lèpre et Pian [PNLUB-LP]). Initially limited resources hampered the progress of program activities, however, collaboration with non-governmental organizations (Handicap International [HI], France; German Leprosy and Tuberculosis Relief Association [DAHW], Germany) enhanced the efficiency of BUD control. In 2007, a five year strategic plan was developed to intensify treatment, case detection, laboratory confirmation and surveillance of BUD, initially focusing on Maritime and Central Region. The Centre Hospitalier Régionale (CHR) Tsévié, Maritime Region, was appointed National Reference Centre for BUD in Togo, and recently the Centre Hospitalier Préfectoral (CHP) Sotouboua, Central Region, was turned into an outpost of the National Reference Centre. The DAHW in particular supports training, treatment and laboratory confirmation of patients with suspected BUD [“Plan Stratégique de Lutte contre L’Ulcère de Buruli, 2008–2012”. Ministère de la Santé, République Togolaise, Lomé 2007; 18].

Whereas microscopic analysis has been locally carried out at CHR Tsévié, facilities for the diagnostic IS*2404* PCR were not available before 2011. Therefore, in 2007 DAHW and the Department of Infectious Diseases and Tropical Medicine, University Hospital, Ludwig-Maximilians University, Munich, Germany (DITM) began a collaboration to analyze diagnostic samples from patients with suspected BUD by PCR at DITM. This study presents a retrospective analysis of the laboratory results of the first cohort of suspected BUD cases from Togo subjected to systematic laboratory analysis. The results of this study also give proof that a collaborative effort of local and international partners allows the successful implementation of a diagnostic system within a relatively short period of time.

Methods

Ethics Statement

Laboratory confirmation and treatment of BUD patients was covered by a skeleton agreement between the DAHW and the Ministry of Health, Togo. As all activities fall under routine patient management, ethical clearance by the Committee of Bioethics in Research, Ministry of Health, Togo, was not required. In accordance with standard practice customary in Togo, from 2007 through 2008 patients with suspected BUD were verbally informed on the need for collection of diagnostic samples and treatment, and verbal consent was obtained from the patients. In 2009, the PNLUB-LP introduced informed consent forms. Written informed consent (signature or thumb print, in case of minors given by legal representatives) was obtained from the majority of patients with suspected BUD attending CHR Tsévié. Publication of pseudonymized data and results obtained during the study period was authorized by the PNLUB-LP.

Case Finding

To integrate active BUD case finding into the existing Togolese network of TB and Leprosy District and Regional Controllers (Contrôleur Lèpre–TB–Buruli, CLT), the DAHW conducted two initial training workshops for CLT and health staff at CHR Tsévié and CHP Sotouboua in 2007, followed by regular re-training from 2008 through 2010 (four workshops in Maritime Region, one workshop in Central and Kara Region each). Supported by CHR Tsévié hospital staff, the CLT teams conducted quarterly sensitization campaigns and outreach activities to identify cases at community level under coordination of the PNLUB-LP. Clinically suspected BUD cases were referred to peripheral health posts (Unité de Soins Périphérique, USP), CHP Sotouboua or CHR Tsévié for collection of diagnostic samples and treatment. Passive case finding included patients presenting at BUD treatment centers (USPs, CHR-Tsévié and CHP Sotouboua).

Study Population

From September 2007 through August 2010, 202 suspected BUD cases from three different study sites in Togo (CHR Tsévié, Maritime Region, n = 187; CHP Sotouboua, Central Region, n = 14, USP Agbetiko, Maritime Region, n = 1) were included in the study (table 1).

Table 1. Case confirmation rates.

Type of lesion[a]	Study site	Suspected cases	MIC[b]			PCR[c]		
			Confirmed cases [N]	Suspected cases subjected to MIC [N]	Case confirmation rate (%)	Confirmed cases [N]	Suspected cases subjected to PCR [N]	Case confirmation rate (%)
Non-ulcerative	Tsévié	49	9	23	(39.1)	38	49	(77,6)
	Sotouboua	2	NA[d]	NA	NA	0	2	(0.0)
	Agbetiko	0	NA	NA	NA	NA	NA	NA
	Total	51	9	23	(39.1)	38	51	(74.5)
Ulcerative	Tsévié	138	34	120	(28.3)	71	138	(51.4)
	Sotouboua	12	0	1	(0.0)	0	12	(0.0)
	Agbetiko	1	NA	NA	NA	0	1	(0.0)
	Total	151	34	121	(28.1)	71	151	(47.0)
All		202	43	144	(29.9)	109	202	(54.0)

Table 1 describes the case confirmation rates, i.e. the number of laboratory confirmed BUD cases divided by the total number of patients with suspected BUD (suspected cases) of whom samples were subjected to a certain diagnostic test; diagnostic samples from 202 suspected BUD cases (suspected cases) from three study sites in Togo (CHR Tsévié, CHP Sotouboua, USP Agbetiko) were collected within three years (September 2007 through August 2010);
[a]Non-ulcerative lesions: FNA (fine needle aspiration) samples, punch biopsy samples and surgical biopsy samples were analyzed; ulcerative lesions: swab samples, FNA (fine needle aspiration) samples, punch biopsy samples and surgical biopsy samples were analyzed;
[b]Test: MIC, microscopic examination for the detection of acid fast bacilli; swab samples and FNA samples were analyzed;
[c]Test: PCR, polymerase chain reaction, gel-based IS*2404* PCR; swab samples, FNA samples, punch biopsy samples and surgical biopsy samples were analyzed;
[d]NA, not available;
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Diagnostic Specimens

Diagnostic samples were collected according to standardized procedures which have been developed in the context of previous studies on laboratory diagnosis of BUD in Ghana [13,19–22]. Briefly, swabs were taken by circling the entire undermined edges of ulcerative lesions. Three millimeter punch biopsies and surgical biopsies with a maximum size of 10×10 mm were taken from the center of non-ulcerative lesions or from undermined edges of ulcerative lesions including necrotic tissue. FNA was performed with 21-gauge needles by trans-dermal aspiration. For non-ulcerative lesions, the needle was inserted into the center of the lesion, for ulcerative lesions, FNA was performed with a maximal distance of 1–2 cm from the margins of the ulcers. If collected from surgical patients, all samples were taken under general anesthesia. Swab samples were collected throughout the entire study period. Most surgical biopsy samples were collected during the first six months of the study period, and then gradually replaced by FNA and punch biopsy samples which were introduced in the first half of 2008.

To facilitate sampling, standardized specimen collection bags including swabs, biopsy punches, syringes and needles, containers with transport media (700 µl CLS® [cell lysis solution, Qiagen, Hilden, Germany] for PCR samples) and data entry forms (BU01 form [1] and a specific laboratory data entry form) were provided to the study sites. Table 2 shows the different types of samples collected according to type of lesion and type of treatment (surgical, non-surgical). However, it was not always possible to collect complete sets of specimens.

Diagnostic Methods and Laboratories

As shown in table 3, swab (n = 115) and FNA samples (n = 115) were subjected to Ziehl-Neelsen smear microscopy at CHR Tsévié and one swab sample was tested at CHP Sotouboua [23]. For external quality assurance (EQA) of microscopic analysis, 85 stained slides were sent to DITM. PCR samples (swabs, n = 152; FNA, n = 167; punch biopsies, n = 172 surgical biopsies, n = 51) with corresponding laboratory data and BU01 forms were shipped to DITM by courier service on a quarterly basis and subjected to gel based IS*2404* PCR (primers MU5 and MU6) according to standardized procedures [13,21,24–26]. To assure that no contamination occurred during extraction and PCR, extraction controls and negative run controls were processed with each extraction procedure and each PCR.

The turnaround time between shipment of samples and availability of results averaged approximately two weeks. Results were communicated by email to DAHW and distributed to the hospitals.

Statistical Analysis

Clinical and epidemiological information derived from laboratory data entry and BU01 forms as well as diagnostic results obtained at DITM and CHR Tsévié were stored in a database (Access 2003, Microsoft Cooperation, Redmond, WA). For analysis, the study period was divided into three observation periods (September 2007 through August 2008, September 2008 through August 2009, September 2009 through August 2010), for clarification selected data are also indicated per calendar year. Beside epidemiological data, the analysis included case confirmation rates (number of laboratory confirmed BUD patients divided by the total number of suspected BUD cases included in the study) per diagnostic test, and positivity rates (number of positive samples divided by the total number of samples tested) per sample type and diagnostic test. Approximative tests (χ2-tests) and t-tests as parametric tests were conducted using Stata software, version 9.0 (Stata Corporation, College Station, TX) and EpiInfo, version 3.3.2 (Centers for Disease Control and Prevention, CDC, Atlanta, GA).

Results

Diagnostic Samples

206 sets of specimens from 202 suspected BUD cases were collected for laboratory confirmation. Fifty-one suspected cases

Table 2. Diagnostic specimens and transport media.

Type of Treatment	Type of lesion	Diagnostic test	Transport medium	Swab	FNA[a]	Punch biopsy	Surgical biopsy
Surgical	Non-ulcerative	MIC[b]	NA[c]	NA	yes	NA	NA
		PCR[d]	CLS[e]	NA	yes	yes	yes
	Ulcerative	MIC	NA	yes	yes	NA	NA
		PCR	CLS	yes	yes	yes	yes
Non-surgical	Non-ulcerative	MIC	NA	NA	yes	NA	NA
		PCR	CLS	NA	yes	yes	NA
	Ulcerative	MIC	NA	yes	yes	NA	NA
		PCR	CLS	yes	yes	yes	NA

Table 2 describes diagnostic specimens and transport media according to diagnostic tests, type of lesion and type of treatment.
[a]FNA, fine needle aspiration;
[b]MIC, microscopic examination for the detection of acid fast bacilli;
[c]NA, not applicable;
[d]PCR, IS*2404* gel-based polymerase chain reaction;
[e]CLS, cell lysis solution (Qiagen, Germany).
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(25.2%) had non-ulcerative lesions, 151 (74.8%) had ulcerative lesions. Four suspected cases (2.0%) had two lesions. From 13 of the 202 study participants 13 sets of follow-up specimens were available.

The patients with suspected BUD originated from ten districts in three regions (Maritime, Central and Plateaux). Most of the suspected cases (82.2%) were detected in districts Zio (n = 89 [44.1%]) and Yoto (n = 77 [38.1%]) of Maritime Region. The age range of the suspected cases was 1–72 years (mean = 24.8 years, median = 17 years) and 39.6% of the suspected cases were in age group 5–14 years, 114 of the suspected cases (56.4%) were male.

Laboratory Confirmed BUD Cases

Out of the 202 patients with suspected BUD 109 were laboratory confirmed as BUD patients. Out of them 43 (39.5%) were confirmed by two and 66 (60.6%) by at least one positive laboratory test. Out of the 13 study participants followed over time twelve were laboratory confirmed at their first presentation at hospital (also the second sample collection rendered positive results). For one of the 13 study participants followed over time neither the first nor the second sample collection rendered positive results.

The overall case confirmation rate by PCR was 54.0% (109/202), and 29.9% (43/144) by microscopy (table 1). Among the 51 suspected BUD cases with non-ulcerative lesions, 38 (74.5%) were confirmed by a positive tissue PCR result (positive FNA PCR result 26/47 [55.3%], positive punch biopsy PCR result 30/44 [68.2%], positive surgical biopsy PCR result 2/3 [66.7%]). FNA and punch biopsy samples were available from 33 out of these 51 suspected cases, thus a comparison of the PCR case confirmation

Table 3. Positivity rates.

		Number of positive samples/total number of samples tested [N(%)]							
Type of lesion	**Study site**	**Swab**		**FNA[a]**		**Punch biopsy**		**Surgical biopsy**	
		MIC [N(%)][b]	**PCR [N(%)][c]**	**MIC [N(%)]**	**PCR [N(%)]**	**MIC [N(%)]**	**PCR [N(%)]**	**MIC [N(%)]**	**PCR [N(%)]**
Non-ulcerative	Tsévié	ND[d]	ND	9/23 (39.1)	27/49 (55.1)	NA[e]	32/50 (64.0)	NA	2/3 (66.7)
	Sotouboua	ND	ND	NA	0/1 (0)	NA	NA	NA	0/2 (0)
	Agbetiko	ND	ND	NA	NA	NA	NA	NA	NA
	Total	ND	ND	9/23 (39.1)	27/50 (54.0)	NA	32/50 (64.0)	NA	2/5 (40.0)
Ulcerative	Tsévié	27/115 (23.5)	63/142 (44.4)	31/92 (33.7)	45/111 (40.5)	NA	49/121 (40.5)	NA	12/44 (27.3)
	Sotouboua	0/1 (0.0)	0/9 (0.0)	NA	0/5 (0.0)	NA	0/6 (0.0)	NA	0/2 (0.0)
	Agbetiko	NA	0/1 (0.0)	NA	0/1 (0.0)	NA	0/2 (0.0)	NA	NA
	Total	27/116 (23.3)	63/152 (41.5)	31/92 (33.7)	45/117 (38.5)	NA	49/129 (38.0)	NA	12/46 (26.1)
All		27/116 (23.3)	63/152 (41.5)	40/115 (34.8)	72/167 (43.1)	NA	81/179 (45.3)	NA	14/51 (27.5)

Table 3 describes the positivity rates, i.e. the number of positive samples divided by the total number of samples tested, of microscopy and IS*2404* gel-based polymerase chain reaction per type of lesion and type of sample; diagnostic samples from 202 patients with suspected BUD from three study sites in Togo (CHR Tsévié, CHP Sotouboua, USP Agbetiko) were collected within three years (September 2007 through August 2010);
[a]FNA, fine needle aspiration;
[b]MIC, microscopic examination for the detection of acid fast bacilli;
[c]PCR, IS*2404*, gel-based polymerase chain reaction;
[d]ND, not done;
[e]NA, not available;
doi:10.1371/journal.pntd.0001228.t003

rates for both types of samples was possible. Among these 33 patients with suspected BUD, the case confirmation rate for punch biopsy samples (30/33; 90.9%) was significantly higher than for FNA samples (23/33; 69.7%) ($p = 0.03$). For 20/33 (60.6%) of these suspected cases both samples had a positive PCR result, 3/33 (9.1%) were confirmed by FNA PCR only, for 10/33 (30.3%) the diagnosis was established by a positive punch biopsy PCR result only, i.e. the additional diagnostic yield of punch biopsy samples for patients with non-ulcerative lesions was 30.3%.

Among the 151 suspected cases with ulcerative lesions, 71/151 (47.0%) were PCR confirmed (table 1). Out of these, 51/131 (38.9%) were confirmed by a positive swab PCR result, and 56/130 (43.1%) had a positive tissue PCR result (positive FNA PCR result 39/104 [37.5%], positive punch biopsy result 39/95 [41.1%], positive surgical biopsy result 4/18 [22.2%]). All types of samples were available from 41 out of these 151 suspected cases, thus a comparison of the PCR case confirmation rates for swab, FNA and punch biopsy samples was possible. Among these 41 suspected cases there was no significant difference in case confirmation rates 31/41 [75.6%] for swab samples, 36/41 [87.8%] for FNA samples and 36/41 [87.8%] for punch biopsy samples ($p = 0.22$).

The positivity rates for microscopy and PCR per type of specimen are shown in table 3.

EQA for microscopy resulted in 23/37 (62.2%) concordant results, 14 slides (37.8%) were false negative.

Epidemiological Baseline Data of Confirmed BUD Cases

Out of the 109 laboratory confirmed BUD patients, 38 (34.9%) had non-ulcerative, 71 (65.1%) had ulcerative lesions, 57 (52.3%) were male, and 65 (59.6%) of them were in age group 5–14 years (age range 2–60 years, mean 17.3 years, median 12 years) (figure 1).

The confirmed BUD patients originated from five districts of Maritime Region (Zio, n = 51; Yoto, n = 49; Vo, n = 5, Golfe, n = 1; Avé, n = 1).

In 90.8% (99/109) the lesions were located on limbs or shoulders. None of the sides was significantly more affected (right side, n = 51 and left side, n = 48).

For all 109 confirmed BUD patients the lesion sizes were known and the lesions were distributed according to WHO categories as follows [1]: category I (single lesion <5 cm in diameter), n = 43 (39.4%); category II (single lesion between 5 and 15 cm in diameter), n = 41 (37.6%); category III (single lesion >15 cm in diameter, multiple lesions, osteomyelitis), n = 25 (22.9%).

Among the BUD patients originating from Maritime Region, five pairs of siblings (two individuals each) were identified, three pairs of siblings developed BUD at the same time. Three pairs of siblings originated from the district of Yoto, two from the district of Zio, all affected families lived close to flowing water bodies (Haho River, Lili River).

Development of PCR Case Confirmation Rates from 2007 through 2010

The PCR case confirmation rate increased with a definite trend from 42.9% (36/84) to 69.2% (36/52) (coefficient of determination, $R^2 = 1$) from the first through the third observation period (figure 2). Calculated per calendar year, the PCR case confirmation rate was 41.7% (10/24) in 2007, 45.8% (38/83) in 2008, 58.9% (33/56) in 2009, and 71.8% (28/39) in 2010 (data not shown).

Discussion

This study describes the results of a collaborative approach to implement systematic laboratory confirmation of BUD in Togo. Whereas previous data reported from Togo were largely based on clinical observations, this study proves the prevalence of laboratory confirmed BUD cases in Maritime Region. From 2007 through 2010 out of 202 suspected BUD cases 109 BUD patients were PCR confirmed, which equals an overall PCR case confirmation rate of 54%. During the decade after the description of the first two laboratory confirmed BUD patients in 1996 [15], more than 1,800 (in most cases clinically suspected but not laboratory confirmed) BUD cases were reported from Togo ["Politique Nationale de Lutte contre L'Ulcère de Buruli." Ministère de la Santé, République Togolaise, Lomé 2007; 16–18]. As recently as 2007, the initiation of a close collaboration between PNLUB-LP and several non-governmental organizations as well as the establishment of the National Reference Centre for BUD at CHR Tsévié, laid the foundations for intensified BUD control activities. In accordance with the objectives for BUD control as defined by the Togolese Health Authorities, emphasis was given to early case detection and laboratory confirmation of cases ["Plan Stratégique de Lutte contre L'Ulcère de Buruli, 2008–2012", Ministère de la Santé, République Togolaise, Lomé 2007; 18]. A collaborative project on laboratory confirmation of patients with

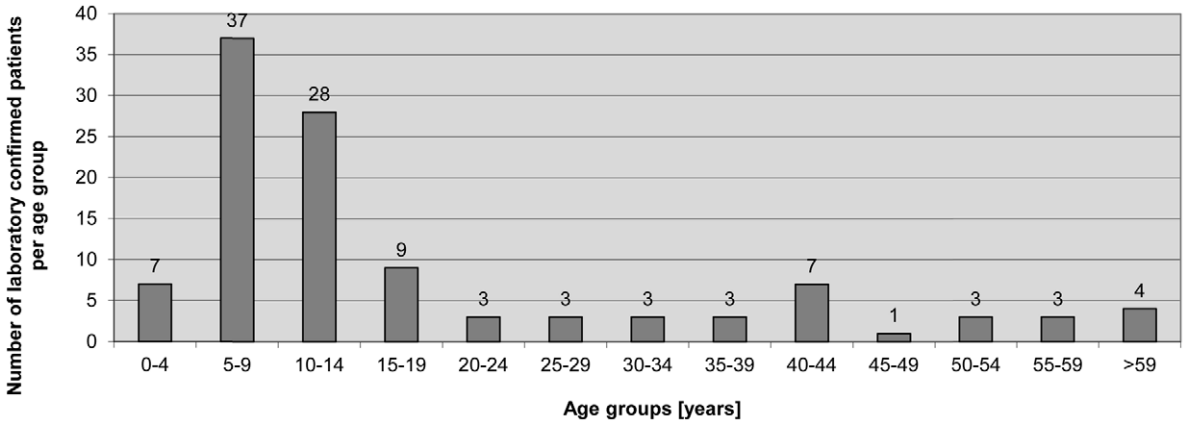

Figure 1. Age distribution of 109 laboratory-confirmed BUD patients. For all patients the age was known and 65 (59.6%) of them were in age group 5–14 years. The age range was 2–60 years with a mean of 17.3 years and the median was 12 years.
doi:10.1371/journal.pntd.0001228.g001

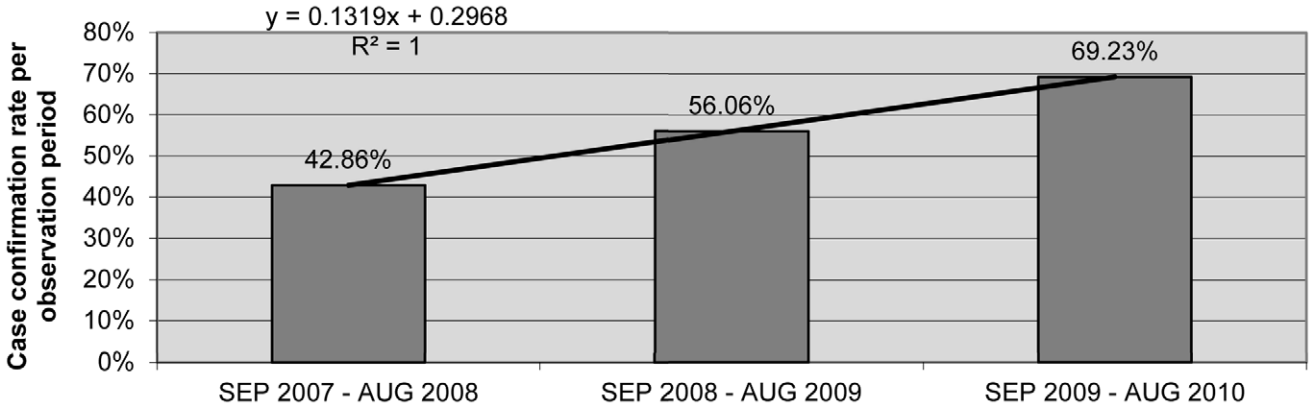

Figure 2. Case confirmation rate per observation period. The PCR case confirmation rate was 36/84 (42.86%) in the first observation period (September 2007–August 2008), 37/66 (56.06%) in the second observation period (September 2008–August 2009) and 36/52 (69.23%) in the third observation period (September 2009–August 2010). The case confirmation rate increased during the three observation periods with a definite trend (coefficient of determination, $R^2 = 1$).
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suspected BUD conducted by DAHW and DITM allowed for the first time continuous data acquisition over a period of three years.

The strategies applied for collection of diagnostic samples and data management were originally developed in the context of an EC funded research project (project no. INCO-CT-2005-015476-BURULICO) conducted in Ghana [13,19–22]. Visits of DAHW staff at the Kumasi Centre for Collaborative Research in Tropical Medicine (KCCR), Kumasi, Ghana, proved to be instrumental in adopting these procedures for the implementation of laboratory confirmation of BUD in Togo, thus provide an example for efficient South-South collaboration in the area of disease control.

Initially the PCR case confirmation rate was below 50%. However, for the second (56.1%) and third (69.2%) observation period as well as for the entire study period (54.0%) the WHO criteria for PCR case confirmation rates have been met [1,11]. These findings are mainly attributable to the intensified and regular training activities for CLTs and health staff in the field of clinical diagnosis and collection of diagnostic samples conducted by DAHW.

As far as punch biopsies are concerned, meanwhile broad consensus has been reached that FNA are equal to punch biopsies for most diagnostic applications, and – in the interest of the patients - the use of punch biopsies should be restricted to a minimum [22,27–30]. Whereas the recently published studies on FNA provide details on diagnostic sensitivities of laboratory analysis of various sample types, the data obtained from the Togolese cohort of patients with suspected BUD focus on case confirmation rates. Among the suspected BUD cases with non-ulcerative lesions from Togo the case confirmation rate for punch biopsy samples was significantly higher than for FNA samples. Moreover, PCR analysis of punch biopsy samples allowed the confirmation of 30% additional patients that were not detected by PCR of FNA samples. These findings suggest that at this point in time replacement of punch biopsies by FNA for suspected BUD cases with non-ulcerative lesions is not advisable. Upcoming training activities have to focus on improvement of FNA sample collection techniques and the use of punch biopsies should be maintained until analysis of diagnostic results provides sufficient evidence that no more case are missed if FNA is applied.

A number of limitations of this study need to be mentioned. During the study period most training workshops were held in Maritime Region – which is reflected in a continuous improvement of the quality of samples and data obtained from the catchment area of CHR Tsévié. In contrast, all diagnostic samples sent from Central Region were negative, therefore this study did not succeed to confirm the prevalence of BUD outside of Maritime Region. Further attempts to verify if the disease occurs in other regions of the country require intensified training in the field of clinical diagnosis and collection of diagnostic samples in the respective areas.

Furthermore, this study did not use specific questionnaires; patient related information was obtained from standardized BU01 forms instead. The current versions of BU01 forms however, do not contain information required for analysis of risk factors to contract the disease (e.g. information on living conditions and contact with water bodies); therefore only baseline data were available for analysis.

As PCR assessment was conducted in an external reference laboratory in Germany, a maximum number of samples were collected per patient to increase the probability for laboratory confirmation and to avoid repeated shipping of samples. To comply with recent WHO recommendations [30], future routine clinical management in Togo will have to reduce the number of diagnostic samples.

Concerning microscopy, beside a low concordance rate and a high percentage of false negative results, for approximately 30% of the patients with suspected BUD microscopy had either not been performed, local results could not be retrieved retrospectively, or a considerable number of slides had been discarded, thus were not available for re-checking at DITM. Improvement of the performance of microscopy requires a more stringent system for external quality assurance including regular supervision of local microscopy laboratories.

Although in general - with a turnaround time of approximately two weeks between shipment of samples and availability of laboratory results - PCR assessment at an external reference laboratory in Germany worked satisfactorily, local PCR capacities are desirable. Therefore, in January 2011 the National Hygiene Institute (INH) in Lomé has assumed the role of a National Reference Laboratory for PCR confirmation and microscopy.

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Author Contributions

Conceived and designed the experiments: GB KLH BK MB FXW AD TL JN. Performed the experiments: KLH EP KH EF JN. Analyzed the data: GB KLH MB EP K-HH KA AB-K KH JN. Contributed reagents/materials/analysis tools: GB KB K-HH FXW AB-K AD TL. Wrote the paper: GB KLH MB K-HH JN. Planning and realization of training workshops for CLTs and health staff in Togo: GB KLH BK EP FXW KA AB-K AD JN. Planning and realization of outreach activities for case finding in Togo: KLH BK EP FXW KA AB-K AD JN. Critical review of manuscript: K-HH TL.

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Implementation of a National Reference Laboratory for Buruli Ulcer Disease in Togo

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Abstract

Background: In a previous study PCR analysis of clinical samples from suspected cases of Buruli ulcer disease (BUD) from Togo and external quality assurance (EQA) for local microscopy were conducted at an external reference laboratory in Germany. The relatively poor performance of local microscopy as well as effort and time associated with shipment of PCR samples necessitated the implementation of stringent EQA measures and availability of local laboratory capacity. This study describes the approach to implementation of a national BUD reference laboratory in Togo.

Methodology: Large scale outreach activities accompanied by regular training programs for health care professionals were conducted in the regions ''Maritime'' and ''Central,'' standard operating procedures defined all processes in participating laboratories (regional, national and external reference laboratories) as well as the interaction between laboratories and partners in the field. Microscopy was conducted at regional level and slides were subjected to EQA at national and external reference laboratories. For PCR analysis, sample pairs were collected and subjected to a dry-reagent-based IS*2404*-PCR (DRB-PCR) at national level and standard IS*2404* PCR followed by IS*2404* qPCR analysis of negative samples at the external reference laboratory.

Principal Findings: The inter-laboratory concordance rates for microscopy ranged from 89% to 94%; overall, microscopy confirmed 50% of all suspected BUD cases. The inter-laboratory concordance rate for PCR was 96% with an overall PCR case confirmation rate of 78%. Compared to a previous study, the rate of BUD patients with non-ulcerative lesions increased from 37% to 50%, the mean duration of disease before clinical diagnosis decreased significantly from 182.6 to 82.1 days among patients with ulcerative lesions, and the percentage of category III lesions decreased from 30.3% to 19.2%.

Conclusions: High inter-laboratory concordance rates as well as case confirmation rates of 50% (microscopy), 71% (PCR at national level), and 78% (including qPCR confirmation at external reference laboratory) suggest high standards of BUD diagnostics. The increase of non-ulcerative lesions, as well as the decrease in diagnostic delay and category III lesions, prove the effect of comprehensive EQA and training measures involving also procedures outside the laboratory.

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Introduction

Buruli ulcer disease (BUD), caused by *Mycobacterium ulcerans*, is an infectious disease affecting skin, soft tissue and bones. If left untreated, extensive destruction of tissue followed by fibrous scarring and contractures may lead to severe functional limitations [1–6]. BUD is treated with rifampicin and streptomycin (or clarithromycin) for eight weeks if necessary followed by surgical interventions; the laboratory confirmation of clinically suspected BUD cases prior to treatment has become an integral part of clinical management. Whereas microscopy is an appropriate and cost-effective first-line test for peripheral laboratories, PCR is

Author Summary

Buruli ulcer disease (BUD), the third most common mycobacterial disease worldwide, is treated with standardized antimycobacterial therapy. According to WHO recommendations at least 50% of cases should be laboratory confirmed by polymerase chain reaction (PCR). In a previous study PCR analysis of clinical samples from suspected BUD cases from Togo and external quality assurance (EQA) for local microscopy were conducted at an external reference laboratory in Germany. The relatively poor performance of local microscopy as well as time and effort associated with shipment of clinical samples abroad necessitated the availability of a local BUD reference laboratory and the implementation of stringent EQA measures. All processes in the laboratories as well as in the field were defined by standard operating procedures, microscopy conducted at regional facilities was subjected to EQA at national and external reference level, and PCR samples were analyzed in parallel at national and external reference laboratories. Inter-laboratory concordance rates of >90% and case confirmation rates of 50% (microscopy) and >70% (PCR) respectively suggest high standards of BUD diagnostics. Furthermore, an increase of non-ulcerative lesions and a decrease in diagnostic delay and category III lesions reflect the impact of comprehensive EQA measures also involving procedures outside the laboratory on the quality of BUD control.

considered the method of choice and WHO recommends PCR confirmation of at least 50% of suspected BUD cases [3,7–13]. Microscopy and various PCR assays have been successfully implemented in other endemic countries and case confirmation rates of 29–78% (microscopy) and 54–83% (PCR) were reported [10,12–32].

Since the early 1990s, close to 2,000 BUD cases were reported from Togo. However, due to the lack of local diagnostic laboratory capacity, the majority of these cases remained unconfirmed [7,13,33–35].

From 2007 through 2010, a joint research project between the German Leprosy and Tuberculosis Relief Organization, Togo office, Lomé, Togo (DAHWT) and the Department of Infectious Diseases and Tropical Medicine (DITM), University Hospital, Ludwig-Maximilians-University, Munich, Germany, allowed the first systematic study on laboratory confirmation of BUD cases from Togo and proved the prevalence of BUD in South Togo (region "Maritime"). The study revealed a relatively poor performance of local Ziehl-Neelsen microscopy, suggesting the need for a stringent system for external quality assurance (EQA) including regular supervision of microscopy laboratories. Intensified training measures in the area of sample collection resulted in a PCR case confirmation rate of 70%. Effort and turnaround time associated with shipment of samples to an external reference laboratory, however, necessitated the availability of local laboratory capacities [13].

In the context of the EC-funded research project "BuruliVac" (FP7/2010–2013; grant agreement N° 241500), the implementation of a national reference laboratory for BUD in Togo was envisaged. Therefore, from January 2011 through April 2012, microscopy and PCR facilities were established at the "Institut National d'Hygiène" (INH), Lomé, Togo.

This study describes the approach to implementation of a national reference laboratory and analyzes the impact of intensified EQA and training measures on laboratory diagnosis and control of BUD in Togo.

Materials and Methods

Ethics

Ethical clearance was obtained through the national Togolese ethics committee ("Comité de Bioéthique pour la Recherche en Santé") at the University of Lomé (14/2010/CBRS) and the study was approved by the "Ministère de la Santé de la République Togolaise" Lomé, Togo (Ref. No. 0009/2011/MS/DGS/DPLET). All samples analyzed in this study were collected for diagnostic purposes within the EC funded research project "BuruliVac". Written informed consent was obtained from all study participants.

Role of participating institutions

This study constitutes a collaborative project between several Togolese and German institutions. Since 2007, the German Leprosy and Tuberculosis Relief Organization (DAHW) has supported the Togolese National Buruli Ulcer Control Program ("Programme National de Lutte contre L'Ulcère de Buruli – Lèpre et Pian" [PNLUB-LP]) in the area of training, laboratory confirmation and treatment of BUD. In this study, the main tasks of DAHWT, as partner of the "BuruliVac" consortium were field work, recruitment of study participants, and collection of diagnostic samples. The tasks of DITM – an accredited laboratory according to DIN EN ISO 15189 - as lead partner for all patient related activities of the "BuruliVac" project consisted of implementation of molecular diagnostic laboratory methods at the designated national Togolese BUD reference laboratory and standardization of all processes through on-site training, standard operating procedures (SOPs), and EQA of microscopy and PCR (by standard gel-based IS*2404* PCR and IS*2404* quantitative real-time PCR [qPCR]) including supervisory visits. Patients with suspected BUD were referred to peripheral health posts ("Unité de Soins Périphérique", USP; operating on district level as point of care facilities with a catchment area of 5,000–9,000 inhabitants depending on the number of facilities per district), or a regional hospital ("Centre Hospitalier Régional [CHR] de Tsévié", region "Maritime", Togo, since 2007 national reference centre for BUD in Togo; catchment area: 2,599,955 inhabitants) for diagnosis and treatment; CHR conducted microscopic analysis. The "Institut National d'Hygiène" (INH), Lomé, Togo – a laboratory accredited by COFRAC ("Comité Français d'Accréditation") according to NF EN ISO/CEI 17025 (version 2005) – constitutes the national Togolese reference laboratory for surveillance of transmissible, especially outbreak prone diseases, and has been nominated national reference laboratory for Buruli ulcer disease in 2010 [13]. In this study, INH resumed EQA for microscopy conducted at regional level and – after installation of a BUD PCR laboratory – PCR assessment of diagnostic samples by means of a dry-reagent-based PCR [21,25,29]. In March 2011, INH joined the WHO network for laboratory confirmation of BUD and – like DITM – participates in the annual program for external quality assessment of molecular detection of *M. ulcerans* in clinical specimens provided by the Mycobacteriology Unit, Microbiology Department, Institute for Tropical Medicine, Antwerp, Belgium, WHO Collaborating Centre for the diagnosis and surveillance of *M. ulcerans* infection [36].

Study area and implementation of outreach programs

In each of the six districts (Golfe, Ave, Zio, Yoto, Vo, Lac) of the region "Maritime", five districts ("Direction de District Sanitaire" [DDS] 1–5) of the region "Lomé Commune" where BUD was proven to be endemic [13] and the four districts of the region "Central" (Blitta, Sotouboua, Tchaoudjo, Thamba), where BUD has been assumed to be endemic, outreach teams ("CLT teams") consisting of district controllers ("Contrôleur Lèpre-TB-Buruli",

CLT), USP staff (“Infirmière du Centre Peripherique”, ICP) and community health workers (“Agent de Santé Communautaire”, ASC), and village nurses were formed and trained by experienced PNLUB-LP, CHR, DITM and DAHW staff. The main tasks of the CLT teams are supervision of USPs, as well as sensitization and screening activities in the field which are mostly conducted under participation of DAHW and CHR staff and in collaboration with PNLUB-LP and the non-governmental organization Handicap International. In particular the ASCs who are trained and continuously supervised by the respective CLTs constitute an integral part of the outreach activities. They organize quarterly sensitization activities and present educational films and information material in villages within proven or assumed areas of endemicity. Villagers are instructed to report to their local ASCs in case of wounds or other lesions suspicious for BUD, thus ASCs represent the primary contact person for the population on community level. Furthermore, ASCs organize regular screening programs in village schools to identify suspected BUD cases in the field. The final decision on referral of suspected BUD cases to USPs or CHR for further diagnosis and treatment lies with a superordinate “BUD team” consisting of medical staff (physician, nurse) from CHR, ASCs, and the regional CLT. Visits to field sites are conducted on demand of district CLT teams according to a schedule elaborated by the ASCs. A routine reporting system between ASCs, ICPs, CLTs and CHR staff has been established and to facilitate communication within and between CLT teams and BUD teams a mobile phone network has been implemented by DAHW in 2010.

Data management

Data collection was conducted by means of the WHO “BU01” form [3] and standardized project specific laboratory data entry forms (Form S1). All clinical, epidemiological and laboratory data including EQA results were entered in a web-based database specifically designed for the “BuruliVac” project.

Sample collection

Diagnostic samples were collected according to standardized procedures. Briefly, swabs were collected by circling the entire undermined edges of ulcerative lesions. Three millimeter punch biopsies and fine needle aspirates (FNA) were collected from the center of non-ulcerative lesions or from undermined edges of ulcerative lesions including necrotic tissue. To facilitate sampling, standardized specimen collection bags including swabs, biopsy punches, syringes and needles, slides, containers with transport media (700 µl [swab and punch biopsy samples], 300 µl [FNA samples] CLS [cell lysis solution, Qiagen, Hilden, Germany] for PCR samples) and data entry forms were provided to the study sites [13,23,25,26,29,37–41].

Samples for PCR analysis were transported in CLS at ambient temperature in an upright position in custom-made specimen collection bags from the field to INH by DAHWT cars within a maximum of 48 hours following sample collection. Upon arrival of PCR samples at INH these were stored at 4–8°C until further processing. Slides for microscopy were transported in slide boxes at ambient temperature to CHR and subsequently to INH.

Laboratory diagnostics

Direct smears for microscopy were prepared from swab and FNA samples at USPs or CHR and subjected to Ziehl-Neelsen staining at CHR. Slides were analyzed according to the WHO recommended grading system [42].

For PCR analysis DNA was prepared using the Gentra Puregene DNA extraction kit (Qiagen, Hilden, Germany) with minor modifications of the manufacturer’s protocol [21].

Three IS*2404* PCR formats (dry-reagent-based [DRB] IS*2404* PCR [INH], standard gel-based IS*2404* PCR and IS*2404* qPCR [DITM]) were applied in this study. Briefly, for DRB-PCR the oligonucleotides MU5 and MU6 were lyophilized in reaction tubes. Illustra PuReTaq Ready-To-Go PCR beads (GE Healthcare, Munich, Germany) were added and dissolved in water before adding template DNA [21,25,26]. Standard IS*2404* PCR was performed according to the protocol described by Stinear et al. [15,17]. IS*2404* qPCR was performed as recently described using a BioRad CFX96 real-time PCR detection system [27,43]. All PCR assays included negative extraction controls, positive, negative (no template) and inhibition controls.

Stepwise approach to implementation of diagnostic laboratory facilities at INH

Implementation of diagnostic laboratory facilities at INH was accomplished in several phases. Before launching the national BUD reference laboratory at INH in January 2011, laboratory assessment of diagnostic samples from “BuruliVac” study participants was conducted at CHR (microscopy) and DITM (PCR) respectively (“initial phase” [phase I] from September 2010 through December 2010). To implement standardized BUD microscopy and PCR services at INH, all required equipment, reagents and consumables were shipped to Togo by DAHWT and installed under supervision of DITM staff from November through December 2010. Subsequently, the transitional phase (phase II) was initiated in January 2011. All relevant laboratory procedures were defined in SOPs (SOP S1–S4). An initial laboratory training workshop was held by DITM staff, and INH staff was familiarized with the principles of standardized documentation of samples and corresponding results (laboratory data entry forms, web-based database), the flow of information between the participating laboratories, and the principles of EQA as outlined below. Whereas during the transitional phase from January 2011 through April 2012 parallel diagnostic samples of all study participants were simultaneously subjected to PCR analysis at INH and DITM, the final phase (phase III) of PCR implementation (ongoing since May 2012) provides for diagnostic PCR conducted independently at INH accompanied by EQA on DNA extracts at DITM. (Figure 1)

External Quality Assurance

During the initial phase EQA was conducted for microscopy only. Slides were read at CHR by two readers, forwarded to DITM for blinded re-reading [13], and both, CHR and DITM results were entered in the web-based database. In case of discordant results between CHR and DITM, slides were subjected to a second re-reading at DITM which determined the consensus result.

During the transitional phase CHR conducted the first reading of slides by two readers, entered a consensus result in a specific result form (Form S1), and forwarded slides and forms to INH (first controller) for blinded re-reading. INH consensus results were also determined by two readers and entered in a specific result form (Forms S2). Finally, CHR and INH results were entered in the web-based database by INH data managers. In case of discordant results the respective slides were re-read by both, CHR and INH staff, and a consensus result was determined. Subsequently, slides were forwarded to DITM (second controller) for blinded re-reading, and DITM results were entered in the web-based database. Slides with discordant results between DITM and INH were re-read by DITM and INH staff during DITM supervisory visits.

For EQA of PCR all clinical samples were collected in pairs and were simultaneously tested at INH (DRB-PCR) and DITM (standard IS*2404* PCR, confirmatory IS*2404* qPCR on negative samples). Results were entered in the web-based database. In

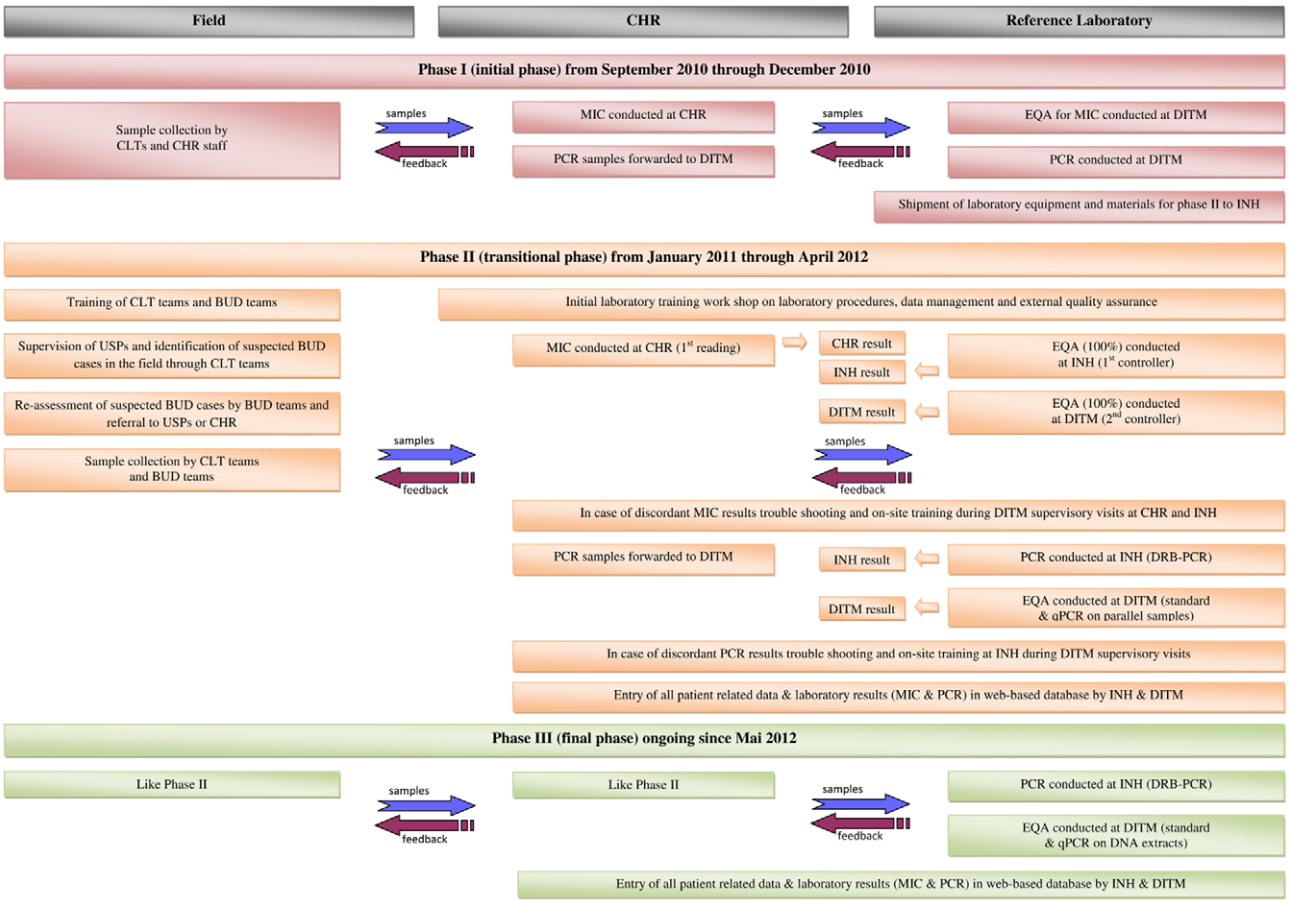

Figure 1. Stepwise approach to implementation of diagnostic laboratory facilities at INH. Figure 1 describes the process of implementation of diagnostic laboratory facilities at INH in three phases and the flow of samples as well as the flow of feedback between the Department for Infectious Diseases and Tropical Medicine (DITM), Ludwig-Maximilians-University, Munich, Germany, the ''Institut National d'Hygiène'' (INH), Lomé, Togo, the ''Centre Hospitalier Régional Maritime'' (CHR), Tsévié, Togo, and field staff. BUD, Buruli ulcer disease; CLT, ''Contrôleur Lèpre-TB-Buruli'' – district controllers; DRB-PCR, dry-reagent-based IS*2404* PCR; EQA, external quality assurance; MIC, microscopic detection of acid fast bacilli by Ziehl-Neelsen staining; PCR, polymerase chain reaction; qPCR, IS*2404* quantitative real-time PCR; standard PCR, conventional gel-based IS*2404* PCR; USP, ''Unité de Soins Périphérique'' – peripheral health posts.
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case of discordant results both laboratories repeated PCR analyses. If the result did not alter, DNA extracts of the respective samples were exchanged and re-tested at both laboratories.

Parameters to determine performance of CHR and INH

In accordance with a previous study on EQA for the laboratory diagnosis of BUD in Ghana [23] microscopy positivity rates (i.e. number of positive samples divided by the total number of samples tested) at CHR, INH, and DITM, PCR positivity rates at INH and DITM, rates of false negative and false positive results compared to DITM results and inter-laboratory concordance rates between CHR/INH/DITM for microscopy and INH/DITM for PCR were determined for the initial and transitional phases. In addition, case confirmation rates (i.e. number of laboratory confirmed BUD patients divided by the total number of suspected BUD cases) were determined for CHR (microscopy), INH and DITM (microscopy and PCR).

Parameters to assess the impact on BUD control

To assess the impact of the local reference laboratory and continuous EQA measures on BUD control, the clinical parameters ''type of lesion'', ''category of lesion'', and ''duration of disease before clinical diagnosis'' (i.e. the mean duration of disease in days based on the time from first recognition of clinical symptoms by patients and availability of the clinical diagnosis BUD) were analyzed and data obtained from the current study cohort from January 2011 through April 2012 after implementation of the national reference laboratory were compared to data obtained in a previous study from September 2007 through December 2010.

Feedback to CHR and field staff

INH forwards all laboratory results directly to CHR, the subsequent reporting chain includes regional CLTs, district CLTs, ICPs, and ASCs. Laboratory confirmed BUD patients are subjected to treatment. In case of negative laboratory results in general the treatment decision is referred to the BUD team. For the purpose of documentation, lesions of all confirmed patients are photographed; the material is available for training and sensitization activities.

Statistical analysis

The study design was non-randomized and cross-sectional.

Table 1. Geographic origin, type and classification of lesions of clinically suspected and laboratory confirmed BUD patients.

Region	District	Clinically suspected BUD cases	Laboratory confirmed BUD patients[a]					
			Total per district[b]	Non-ulcerative lesions	Ulcerative lesions	Category I[c]	Category II[d]	Category III[e]
Central	Sotouboua	4.9% (4/82)	0% (0/64)	0% (0/64)	0% (0/64)	0% (0/64)	0% (0/64)	0% (0/64)
Maritime	Golfe	2.4% (2/82)	1.6% (1/64)	0% (0/64)	1.6% (1/64)	0% (0/64)	0% (0/64)	1.6% (1/64)
	Yoto	48.8% (40/82)	57.8% (37/64)	31.3% (20/64)	26.6% (17/64)	32.8% (21/64)	18.8% (12/64)	6.3% (4/64)
	Vo	1.2% (1/82)	1.6% (1/64)	1.6% (1/64)	0% (0/64)	1.6% (1/64)	0% (0/64)	0% (0/64)
	Zio	36.6% (30/82)	34.4% (22/64)	18.8% (12/64)	15.6% (10/64)	9.4% (6/64)	18.8% (12/64)	6.3% (4/64)
Plateaux[f]	Amoú	1.2% (1/82)	0% (0/64)	0% (0/64)	0% (0/64)	0% (0/64)	0% (0/64)	0% (0/64)
	Anié	1.2% (1/82)	1.6% (1/64)	0% (0/64)	1.6% (1/64)	0% (0/64)	1.6% (1/64)	0% (0/64)
	Haho	1.2% (1/82)	0% (0/64)	0% (0/64)	0% (0/64)	0% (0/64)	0% (0/64)	0% (0/64)
	Ogou	1.2% (1/82)	1.6% (1/64)	0% (0/64)	1.6% (1/64)	0% (0/64)	0% (0/64)	1.6% (1/64)
Savanes[f]	Dapaong	1.2% (1/82)	1.6% (1/64)	0% (0/64)	1.6% (1/64)	0% (0/64)	1.6% (1/64)	0% (0/64)
Total		**100% (82/82)**	**100% (64/64)**	**51.6% (33/64)**	**48.4% (31/64)**	**43.8% (28/64)**	**40.6% (26/64)**	**15.6% (10/64)**

Table 1 shows the geographic origin of all suspected and confirmed BUD patients, and type/category of lesions of confirmed BUD patients who presented from September 2010 through April 2012 in Togo. More than 85% of confirmed BUD patients originated from the districts Yoto and Zio of region ''Maritime''.
[a]Patients were confirmed by dry-reagent-based IS*2404*, standard gel-based IS*2404* PCR and/or IS*2404* quantitative real-time PCR. BUD, Buruli ulcer disease.
[b]Number of confirmed BUD patients per district.
[c]Category I, single lesion <50 mm in diameter.
[d]Category II, single lesion between 50 and 150 mm in diameter.
[e]Category III, single lesion >150 mm in diameter or multiple lesions, osteomyelitis or lesions at critical sites.
[f]Laboratory confirmed BUD patients were referred to CHR, Tsévié, for antimycobacterial treatment.
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Approximative tests (χ^2-tests) including analysis for linear trends in proportions and t-tests as parametric test were conducted using Stata software, version 9.0. (Stata Corporation, College Station, TX) and EpiInfo, version 3.3.2. (Centers for Disease Control and Prevention, Atlanta, GA). Significant differences were defined as not overlapping of 95 percent confidence intervals (95% CI) of proportions.

Results

Training measures for field staff and outcome of outreach programs

Altogether 16 workshops with 559 participants (''CLT teams'' as well as other medical and paramedical staff) addressing clinical picture, laboratory diagnosis and treatment of BUD were held in the regions ''Maritime'' and ''Central''. Since 2011, the CLT teams conducted sensitization activities in 1027 villages and screened a population of approximately 110,000. Out of 192 persons with lesions suspicious for BUD identified in the field, 82 suspected BUD cases were finally referred to USPs or CHR. (Table 1)

Number of samples analyzed by microscopy

During the initial phase, 17 slides (swab, n = 6; FNA, n = 11) obtained from 16 suspected BUD cases (ten non-ulcerative lesions: one FNA sample per lesion; six ulcerative lesions, one swab sample per lesion and one additional FNA sample from one lesion with scarred edges) were analyzed at CHR and subjected to EQA at DITM.

Table 2. Clinical samples analyzed by microscopy for *M. ulcerans*.

	No. of suspected BUD cases	No. of swab samples subjected to MIC[a]		No. of FNA samples subjected to MIC[a]		Total[b]
		CHR/DITM	CHR/INH/DITM	CHR/DITM	CHR/INH/DITM	
Phase I[c]	16	6	N/A	11	N/A	17
Phase II[d]	66	N/A	24	N/A	48	72
Total	82	30		59		89

Table 2 indicates all slides prepared from swab or FNA samples and subjected to Ziehl-Neelsen staining at ''Centre Hospitalier Régional'' (CHR) for the detection of acid fast bacilli. Slides were analyzed consecutively at CHR and the Department of Infectious Diseases and Tropical Medicine (DITM), Ludwig-Maximilians-University during initial phase (phase I) or CHR, at the ''Institut National d'Hygiène'' (INH) and DITM during transitional phase (phase II). N/A, not applicable.
[a]MIC, microscopic detection of acid fast bacilli.
[b]Total, number of slides prepared from swab and FNA samples and subjected to reading at CHR/DITM or CHR/INH/DITM.
[c]Phase I, initial phase of implementation of the national reference laboratory at INH from September 2010 through December 2010; slides were read at CHR and forwarded via DAHWT to DITM for EQA.
[d]Phase II, transitional phase of implementation of the national reference laboratory at INH from January 2011 through April 2012; slides were read at CHR, followed by blinded re-reading at INH and DITM.
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Table 3. Results of external quality assurance for microscopy.

	CHR			INH			DITM		
	Swab[a]	FNA[b]	Total	Swab[a]	FNA[b]	Total	Swab[a]	FNA[b]	Total
Phase I									
Positivity rate[c]	50.0% (3/6)	36.4% (4/11)	41.2% (7/17)	N/A	N/A	N/A	66.7% (4/6)	36.4% (4/11)	47.1% (8/17)
False negative results[d]	16.7% (1/6)	0% (0/11)	5.9% (1/17)	N/A	N/A	N/A	N/A	N/A	N/A
False positive results[e]	0% (0/6)	0% (0/11)	0% (0/17)	N/A	N/A	N/A	N/A	N/A	N/A
Discordance rate[f]	16.7% (1/6)	0% (0/11)	5.9% (1/17)	N/A	N/A	N/A	N/A	N/A	N/A
Concordance rate for CHR/DITM[g]	83.3% (5/6)	100% (11/11)	94.1% (16/17)	N/A	N/A	N/A	N/A	N/A	N/A
Case confirmation rate[h]	31.3% (5/16)			N/A			37.5% (6/16)		
Phase II									
Positivity rate[c]	50.0% (12/24)	45.8% (22/48)	47.2% (34/72)	50.0 (12/24)	47.9% (23/48)	48.6% (35/72)	58.3% (14/24)	54.2% (26/48)	55.6% (40/72)
False negative results[d]	8.3% (2/24)	10.4% (5/48)	9.7% (7/72)	8.3% (2/24)	6.3% (3/48)	6.9% (5/72)	N/A	N/A	N/A
False positive results[e]	0% (0/24)	2.1% (1/48)	1.4% (1/72)	0% (0/24)	0% (0/48)	0% (0/72)	N/A	N/A	N/A
Discordance rate[f]	8.3% (2/24)	12.5% (6/48)	11.1% (8/72)	8.3% (2/24)	6.3% (3/48)	6.9% (5/72)	N/A	N/A	N/A
Concordance rate for CHR/INH[g]	100% (24/24)	91.7% (44/48)	94.4% (68/72)	N/A	N/A	N/A	N/A	N/A	N/A
Concordance rate for CHR/DITM[g]	91.7% (22/24)	87.5% (42/48)	88.9% (64/72)	N/A	N/A	N/A	N/A	N/A	N/A
Concordance rate for INH/DITM[g]	N/A	N/A	N/A	91.7% (22/24)	93.7% (45/48)	93.1% (67/72)	N/A	N/A	N/A
Case confirmation rate[h]	43.9% (29/66)			47.0% (31/66)			53.0% (35/66)		
Concordance rate[g] for CHR/DITM – Phase I and II	89.9% (80/89)			N/A			N/A		
Case confirmation rate[h] – Phase I and II	41.5% (34/82)			N/A			50.0% (41/82)		

Table 3 shows results of Ziehl-Neelsen microscopy conducted at CHR and corresponding results of EQA conducted at INH and DITM during the initial (phase I, September 2010 through December 2010) and transitional (phase II, January 2011 through April 2012) phases. N/A, not applicable.

[a]Swab, slides were prepared as direct smears from swab samples.

[b]FNA, slides were prepared as direct smears from fine-needle aspirate samples.

[c]Positivity rate, number of positive samples divided by the total number of samples tested.

[d]Rate of false negative results at CHR and INH, respectively, compared to DITM results.

[e]Rate of false positive results at CHR and INH, respectively, compared to DITM results.

[f]Rate of false negative and false positive results at CHR and INH, respectively, compared to DITM results.

[g]Rate of concordant results between CHR/INH, CHR/DITM and INH/DITM.

[h]Confirmation rate, number of laboratory confirmed BUD patients divided by the total number of suspected BUD cases.

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Table 4. Clinical samples analyzed by PCR for *M. ulcerans*.

	No. of suspected BUD cases	Samples analyzed by PCR					
		Laboratory	IS*2404* PCR assay	Swab[a]	FNA[b]	Punch[c]	Total
Phase I[d]	16	DITM	Standard PCR	6	16	13	35
			qPCR[e]	3/6	6/16	3/13	12/35
		Total[f]		6	16	13	35
Phase II[g]	66	INH	DRB PCR	33	44	22	99
		DITM	Standard PCR	33	44	22	99
			qPCR[e]	6/33	15/44	9/22	30/99
		Total[f]		66	88	44	198
Total -phase I and II	**82**			**72**	**104**	**57**	**233**

Table 4 indicates all samples tested by PCR at IHN and DITM. During the initial phase (phase I) samples were analyzed by standard gel-based IS*2404* PCR at DITM. During the second phase (phase II) parallel samples were subjected to IS*2404* dry-reagent based (DRB) PCR at INH and standard IS*2404* PCR at DITM. During both phases all samples tested negative in standard PCR were subjected to re-testing by IS*2404* quantitative real-time PCR (qPCR) at DITM.
[a]Swab, DNA extracts prepared from swab samples.
[b]FNA, DNA extracts prepared from fine-needle aspirate samples.
[c]Punch, DNA extracts prepared from 3 mm punch biopsy samples.
[d]Phase I, initial phase of implementation of the national reference laboratory at INH from September through December 2010.
[e]Only samples tested negative in standard IS*2404* PCR were subjected to IS*2404* qPCR at DITM.
[f]Total amount of samples tested by DRB- and Standard PCR during the corresponding phases.
[g]Phase II, transitional phase of implementation of the national reference laboratory at INH from January 2011 through April 2012.
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During the transitional phase, 72 slides (swab, n = 24; FNA, n = 48) obtained from 66 suspected BUD cases (38 non-ulcerative lesions: one FNA sample per lesion; 28 ulcerative lesions: one swab sample each from 18 lesions, one swab and one FNA sample each from six lesions, one FNA sample each from four lesions) were analyzed at CHR and subjected to EQA at INH and DITM. (Table 2)

External quality assurance of microscopy

During the initial phase positivity rates of microscopy were 41.2% (7/17) at CHR and 47.1% (8/17) at DITM with 5.9% (1/17) false negative results from CHR, and an inter-laboratory concordance rate of 94.1% (16/17) between CHR and DITM.

During the transitional phase positivity rates of microscopy were 47.2% (34/72) at CHR, 48.6% (35/72) at INH and 55.6% (40/72) at DITM. The rate of false negative test results was 9.7% (7/72) at CHR and 6.9% (5/72) at INH, and 1 out of 72 slides (1.4%) was read false positive at CHR. Concordance rates between laboratories were 94.4% (68/72) for CHR/INH, 88.9% (64/72) for CHR/DITM and 93.1% (67/72) for INH/DITM.

The concordance rate between CHR and DITM for both phases was 89.9% (80/89). (Table 3)

Number of samples analyzed by PCR

During the initial phase, 35 samples (swab, n = 6; FNA, n = 16; punch biopsy, n = 13) obtained from 16 suspected BUD cases were subjected to standard PCR at DITM, all negative samples (n = 12) were additionally subjected to qPCR.

During the transitional phase, 99 sample pairs (swab, n = 33; FNA, n = 44; punch biopsy, n = 22) obtained from 66 suspected BUD cases were subjected to PCR at INH and DITM, which equals a mean rate of 3.0 (198/66) samples tested per patient. All negative samples (n = 30) were additionally subjected to qPCR. (Table 4)

External quality assurance of PCR

During the initial phase the positivity rate of standard PCR at DITM was 65.7% (23/35). Confirmation of two out of 12 negative samples by qPCR provided an additional diagnostic yield of 5.7%.

During the transitional phase positivity rates of conventional PCR assays were 65.7% (65/99) at INH and 69.7% (69/99) at DITM. The rate of false negative test results at INH was 4.0% (4/99; 1 swab sample and 3 FNA samples), there were no false positive results, and the inter-laboratory concordance rate was 96.0% (95/99). Confirmation of 6 out of 30 negative samples by qPCR provided an additional diagnostic yield of 6.1%. (Table 5)

Laboratory confirmed patients

The case confirmation rates for microscopy were 31.3% (5/16) at CHR and 37.5% (6/16) at DITM during the initial phase, and 43.9% (29/66) at CHR, 47.0% (31/66) at INH, and 53.0% (35/66) at DITM during the transitional phase. In total 50.0% (41/82) of the suspected BUD cases were confirmed by microscopy. (Table 3)

The case confirmation rates for PCR were 75.0% (12/16) at DITM during the initial phase, and 71.2% (47/66) at INH and 78.8% (52/66) at DITM (including two cases additionally confirmed by qPCR) during the transitional phase. In total 78.1% (64/82) of the suspected BUD cases were confirmed by PCR. (Table 5)

Epidemiological baseline and treatment data of confirmed BUD cases

Out of 64 laboratory confirmed BUD patients, 51.6% (33/64) had non-ulcerative lesions (plaque, n = 17; nodule, n = 10; papule, n = 1; edema, n = 5) and 48.4% (31/64) had ulcerative lesions, 48.4% (31/64) were male, and 48.4% (31/64) were in age group 5–14 years (age range 2–68 years, mean 18.1 years, median 13 years). Figure 2

The confirmed BUD patients originated from four districts of region "Maritime" (Yoto, n = 37; Zio, n = 22; Vo, n = 1; Golfe, n = 1), two districts of region "Plateaux" (Anié, n = 1; Ogou, n = 1) and one district of region "Savanes" (Dapaong, n = 1). The categories of lesions according to WHO classification [3] were as follows: 43.8% (28/64) category I, 40.6% (26/64) category II and 15.6% (10/64) category III. (Table 1)

All patients with suspected BUD (n = 82) who presented in Togo during the study period were included (no refusals to participate)

Table 5. Results of EQA for PCR.

		Swab samples[a]			FNA samples[b]			Punch biopsy samples[c]			Total[d]			
		INH[e]	DITM[f]		INH[e]	DITM[f]		INH[e]	DITM[f]		INH[e]	DITM[f]		
		DRB-PCR[e]	Standard PCR[f]	qPCR[f]	DRB-PCR[e]	Standard PCR[f]	qPCR[f]	DRB-PCR[e]	Standard PCR[f]	qPCR[f]	DRB-PCR[e]	Standard PCR[f]	qPCR[f]	Final result[g]
Phase I[h]	**Positivity rate[i]**	N/A	50.0% (3/6)	0% (0/3)	N/A	62.5% (10/16)	16.7% (1/6)	N/A	76.9% (10/13)	33.3% (1/3)	N/A	65.7% (23/35)	16.7% (2/12)	71.4% (25/35)
	Case confirmation rate[j]	N/A	18.8% (3/16)	0% (0/16)	N/A	43.8% (7/16)	0% (0/16)	N/A	12.5% (2/16)	0% (0/16)	N/A	75.0% (12/16)	0% (0/16)	75.0% (12/16)
Phase II[m]	**Positivity rate[i]**	78.8% (26/33)	81.8% (27/33)	16.7% (1/6)	59.1% (26/44)	66.0% (29/44)	33.3% (5/15)	59.1% (13/22)	59.1% (13/22)	0.0% (0/9)	65.7% (65/99)	69.7% (69/99)	20.0% (6/30)	75.8% (75/99)
	False negative[k]	3.0% (1/33)	N/A	N/A	6.8% (3/44)	N/A	N/A	0.0% (0/22)	N/A	N/A	4.0% (4/99)	N/A	N/A	N/A
	Concordance rate[l]	97.0% (32/33)		N/A	93.2% (41/44)		N/A	100% (22/22)		N/A	96.0% (95/99)		N/A	N/A
	Case confirmation rate[j]	30.3% (20/66)	30.3% (20/66)	0.0% (0/66)	34.8% (23/66)	39.4% (26/66)	3.0% (2/66)	6.1% (4/66)	6.1% (4/66)	0.0% (0/66)	71.2% (47/66)	75.8% (50/66)	3.0% (2/66)	78.8% (52/66)
Total – phase I and II[n]	**Positivity rate[i]**	N/A	76.9% (30/39)	11.1% (1/9)	N/A	65.0% (39/60)		N/A	65.7% (23/35)	8.3% (1/12)	N/A	68.7% (92/134)	19.0% (8/42)	74.6% (100/134)
	Case confirmation rate[j]	N/A	28.1% (23/82)	0% (0/82)	N/A	40.2% (33/82)	2.4% (2/82)	N/A	7.3% (6/82)	0% (0/82)	N/A	75.6% (62/82)	2.4% (2/82)	78.1% (64/82)

Table 5 shows results of external quality assurance for PCR. During the initial phase (phase I) PCR samples were analyzed at DITM by IS*2404* standard PCR and IS*2404* quantitative real-time PCR (qPCR). During the transitional phase (phase II) diagnostic sample pairs where analyzed in parallel at INH (IS*2404* dry-reagent-based [DRB] PCR) and DITM as described for phase I. Positivity rates and case confirmation rates are provided for IS*2404* DRB- and standard PCR, and additional diagnostic yields were calculated for IS*2404* qPCR. N/A, not applicable.

[a]Swab samples, DNA extract were prepared from swab samples.

[b]FNA samples, DNA extract were prepared from fine-needle aspirate samples.

[c]Punch samples, DNA extract were prepared from 3-mm punch biopsy samples.

[d]Total per phase and laboratory.

[e]INH applied IS*2404*-DRB-PCR. [21]

[f]DITM applied standard, gel-based, IS*2404* PCR [17] and IS*2404* qPCR [27,42] on all DNA extracts tested negative with standard PCR. For qPCR the additional diagnostic yield (i.e. the deviation of total final result from total result of standard PCR) were 5.7% (phase I) and 6.1% (phase II).

[g]Final result of standard PCR and qPCR.

[h]Phase I, initial phase of implementation of the national reference laboratory at INH from September through December 2010.

[i]Positivity rate, number of positive samples divided by the total number of samples tested.

[j]Case confirmation rate, number of laboratory confirmed BUD patients divided by the total number of suspected BUD cases.

[k]Rate of false negative results at INH as determined by re-testing of DNA extracts at DITM by standard PCR.

[l]Rate of concordant results from sample pairs at INH and DITM.

[m]Phase II, transitional phase of implementation of the national reference laboratory at INH from January 2011 through April 2012.

[n]Total results of the initial and the transitional phase.

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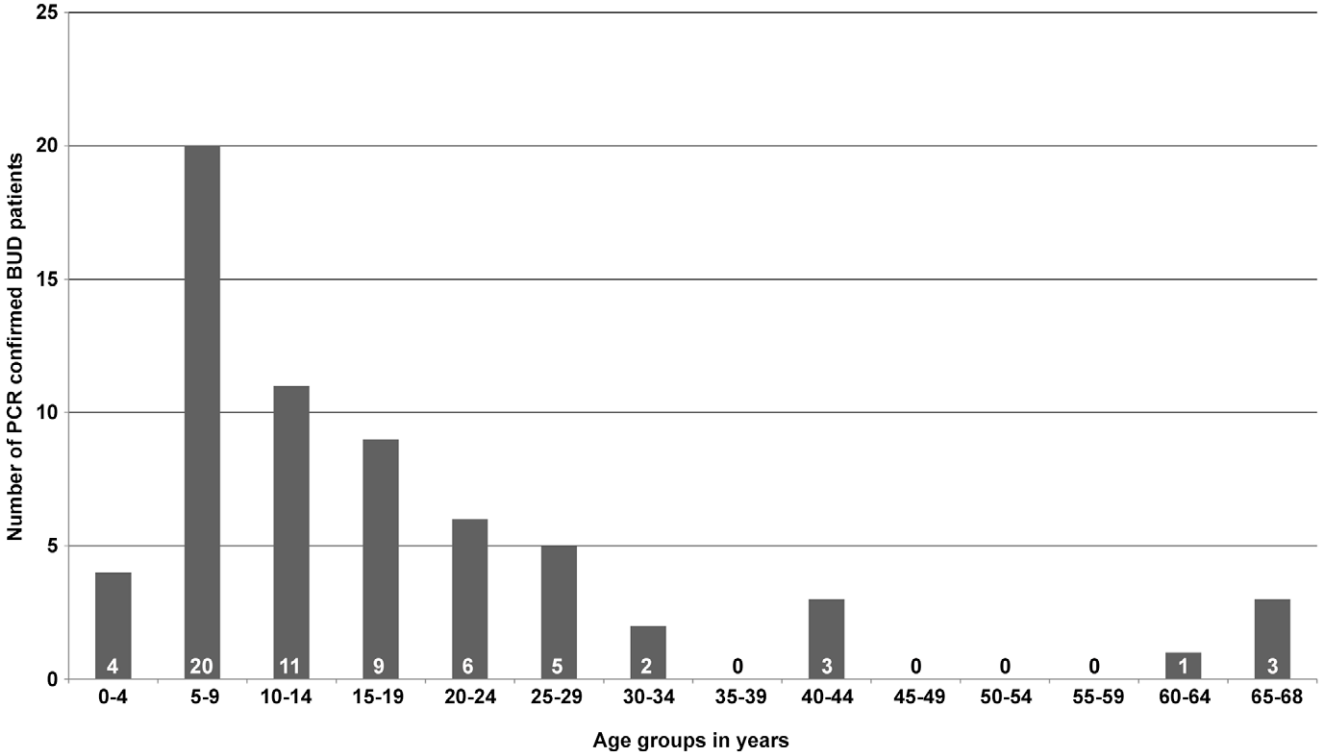

Figure 2. Age distribution of laboratory confirmed BUD patients. Age distribution of 64 laboratory confirmed BUD patients recruited from September 2010 through April 2012. The age of all patients was known and 48.4% (31/64) were in age group 5–14 years. The age range was 2–68 years with a mean of 18.1 years and a median of 13 years.
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and clinical samples were collected and analyzed from all of them. All laboratory confirmed BUD patients (n = 64) received a full course of treatment with rifampicin and streptomycin; in addition, six patients, despite negative laboratory results, were subjected to antimycobacterial treatment based on strong clinical suspicion of BUD. Although no regular outreach activities were conducted in region ''Plateaux'' and ''Savanes'' patients from both regions were referred to CHR for treatment.

Impact on quality of BUD control

The number of patients with non-ulcerative lesions among all PCR-confirmed patients increased significantly ($p<0.01$) from 37.0% (as determined for the study cohort from 2007–2010, 119 patients) to 50.0% (current study cohort from January 2011 through April 2012, 52 patients).

Compared to the previous study category I lesions increased from 36.9% (95% CI: 28.3–45.6) to 44.2% (95% CI: 30.7–57.7), category II lesions increased from 32.8% (95% CI: 24.3–41.2) to 36.6% (95% CI: 23.5–49.6) and category III lesions decreased from 30.3% (95% CI: 22.0–38.5) to 19.2% (95% CI: 8.5–29.9).

The mean duration of disease before clinical diagnosis decreased from 51.8 (95% CI: 19.0–84.7) to 35.0 (95% CI: 23.5–46.5) days (no significant difference) among patients with non-ulcerative lesions, and significantly from 182.6 [95% CI: 119.2–245.9] to 82.1 [95% CI: 51.3–112.8] days among patients with ulcerative lesions. (Table 6)

Discussion

Laboratory confirmation of suspected BUD cases, in particular by molecular diagnostic tests, plays a crucial role for clinical management, disease control and research on *M. ulcerans*.

To achieve the targeted PCR confirmation rate of more than 50% of suspected BUD cases worldwide, WHO has set up a network of external and local PCR reference laboratories [36]. Whereas until the early 2000s laboratory diagnostic services for endemic countries were mainly provided by external reference laboratories, until 2011 six African countries (Ivory Coast, Ghana, Benin, Cameroon, Central African Republic, Democratic Republic of Congo) installed their own reference laboratories upon increasing demand for local diagnostic capacity [6,10,11,18,20–26,29,30,32,37,44–46]. Due to the absence of laboratory facilities a number of countries still require support from external reference laboratories; in general however, the role of external reference laboratories has shifted to development of improved laboratory techniques for application in endemic countries, technical support and training of local laboratory staff, as well as external quality assurance for newly established reference laboratories [6,11,21,23–32,37–40,43].

As well known from other studies, the implementation of reference level laboratory facilities necessitates multiple provisions in terms of logistics, trained personnel and quality management [11,23,47,48]. In the case of Togo, extensive preparatory work conducted in the context of previous research projects by DAHWT and DITM [13], vast expertise gained from a long-standing cooperation with partners in Ghana [21,23,25,26,29,40], as well as continuous exchange of information with other ''BuruliVac'' partners [6,32] facilitated the implementation of a national reference laboratory considerably.

Excellent technical skills of INH laboratory staff in conventional and molecular microbiological diagnostic techniques allowed starting laboratory training at an advanced level. All training activities took place at INH; basic laboratory training according to the concept of

Table 6. Impact of local reference laboratory and external quality assurance measures on BUD control.

Year of clinical presentation	2007	2008	2009	2010	2011	2012	2007–2010	2011–2012
No. of confirmed BUD patients[a]	10	38	33	38	41	11	119	52
No. of confirmed patients with non-ulcerative lesion	3	6	12	23	21	5	44	26
No. of confirmed patients with ulcerative lesions	7	32	21	15	20	6	75	26
Rate of confirmed BUD patients with non-ulcerative lesions[b]	30.0% (3/10)	15.8% (6/38)	36.4% (12/33)	60.5% (23/38)	51.2% (21/41)	45.5% (5/11)	37.0% (44/119)	50.0% (26/52)
Rate of confirmed BUD patients – category I[c]	20.0% (2/10)	50.0% (19/38)	24.2% (8/33)	39.5% (15/38)	46.3% (19/41)	36.4% (4/11)	36.9% (44/119)	44.2% (23/52)
95% confidence interval	0–44.8	34.1–65.9	9.6–38.9	23.9–55.0	31.1–61.6	7.9–64.8	28.3–45.6	30.7–57.7
Rate of confirmed BUD patients – category II[d]	30.0% (3/10)	36.8% (14/38)	30.3% (10/33)	31.6% (12/38)	34.2% (14/41)	45.5% (5/11)	32.8% (39/119)	36.6% (19/52)
95% confidence interva	1.6–58.4	21.5–52.2	14.6–46.0	16.8–46.4	19.6–48.7	16.0–74.9	24.3–41.2	23.5–49.6
Rate of confirmed BUD patients – category III[e]	50.0% (5/10)	13.2% (5/38)	45.5% (15/33)	29.9% (11/38)	19.5% (8/41)	18.1% (2/11)	30.3% (36/119)	19.2% (10/52)
95% confidence interval	19.0–81.0	2.4–23.9	28.5–62.4	14.5–43.4	7.4–31.6	0–41.0	22.0–38.5	8.5–29.9
Mean duration of disease before clinical diagnosis in days[f]								
Patients with non-ulcerative lesions	318.7	74.0	25.8	24.8	30.3	54.6	51.8	35.0
95% confidence interval	0–718.2	16.7–131.4	12.6–38.9	16.6–33.1	18.7–41.9	23.2–86.0	19.0–84.7	23.5–46.5
Patients with ulcerative lesions	386.0	239.2	107.6	71.8	87.5	64.0	182.6	82.1
95% confidence interval	78.3–693.7	118.2–360.1	59.6–55.6	45.6–98.0	48.0–27.0	45.0–83.0	119.2–245.9	51.3–112.8
All patients	365.8	213.1	77.8	43.4	58.2	59.7	134.2	58.5
95% confidence interval	130.8–600.8	109.3–316.9	44.3–111.3	29.9–56.8	36.4–80.0	42.8–76.6	91.1–177.4	41.1–76.0

Table 6 shows analyses of clinical parameters (i.e. ''type of lesion'' and ''duration of disease before clinical diagnosis'') among PCR confirmed BUD new cases to assess impact of the local reference laboratory and external quality assurance measures on BUD control in Togo. Therefore, data from a previous study (September 2007 through December 2010) prior to implementation of the national reference laboratory at INH were analyzed and compared with data obtained in the present study (January 2011 through April 2012). Analysis for linear trends in proportions revealed a significant ($p<0.01$) increase of patients presenting with non-ulcerative lesions from 37.0% (2007–2010) to 50.0% (2011–2012). The mean duration of disease among patients with non-ulcerative lesions before presentation and establishment of clinical diagnosis decreased not significantly from 51.8 (95% CI: 19.0–84.7) to 35.0 (95% CI: 23.5–46.5) days during the two observation periods. However, the mean duration of disease among patients with ulcerative lesions before presentation of patients and establishment of clinical diagnosis decreased significantly from 182.6 (95% CI: 119.2–245.9) to 82.1 (95% CI: 51.3–112.8) days during the two observation periods. Furthermore, analysis of the development of categories of lesions showed a statistically non significant decrease from 30.3% (95% CI: 22.0–38.5) to 19.2% (95% CI: 8.5–29.9) of category III lesions. BUD, Buruli ulcer disease; CI, confidence interval.

[a]Number of confirmed BUD patients, laboratory confirmation was conducted by standard IS*2404* PCR, IS*2404* DRB-PCR and/or IS*2404* qPCR.

[b]Rate of confirmed BUD patients with non-ulcerative lesions among all confirmed BUD patients per observation period.

[c]Category I, single lesion <50 mm in diameter.

[d]Category II, single lesion between 50 and 150 mm in diameter.

[e]Category III, single lesion >150 mm in diameter or multiple lesions, osteomyelitis or lesions at critical sites.

[f]Mean duration of disease in days based on the time from first recognition of clinical symptoms by patients and availability of the clinical diagnosis ''BUD''. Only data from PCR confirmed BUD patients were analyzed.

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short-term ''training of trainers'' workshops in Europe as successfully applied by other external reference laboratories was not required.

In consideration of the existing quality management systems at DITM and INH, special emphasis was given to standardization of all relevant procedures. SOPs defined the interaction of the laboratory with external partners in the field and the external reference laboratory in Germany, as well as all processes within the laboratory, and granted a smooth workflow from the beginning of the project. Standardized documentation of all analyses and results in standardized laboratory forms and the project-specific web-based database facilitated rapid retracing of errors for local and external reference laboratory and allowed targeted training measures.

To measure the quality of diagnostics conducted at INH, we determined concordance rates between local and external reference laboratories. Compared to a previous study [13], the concordance rate for microscopic analysis between CHR and DITM (initial and transitional phase) increased from less than 70% to 90%, and the concordance rate between INH and DITM was over 90% during the transitional phase, suggesting a high standard of microscopy at both, CHR and INH. Compared to previous findings [13], also the case confirmation rate for microscopy increased from 30% (CHR) to 43% (CHR) and 47% (INH), respectively. Likewise, concordance rates between INH and DITM for PCR of swab and punch biopsy samples were over 95%. In this study, instead of testing the same sample subsequently at both laboratories, sample pairs were collected and one sample each was sent to DITM and INH to allow quality control for both, extraction efficiency and amplification. As

already observed in other studies, parallel samples – even if collected from the same site of the lesion - may show an inhomogeneous distribution of mycobacteria and may increase the normal inter-laboratory variation regularly observed for weakly positive samples ([23,49], unpublished data). Therefore, the findings suggest high quality of PCR conducted at INH. With 93% the inter-laboratory concordance rate for FNA samples was slightly lower which may be attributable to dividing FNA samples in two pieces for microscopy and PCR at INH (whereas the entire parallel sample was subjected to PCR at DITM). Consequently, also the case confirmation rate at INH was a little lower (71%) than at DITM (76%). Future EQA of PCR diagnostics is conducted on DNA extracts only, therefore both confounders (sample pairs and divided samples) are excluded.

In addition to conventional gel-based PCR, DITM applied IS*2404* qPCR on negative samples which resulted in laboratory confirmation of two additional cases. As real-time PCR facilities are available at INH, implementation of IS*2404* qPCR is envisaged for 2013. Laboratories in endemic countries without access to real-time PCR may consider forwarding at least samples from patients with strong clinical suspicion but negative conventional PCR result to an external reference laboratory for confirmatory IS*2404* qPCR.

The study also attempted to measure the impact of local laboratory capacity and quality management on BUD control. The increase of the rate of non-ulcerative lesions by 13%, the significant reduction of the diagnostic delay by more than 100 days for patients with ulcerative lesions as compared to a previous study [13] and the reduction of category III lesions from 30.3% to 19.2% may be attributed to an extended quality management system also comprising patient related procedures outside the laboratory and intensified training measures.

Already during the previous study period from 2007 through 2010 CLTs, ICPs, ASCs and other field staff had been trained in 28 workshops with 152 participants. Since 2011, however, training measures achieved a roughly five-fold increase in coverage, and training of teams instead of individuals resulted in a multiplier effect in terms of knowledge transfer which became noticeable also in areas without regular outreach activities through referral of patients to CHR. The availability of trained CLT teams in 11 districts, in particular the ASCs, increased the coverage of sensitization activities and allowed to conduct extensive "information, education and communication" (IEC) campaigns under the guidance of DAHWT and PNLUB-LP in regions "Maritime" and "Central" accompanied by regular outreach activities to identify suspected BUD cases in the field. Finally, supervision of CLT teams by the CHR BUD team in terms of re-examining these patients provided continuous on-site training for CLT teams and enhanced the diagnostic skills of all field staff involved. Feed- back of laboratory results through a newly established reporting chain from INH to community level not only provides the basis for targeted case finding activities in the environment of confirmed patients, but is also conceived as confidence-building measure by ASCs as well as patients and their families. Altogether, the outreach system implemented in 2011 allowed to realize key components of BUD control in the field of early case detection, diagnosis and treatment as defined by the WHO [7], and more than 90% of BUD cases are currently detected through active case finding (opposed to roughly 60% in the previous study).

Whereas these outreach activities resulted in a constant flow of diagnostic samples from suspected BUD cases from peripheral health facilities in region "Maritime" via the regional hospital (CHR) to INH, and the first cases from region "Plateaux" and "Savanes" have been identified, to date no cases from region "Central" have been confirmed.

Since June 2012, a cooperation agreement between the "Faculté Mixte de Médécine et de Pharmacie" of the University of Lomé, Togo and the Faculty of Medicine of the Ludwig-Maximilians-University, Munich, Germany, has reinforced the existing diagnostic network through initiation of a collaboration with the "Laboratoire de Biologie Moléculaire et d'Immunologie" (BIOLIM), "Département des Sciences Fondamentales et Biologiques". BIOLIM will support ongoing EQA measures in the field of quality control, academic and in-service training of local laboratory staff, thus contribute to maintaining sustainable standards in laboratory confirmation of BUD. Furthermore, access to a nationwide laboratory network established in the context of research on HIV and other infectious diseases conducted by BIOLIM will enable operational research on decentralised diagnostics and increase the efficiency of BUD control. [7,48,50]

Supporting Information

Form S1 BuruliVac laboratory data entry form.
(PDF)

Forms S2 BuruliVac MIC result forms – CHR, Tsévié, and INH, Lomé.
(PDF)

SOP S1 Collection, transport and storage of diagnostic specimens.
(PDF)

SOP S2 Microscopic analysis for the detection of acid fast bacilli.
(PDF)

SOP S3 Extraction of mycobacterial DNA from clinical specimens.
(PDF)

SOP S4 IS*2404*-DRB-PCR for detection of *M. ulcerans* DNA.
(PDF)

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Author Contributions

Clinical management: EP JN KA AD BK. Designed analysis software & data management: MB KLH IM FV BB KSA KH JN KA AD BK GB. Conceived and designed the experiments: MB KLH KB WAH IM BB KSA KH CM JN KA FXW AD BK ABK MPD TL GB. Performed the experiments: MB KLH IM FV BB KSA KH CM KHH. Analyzed the data: MB KLH KB WHA IM FV BB KSA EP KH CM JN KA FXW AD BK KHH ABK MPD TL GB. Contributed reagents/materials/analysis tools: KB WAH FV EP FXW AD BK KHH ABK TL GB. Wrote the paper: MB KLH KB WHA JN KA FXW KHH ABK MPD TL GB.

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PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Treatment Outcome of Patients with Buruli Ulcer Disease in Togo

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Abstract

Background

Following introduction of antimycobacterial treatment of Buruli ulcer disease (BUD), several clinical studies evaluated treatment outcomes of BUD patients, in particular healing times, secondary lesions and functional limitations. Whereas recurrences were rarely observed, paradoxical reactions and functional limitations frequently occurred. Although systematic BUD control in Togo was established as early as 2007, treatment outcome has not been reviewed to date. Therefore, a pilot project on post-treatment follow-up of BUD patients in Togo aimed to evaluate treatment outcomes and to provide recommendations for optimization of treatment success.

Methodology/Principal Findings

Out of 199 laboratory confirmed BUD patients, 129 could be enrolled in the study. The lesions of 109 patients (84.5%) were completely healed without any complications, 5 patients (3.9%) had secondary lesions and 15 patients (11.6%) had functional limitations. Edema, category III ulcers >15cm, healing times >180 days and a limitation of movement at time of discharge constituted the main risk factors significantly associated with BUD related functional limitations ($P<0.01$). Review of all BUD related documentation revealed major shortcomings, in particular concerning medical records on adjuvant surgical and physiotherapeutic treatment.

Conclusions/Significance

This study presents the first systematic analysis of treatment outcome of BUD patients from Togo. Median times to healing and the absence of recurrences were in line with findings reported by other investigators. The percentage of functional limitations of 11.6% was lower than in other studies, and edema, category III ulcers, healing time >180 days and limitation of movement at discharge constituted the main risk factors for functional limitations in

Competing Interests: The authors have declared that no competing interests exist.

Togolese BUD patients. Standardized treatment plans, patient assessment and follow-up, as well as improved management of medical records are recommended to allow for intensified monitoring of disease progression and healing process, to facilitate implementation of therapeutic measures and to optimize treatment success.

Author Summary

Buruli ulcer disease (BUD) is a mycobacterial skin disease which leads to large ulcerations and causes disabilities in approximately 25% of the patients. Treatment consists of antimycobacterial drugs, complemented by surgery and physiotherapy if necessary. Available data on treatment outcome of BUD patients suggest that recurrences are rare; paradoxical reactions and functional limitations, however, frequently occur. BUD control in Togo was introduced already in 2007, but treatment outcome has not yet been reviewed. Therefore, a clinical follow-up study assessed a cohort of 129 BUD patients at least six months after the end of treatment. The lesions of 84.5% of the patients were healed without complications, 3.9% had secondary lesions, and 11.6%, a lower proportion than in other studies, had functional limitations. Hereby, edema, category III ulcers, healing times >180 days, and limitation of movement at discharge constituted the main risk factors. Review of all BUD related documentation revealed a number of shortcomings, in particular concerning medical records. In view of these findings, standardization of procedures for creating of therapy plans, patient assessment and follow-up, as well as improved management of medical records are recommended to facilitate implementation of therapeutic measures to optimize treatment outcome and to allow for further evaluation.

Introduction

Buruli ulcer disease (BUD), caused by *Mycobacterium ulcerans*, is a chronic, necrotizing skin disease which has been reported from more than 30 countries worldwide with a focus in West Africa [1]. BUD predominantly affects impoverished inhabitants of remote rural areas, approximately 50% of the cases are children <15 years [1–2]. Initially BUD manifests as painless nodule, plaque, papule, or edema followed by large, painless ulcerations with characteristically undermined edges [1–3]. Also cases with osteomyelitis occur [1–2, 4–5]. Lesions are divided into three categories (I: single lesions, <5 cm diameter; II: single lesions, 5–15 cm diameter; III: single lesions, >15 cm diameter, multiple lesions, lesions at critical sites, osteomyelitis) [2]. As a result of scarring and contractures emerging during the healing process, especially patients who are not treated early suffer long-term functional disability [1, 6]. As the mode of transmission of BUD has not been elucidated to date, proven strategies of prevention do not exist [1]. Early diagnosis and treatment are therefore core elements of BUD control which requires strong commitment of health workers at community level, laboratory confirmation of 70% of suspected cases by standardized diagnostic methods (preferably IS*2404* PCR), and standardized antimycobacterial treatment (rifampicin [R] in combination with streptomycin [S], alternatively clarithromycin [C] for eight weeks), if necessary complemented by surgery and/or physiotherapy [2, 7–9]. The WHO classified BUD as one of the currently five neglected tropical diseases (NTDs) in line for the "innovative and intensified disease management (IDM)" approach, demanding a major scaling up of active case detection, treatment, monitoring and surveillance [10].

Since the introduction of antimycobacterial combination therapy a number of clinical studies investigated the treatment outcome of BUD patients, in particular healing times, secondary lesions and functional limitations. Whereas several authors observed healing of lesions of more than 90% of patients receiving various antimycobacterial treatment regimens (RS8, RS4/RC4, RS2/RC6) within twelve months [11–13], information on the time to healing varies. Nienhuis et al. reported median healing times of category I lesions of 18 weeks, and 30 weeks for category II and III lesions respectively [12]. Sarfo et al. further specified median healing times for nodules of 8 weeks, for ulcers of overall 12 weeks (category I: 12 weeks; category II: 11 weeks; category III: 15.5 weeks), and edema ranging from 2–48 weeks [11], Phillips et al. described median healing times of 14 weeks (RS8) and 16 weeks (RS2RC6) [13], and Vincent et al. observed median healing times of 12.6 weeks [5]. Available data from various studies also suggest that healing of up to two thirds of patients occurs within about 25 weeks after onset of treatment [5, 12–14].

Whereas proven recurrences were non-existent [11–13] or below 2% [15], paradoxical reactions in terms of deterioration of lesions on antibiotic treatment or the appearance of secondary lesions during or after treatment, were described for individual patients [16–18] and for larger patient cohorts. Nienhuis et al. found an increase in lesion size in up to 80%, and secondary lesions in 6% of the patients participating in the BURULICO antimicrobial trial in Ghana [12, 19], O'Brien et al. described paradoxical reactions in 21% of an Australian patient cohort [20], and Phillips et al. reported 9% of paradoxical reactions in a Ghanaian patient cohort participating in a recent antimicrobial trial (RS2/RC6) [13]. Increases in lesion size were commonly observed during the first three months after onset of treatment [19–20], but also delayed paradoxical reactions in terms of new lesions occurring up to thirteen months after the end of antibiotic treatment are known [17–18].

Functional limitations were frequently observed. Data from two cohorts of laboratory confirmed BUD patients from Ghana treated between 2003 and 2005 (surgery with or without concomitant antibiotic treatment), and between 2004 and 2009 (antimycobacterial treatment with or without surgical intervention), suggested functional limitations in 27% and 33.3% of the patients [21–22]. A comparison of two patient cohorts from the Democratic Republic of the Congo treated between 2002 and 2004 (surgical treatment only) and 2005–2007 (the majority of patients underwent surgery, more than 50% also received antimycobacterial therapy) showed that 23.4% and 19.5% of the patients healed with complications [4]. A recent study from Benin analyzed a cohort of more than 1000 BUD patients treated between 2005 and 2011 with antimycobacterial combination therapy and surgery if required, and reported 22% permanent functional limitations one year after treatment [5].

Since the early 2000s, several investigators conducted in-depth assessments of functional limitations and identified important risk factors for their development, in particular location on joints and extremities of limbs, lesions >15 cm, and lesions at head and neck [21–28]. Beyond that, Vincent et al. recently established a specific profile of risk factors for BUD patients from Benin (edema, osteomyelitis, lesions >15 cm in diameter, multifocal lesions, healing times >107 days) and introduced the operational definition "severe Buruli ulcer" to earmark patients at risk for functional limitations for specific disability prevention measures [5].

In Togo, systematic BUD control was initiated in 2007. Whereas case finding, laboratory confirmation and antimycobacterial treatment have been fully implemented [29–30], accompanying POD (prevention of disability) measures as outlined by the WHO [6] are not yet sufficiently embedded in routine procedures, and treatment outcome has not been monitored.

This study presents the first analysis of treatment outcome of BUD patients in Togo, critically reviews procedures with a possible impact on the occurrence of complications, and provides recommendations for optimization of treatment success.

Materials and Methods

Ethical considerations

Ethical clearance was obtained through the national Togolese ethics committee ("Comité de Bioéthique pour la Recherche en Santé") at the University of Lomé (14/2010/CBRS) and the study was approved by the "Ministère de la Santé de la République Togolaise" Lomé, Togo (Ref. No. 0009/2011/MS/DGS/DPLET). Written informed consent (IC) was obtained in French, if necessary translated into local languages, from all study participants and/or their legal representatives if aged below 18 years.

Management of BUD in Togo

In Togo, BUD control mainly operates through a network of district based CLTs ("Contrôleurs Lèpre-TB-Buruli") and community based ASCs ("Agent Santé Communitaire"). CLTs regularly conduct sensitization activities in villages and schools, furthermore perform active case finding supported by ASCs who report patients with suspected BUD lesions to their corresponding CLT. Due to extended coverage of sensitization activities, self-referrals of patients to the nearest health post ("Unité de Soins Périphérique [USP]") are on the rise. CLTs as well as USP head nurses ("[ICP] Infirmier Chef Poste") refer clinically suspected BUD cases to the regional reference hospital ("Centre Hospitalier Régionale [CHR] Tsévié"). At CHR-Tsévié a specifically trained medical assistant ("point focal" [PF]) is in charge of further proceedings, such as physical examination, documentation on the WHO recommended BU 01.N and BU 01.R forms in case of suspected recurrences [31], sample collection and initiation of laboratory confirmation at the national hygiene institute ("Institute Nationale d'Hygiène [INH]") according to standardized procedures [29–30]. Whereas most patients with category I (partially also category II) lesions are referred for outpatient treatment to USPs, the majority of patients with severe category II and category III lesions, and children <15 years in general are treated at CHR-Tsévié. Antimycobacterial treatment follows WHO recommendations and is complemented by surgical intervention if required [2]. Wound management at CHR-Tsévié is performed by nurses, at the USPs by the ICP, in both cases according to instructions of the PF. Patients who, according to the judgment of the PF, require physical therapy are referred to physiotherapists who provide treatment at the reference hospital, the USPs and also the patients homes. Currently 7 state examined physiotherapists are seconded to treatment of BUD patients.

Study population

Eligible for the study were 199 PCR confirmed BUD patients originating from regions "Maritime", "Savanes", "Plateaux" and "Centrale" who were treated within the period from September 2007 to November 2013 with more than 6 months elapsed since the end of treatment. Inclusion and exclusion criteria are shown in Table 1.

Clinical, epidemiological and treatment data were retrospectively compiled from existing databases of previous studies which contained information retrieved from standardized WHO BU 01.N and project specific laboratory data entry forms, and cross-checked with original paper forms [29–30].

Study forms

To collect standardized data on treatment outcome a study specific form (S1 Form) was employed which consisted of several sections: A-D) clinical/epidemiological baseline and treatment data (taken from existing databases, prefilled prior to follow-up visits); E-F) information on location of suspected secondary lesions (recorded in the field) and clinical samples collected

Table 1. Inclusion and exclusion criteria.

Inclusion criteria	Exclusion criteria
IS*2404* PCR confirmed BUD patient of any age	Laboratory unconfirmed cases
Antimycobacterial treatment (RS8) was administered (with or without completion of 56 doses) with or without surgical interventions	Antimycobacterial treatment initiated before September 2007 or after November 2013
>6 months elapsed since the end of antimycobacterial treatment	<6 month elapsed since the end of antimycobacterial treatment
written informed consent provided	written informed consent not provided

Table 1 shows inclusion and exclusion criteria for study participants.

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for laboratory diagnosis; G-H) assessment of limitations of movement (LOM) and impairment in daily activities (based on the questionnaire developed by Simonet V [32]; documented in the field); I) recommendations for further medical treatment (issued after data analysis). In addition, for patients with open wounds at follow-up the BU 01.R form and a clinical record form (as used routinely in Togo; S2 Form [30]) were filled in the field. S1 Table summarizes all parameters collected for analysis.

Additional source materials

For patients with secondary lesions and functional limitations case histories were retrospectively retrieved from medical records, where available. The distance from the patients' location of residence to CHR was obtained from logbooks of DAHW-T cars. In addition, documentation on physiotherapy was retrospectively reviewed, as far as accessible ("Fiche de bilan des patients atteints de l'ulcère de Buruli" and "Prévention des incapacités liées à l'UB—formulaire de base"; see S3 and S4 Forms).

Follow-up visits

A total of 25 follow-up visits to 29 USPs (corresponding to the catchment area of 61 villages) were conducted in January-April 2013 (110 patients) and May-June 2014 (19 patients). In advance, patients were grouped according to location of residence and accessibility of the nearest USP and summoned by the responsible ICP upon instructions of the PF at a specific date. A field team (surgeon, physiotherapist, medical assistant and PF) enrolled study participants at the USPs and performed clinical examination and questioning according to the above described study form (S1 Form) if informed consent was provided. For patients unavailable to attend an attempt was made to retrieve them in their villages at a later date.

Patients whose lesions were healed without complications were discharged. From patients with open wounds at examination (in that context referred to as secondary lesions) lesions were measured and categorized according to WHO guidelines, and clinical samples were collected for microbiological analysis. Patients with anatomical impairment (including excessive scars and open wounds) were subjected to goniometric measurements according to the sagittal, frontal, and transverse rotation method (SFTR) [33] and scars were measured by the surgeon and medical assistant of the field team. Furthermore, these patients were questioned about the functional impairment in daily life according to the questionnaire in part H of the study form (S1 Form). Functional limitations were defined as BUD related anatomical impairment as determined by goniometry and/or measurement of scars and were classified in type I (i.e. anatomical impairment not hampering daily activities) and type II (i.e. anatomical impairment hampering daily activities).

Microbiological analyses

Collection of swab samples, fine-needle aspirates or 3mm punch biopsies as well as microscopy and IS*2404* qPCR followed standardized procedures at the laboratories of the INH, Lomé accompanied by external quality assurance conducted at the Department for Infectious Diseases and Tropical Medicine, Munich, Germany, as recently described [30]. For regular bacteriological analysis, conducted in the accredited (COFRAC, "Comité Français d'Accréditation" according to NF EN ISO/CEI 17025 [version 2005]) bacteriology unit of the INH, swab samples were inoculated on Chapman (mannitol-salt [MSA]) agar, blood agar and nutrient broth (BioRad, Munich, Germany). Colonies indicative for *Staphylococcus aureus* were isolated from MSA agar, analyzed by Gram staining, catalase and coagulase test, and subjected to susceptibility testing using the Kirby-Bauer disc diffusion method (15 antimicrobials) on Müller-Hinton agar (BioRad) [34].

Statistical analysis

The study design was a non-randomized clinical cohort study. Statistical analysis (chi-square test, including Fisher exact test) was carried out by EPIINFO 3.3.2. (CDC, Atlanta, GA, USA). The results of statistical analyses were presented by *P*-values. Significant differences were defined as *P*-values below 0.05.

Results

Patients enrolled and baseline data

Out of 199 BUD patients eligible for the study, 129 (64.8%) could be retrieved and enrolled as follow-up patients in the study. Among the 129 follow-up BUD patients, 46.5% were male. At the time of initial diagnosis 90 of 129 follow-up patients (69.8%) were below 15 years of age (range 2–68 years, median 10 years, interquartile range [IQ] 7–16 years). The patients originated from 6 districts of region "Maritime". The distance from the place of residence to CHR-Tsévié was known for 120 patients (93.0%) and was 1–23 km for 47 patients (39.2%) and 24–135 km for 73 patients (60.8%). The duration of disease before clinical diagnosis was known for 128 patients (99.2%) and was 0–42 days for 81 patients (63.3%), and 43–3.600 days for 47 (36.7%) patients. Baseline data and details on statistical analyses are provided in S2 Table.

Drop-out patients and baseline-data

Out of 199 BUD patients eligible for the study, 70 patients (35.2%) could not be enrolled (drop-outs). Forty-three patients (61.4%) had moved to an unknown address, 24 (34.3%) were not found and 3 were deceased (4.3%). Among the 70 drop-out patients, 52.9% were male (no significant difference with the follow-up patients). At the time of initial diagnosis 36 of 70 drop-out patients (51.4%) were above 15 years of age (range 2–65 years, median 15.5 years, IQ 8.3–28 years) and significantly older than the follow-up patients ($P<0.01\%$). The drop-out patients originated from 6 districts of region "Maritime", 2 districts of region "Plateaux", 1 district of region "Centrale" and 1 district of region "Savanes". The distance from the place of residence to CHR-Tsévié was known for 60 drop-out patients (85.7%) and was 1–23 km for 34 patients (56.7%) and 24–135 km for 26 patients (43.3%); the drop-out patients lived significantly closer to CHR Tsévié than the follow-up patients ($P = 0.03\%$). The duration of disease before clinical diagnosis was known for 69 drop-out patients (98.6%) and was 0–42 days for 21 patients (30.4%) and 43–3.600 days for 48 (69.6%) patients; the drop-out patients had a significantly longer duration of disease than the follow-up patients ($P<0.01$). Baseline data and details on statistical analyses are provided in S2 Table.

Characteristics of initial lesions

At the time of initial diagnosis 73 of the 129 follow-up patients (56.6%) had ulcers and 56 patients (43.4%) had non-ulcerative lesions (nodule, n = 19 [33.9%]; plaque, n = 26 [46.4%]; edema, n = 11 [19.6%], 10 edemas evolved into an ulcer). Fifty-nine patients (45.7%) had category I lesions, 44 patients had category II lesions (34.1%) and 26 patients (20.2%) had category III lesions. Four of the patients with category III lesions had multiple lesions [multiple ulcers, n = 2; ulcer and nodule, n = 1; ulcer and plaque, n = 1]). The localization of lesions was as follows: upper limbs, n = 51 (39.5%); lower limbs, n = 50 (39.8%); trunk/head, n = 28 (21.7%). Lesions of 45 patients (34.9%) involved joints (category I, n = 23 [51.1%]; category II, n = 18 [40.0%]; category III, n = 4 [8.9%]). LOM at time of initial diagnosis were not documented.

Treatment and time to healing

Fourty-nine patients (38.0%; category I, n = 13; category II, n = 17; category III, n = 19) received antimycobacterial treatment at CHR-Tsévié, 35 of these patients (71.4%) underwent also surgery (excision and grafting, n = 11 [31.4%]; grafting, n = 23 [65.7%]; reconstructive surgery, n = 1 [2.9%]). Out of these 35 patients, 10 had category I lesions, 8 had category II lesions, and 17 had category III lesions. Eighty patients (62.0%) were referred to USPs (category I, n = 46; category II, n = 27; category III, n = 7) for antimycobacterial therapy.

Among the 129 follow-up BUD patients, 126 patients completed antibiotic therapy (97.7%), three patients (2.3%) did not (two patients were incompliant and for the third the reason was not known). LOM after the end of treatment were documented for 126 patients (97.7%). Out of them, 17 patients (13.5%; category I, n = 2; category II, n = 6; category III, n = 9) were discharged with LOM.

The time to healing was known for 124 patients (96.1%) and ranged from 1–146 days for 63 patients (50.8%; significant correlation with category I lesions [$P<0.01$]), and 147–784 days for 61 patients (49.2%; significant correlation with category III lesions [$P<0.01$]). Stratified into categories of lesions, 57 patients (46.0%) with category I lesions had a median healing time of 108 days (IQR: 93.5–149.5), 42 patients (33.9%) with category II lesions had a median healing time of 151 days (IQR: 125.8–208), and 25 patients (20.1%) with category III lesions had a median healing time of 256 days (IQR: 177–314). Among 41 patients with healing times of more than 180 days, we also observed a correlation with functional limitations ($P<0.01$).

Physiotherapy

According to the BU 01.N forms, 117 out of the 129 follow-up BUD patients (90.7%) received physiotherapy; however for 76 of these patients (65.0%) detailed documentation and physiotherapeutic treatment protocols were not available. Nine follow-up BUD patients (7.0%) did not receive physiotherapy for unknown reasons and for three patients (2.3%) this information was not available. Eighteen patients (23.1%) were treated at CHR-Tsévié only, 60 patients (76.9%) at USPs/patients homes and 39 patients (33.3%) at both locations. The number of sessions was documented for 95 patients (81.2%): 24–99 sessions, n = 46 (48.4%); 100–520 sessions, n = 49 (51.6%). Detailed physiotherapeutical treatment protocols, however, did not exist.

Findings at follow-up

Among the 129 follow-up BUD patients, the lesions of 109 patients (84.5%) were completely healed without any complications. Five patients (3.9%) had secondary lesions (2 of them in combination with functional limitations). Whereas *M. ulcerans* DNA was not detected in any of the lesions, strains of *S. aureus* were isolated from two patients (one of them revealed a

methicillin resistant *S. aureus* [MRSA]), in four cases the etiology of secondary lesions remained unclear. Fifteen patients (11.6%) had functional limitations (type I, n = 5 [3.9%]; type II, n = 10 [7.8%]; two of them in combination with secondary lesions). From 80 patients (62.0%) scars were measured. Out of them, 22 patients (27.5%) had scars with a diameter of <5cm, 33 (41.3%) had scars with a diameter of 5–15cm, and 25 (31.3%) had scars with a diameter of >15cm.

Risk factors for functional limitations

Among the clinical findings, functional limitations were significantly associated with healing times >180 days ($P<0.01$), edema ($P<0.01$), and category III lesions (ulcers >15cm or multiple lesions; $P<0.01$), and a documented LOM at time of discharge ($P<0.01$). Treatment related factors significantly associated with functional limitations were surgery ($P<0.01$) and hospitalization at CHR-Tsévié ($P<0.01$). S2 Table provides detailed risk factor analyses.

Discussion

This study provides the first analysis of treatment outcome of BUD patients in Togo. The median times to healing as determined for various categories of patients lie within the range of values reported by other authors. Likewise, our data also suggest that the lesions of approximately two third of the patients healed within about 25 weeks as reported by other authors [5, 11–14]. The absence of proven recurrences in our study is also in line with the low or non-existing recurrence rate as observed by other investigators [11–13, 15]. As previously published, one patient of our study cohort had developed a delayed paradoxical reaction 10 months after the end of antimycobacterial treatment [18]. At the time of follow-up initial and secondary lesions were completely healed, the patient was therefore not included in the group of patients with complications. Five patients had secondary lesions at the time of clinical examination which may be related to delayed type paradoxical reactions—this is however purely speculative as the patients could not precisely indicate time of occurrence and clinical course of the lesions. From the lesions of two of these patients *S. aureus* strains, one of them MRSA, were isolated. Although this is the first reported case of MRSA from Togo, this finding was to be expected as investigators from the neighboring countries Ghana and Benin have recently shown that a high proportion of BUD lesions are colonized with *S. aureus*, and MRSA is frequently isolated [35–37]. The Togolese MRSA patient was treated with vancomycin and reportedly healed under antibiosis. It became however apparent that follow-up procedures for identification of such complications are lacking, furthermore, a concept for antibiotic management of super-infected BUD lesions does not exist.

A drawback of this study was that almost 35% of laboratory confirmed patients treated with standardized antimycobacterial treatment could not be retrieved at follow-up visits. According to medical records or BU 01.N forms, 60 of the drop-out patients (85.7%, out of them 58 patients without LOM [96.7%]) were completely healed at discharge. We could however not assess long-term sequelae among the drop-out cohort. To avoid these lost to follow-ups, which are likely to occur in mobile populations such as in Togo, we strongly recommend the introduction of standardized follow-up procedures for BUD patients in Togo.

Among the cohort of BUD patients retrieved for follow-up the percentage of functional limitations of 11.6% was lower than in other studies [4–5, 21–22]. However, we need to mention that, in the absence of formal definitions, we introduced an operational definition of type I and II functional limitations, therefore a direct comparison between our data and other studies may not be possible without restrictions. Our data suggest that edema and category III ulcers, a healing time >180 days as well as LOM at discharge constitute the main risk factors for

functional limitations in Togolese BUD patients. The finding that hospitalization and surgical treatment at CHR Tsévié were also associated with functional limitations can be explained by the fact that 73% of patients with category III lesions were hospitalized at the reference center and 89% of them underwent surgery.

In analogy with the operational definition of a "severe Buruli ulcer" as established by Vincent et al. [5], we suggest to introduce criteria for the systematic identification of patients with increased risk for functional limitations also into clinical management of BUD in Togo. We propose to draw special attention to patients initially presenting with edema and category III ulcers, furthermore—although our data did not show a significant correlation—joint involvement as shown by other authors [21–23, 25–26].

A recent study emphasized the special importance of wound care for the prevention of BUD related functional limitations [38]. Although according to our data most Togolese patients with "severe Buruli ulcer" have already been hospitalized in CHR-Tsévié and received advanced wound management, we recommend making it a general rule. Optimal wound management should consist of daily cleansing with saline solution (in cases of severe exudation twice a day), removal of necrotic tissue, and use of vaseline dressing for prevention of drying of the wound. In addition, consistent implementation of the POD related essential health interventions as outlined by the WHO are required and necessitate intensified training programs for hospital staff, CLTs, ICPs and physiotherapists [6].

This study provided an excellent opportunity to review all BUD related documentation. Clinical, epidemiological and treatment records on BU 01.N forms were for the most part complete. The status of LOM at admission was however not documented, and information on evolution of wounds during treatment was not available. For that reason we were not able to retrospectively analyze the prevalence of early paradoxical reactions in terms of enlargement of wounds. Likewise, extensions of lesions were only known for the time of admission and it was impossible to keep track of lesions expanding over joints subsequent to initial diagnosis, which may explain the absence of a significant correlation between lesions over joints and functional limitations in our study cohort. Concerning surgery, operation reports were not available, and information on indication, type and frequency of surgical interventions was largely retrieved from handwritten notes and oral reports of PF and surgeons.

For more than 60% of the patients who allegedly had received physical therapy, written documentation was absent, and treatment protocols indicating the type of exercises performed did not exist. Therefore, conclusions on the impact of physical therapy on prevention and clinical improvement of functional limitations could not be drawn in this study.

In view of these findings, optimization of procedures accompanying or following antimycobacterial treatment are highly recommended. Improvement of documentation of surgical and physiotherapeutic interventions is required and shall be facilitated through filing maps.

Furthermore, to standardize concomitant physiotherapeutic measures, at the time of admission each patient should be seen by a physical therapist to decide on the general requirement of physical therapy and to prepare a treatment schedule, if applicable. Upon completion of antimycobacterial treatment, the PF at CHR-Tsévié and specially trained CLTs at the USPs respectively, should conduct a standardized assessment for each patient to decide on discharge and/or further therapeutic measures. The individual package of measures for each patient shall be defined in a treatment schedule which is regularly monitored by PF and CLTs. As a general rule, all patients should be followed until complete healing of the wound, afterwards at least once per year for a five year period, thus facilitating timely recognition of two further risk factors for functional limitations, i.e. prolonged healing times and LOM at/after discharge, as well as delayed paradoxical reactions. Regular feedback on fulfillment of treatment measures and results of follow-up visits to the PNLUB-LP ("Programme National de Lutte contre l'Ulcère de

Buruli, la Lèpre et le Pian") is considered mandatory to enhance the transparency of the system and to allow for further evaluation and improvement.

Supporting Information

S1 Table. List of parameters collected for analysis.
(DOCX)

S2 Table. Data of the study cohort comprising 199 PCR confirmed BUD patients.
(DOCX)

S1 Form. Clinical follow-up form.
(PDF)

S2 Form. BUD clinical record and laboratory data entry form.
(PDF)

S3 Form. "Fiche de bilan des patients atteints de l'ulcère de Buruli".
(PDF)

S4 Form. "Prévention des incapacités liées à l'UB—formulaire de base".
(PDF)

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Author Contributions

Conceived and designed the experiments: MBe NA FW JN GB. Performed the experiments: MBe NA EP BK KB ABK. Analyzed the data: MBe NA FW BK MBa KHH TL JN GB. Contributed reagents/materials/analysis tools: FW KHH KB ABK TL GB. Wrote the paper: MBe MBa TL JN GB. Recruitment and patient management: NA FW EP BK JN. Laboratory analyses: MBe KB ABK GB. Critical revisions of manuscript: MBe KHH TL GB.

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