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Untersuchungen zur Detektion resistenzassoziierter Mutationen des *rpoB- und rpsL*-Gens in *Mycobacterium ulcerans* Isolaten von Patienten mit PCR bestätigtem Buruli Ulkus aus Ghana.

Dissertation
zum Erwerb des Doktorgrades der Medizin
an der Medizinischen Fakultät der
Ludwig-Maximilians Universität zu München

vorgelegt von
Marcus Wilfried Beißner
aus
Bad Oldesloe
2011

Mit Genehmigung der Medizinischen Fakultät
der Universität München

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Tag der mündlichen Prüfung:	07.07.2011

meinen Eltern

Die vorliegende kumulative Dissertation umfasst zwei Manuskripte, die mit Genehmigung des Promotionsausschusses der Medizinischen Fakultät der Ludwig-Maximilians Universität München vorab publiziert wurden.

Herbinger KH, Adjei O, Awua-Boateng NY, Nienhuis WA, Kunaa L, Siegmund V, Nitschke J, Thompson W, Klutse E, Agbenorku P, Schipf A, Reu S, Racz P, Fleischer B, **Beissner M**, Fleischmann E, Helfrich K, van der Werf TS, Löscher T, Bretzel G. Comparative study of the sensitivity of different diagnostic methods for the laboratory diagnosis of Buruli ulcer disease.
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Inhaltsverzeichnis

1. Abkürzungsverzeichnis

AFB	säurefeste Stäbchen (engl.: **a**cid **f**ast **b**acilli)
bp	Basenpaar(e)
BUD	Buruli Ulkus Erkrankung (engl.: **B**uruli **u**lcer **d**isease)
bzw.	beziehungsweise
°C	Grad Celsius
CLT	Clarithromycin
d.h.	das heißt
DNA	Desoxyribonukleinsäure (engl.: **d**esoxyribo**n**ucleic **a**cid)
His	Histidin
i.S.	im Sinne
IS	Insertionssequenz
kPa	Kilopascal
M.	Mycobacterium
Mbp	Millionen Basen Paare
mind.	mindestens
mm	Millimeter
MPM	Mykolakton produzierende Mykobakterien
N	Anzahl (engl.: **n**umber)
NTM	nicht-tuberkulöse Mykobakterien (engl.: **n**on-**t**uberculous mycobacteria)
rpoB	Gen für die Beta-Untereinheit der RNA-Polymerase
rpsL	Gen für das ribosomale Protein S12
pH	pH-Wert (lat.: **p**otentia **h**ydrogenii)
PCR	Polymerase-Kettenreaktion (engl.: **p**olymerase **c**hain **r**eaction)
RMP	Rifampicin
Ser	Serin
SM	Streptomycin
TAG	Technische Beratungsgruppe (engl.: **T**echnical **A**dvisory **G**roup)
u.a.	unter anderem
u.U.	unter Umständen
v.a.	vor allem
WHO	Weltgesundheitsorganisation (engl.: **W**orld **H**ealth **O**rganisation)

2. Einleitung – Das Buruli Ulkus

2.1 Historischer Hintergrund

Erste Beschreibungen großflächiger Hautulzerationen, die mit dem heute bekannten klinischen Bild des Buruli Ulkus in Einklang zu bringen sind, gehen auf die Entdeckungen des britischen Arztes Sir Albert Cook, im Jahre 1897 in Kampala, Uganda zurück (102). Ein halbes Jahrhundert später gelang Peter MacCallum und Kollegen in der Region um Bairnsdale (Victoria) im südlichen Australien die Isolation und nähere Charakterisierung des ätiologischen Agens, *Mycobacterium ulcerans* (51). In Australien wird die Erkrankung auch heute als „Bairnsdale ulcer" bezeichnet. In den sechziger und siebziger Jahren des 20. Jahrhunderts wurden Fälle aus dem „Buruli Distrikt" (heute Nakasongola Distrikt) in Uganda berichtet – die Erkrankung wurde fortan als „Buruli ulcer disease" (BUD) bezeichnet (6).
Seit 1980 tritt das Buruli Ulkus zunehmend in großen Teilen der Welt in Erscheinung mit einem Hauptfokus und jährlich steigenden Fallzahlen in den subsaharischen Ländern West-Afrikas (102). Im Jahre 1998 gründete die WHO die „Global Buruli Ulcer Initiative" (GBUI), um mehr Aufmerksamkeit auf die, in Relation zu den steigenden Fallzahlen, recht unbekannte Krankheit zu richten. Während der ersten internationalen Konferenz der GBUI wurde die „Yamoussoukro-Deklaration" verabschiedet, die aufgrund des zum damaligen Zeitpunkt verfügbaren Erkenntnisstandes die Transmission und Prävention der Erkrankung als Forschungsschwerpunkte definiert, sowie die Einrichtung eines Surveillance Systems empfiehlt. (110, 112)
Die Notwendigkeit der Verbesserung von Interventions- und Kontrollmaßnahmen durch Förderung intensivierter Forschung zur Etablierung neuer diagnostischer Verfahren und zur Verbesserung der Therapie und Präventionsmaßnahmen wurde im Jahre 2004 durch eine Resolution der „World Health Assembly" (WHA) bestätigt. Diese Inhalte wurden im Jahre 2009 durch die „Cotonou-Deklaration" bekräftigt, die insbesondere herausstellt, dass internationale wissenschaftliche Arbeiten zur Etablierung neuer Testverfahren gezielt gefördert werden sollten, um Diagnostik, Therapie und somit das klinische Management zu verbessern. Das Buruli-Ulkus steht gegenwärtig auf der Liste der 14 „neglected tropical diseases". (99, 100, 107, 108)

2.2 Der Erreger – Mycobacterium ulcerans

Mycobacterium ulcerans gehört zur Familie der Mycobacteriaceae (Gattung Mycobacterium) und ist ein langsam wachsendes, nicht-chromogenes, aerobes, säurefestes Stäbchenbakterium (**AFB**, engl. **a**cid **f**ast **b**acilli) aus der Gruppe der atypischen Mykobakterien (**NTM**, engl. **n**on-**t**uberculous **m**ycobacteria; Synonym: **MOTT**, engl. **m**ycobacteria **o**ther **t**han **t**uberculosis) (69).
Das Genom von *M. ulcerans* (ca. 5,8 Mbp) ist gekennzeichnet durch einen hohen Gehalt (ca. 70%) der Basen Guanin und Cytosin und weist über 98% Sequenzhomologie zu dem fischpathogenen *M. marinum* auf, weshalb evolutionsbiologisch eine phylogenetische Abspaltung von dieser Spezies durch lateralen Gentransfer angenommen wird (65, 85, 86). Weiterführende phylogenetische Analysen ergaben den Hinweis auf die Evolution zweier unterschiedlicher Abstammungslinien: die „ancestral lineage" (Stämme aus Asien, Mittel- und Südamerika), und die „classical lineage" (Stämme aus Australien und Afrika) (46, 73). Gegenwärtig können mittels VNTR-Analysen (**VNTR**, engl. **v**ariable **n**umber **t**andem **r**epeats) vier unterschiedliche genetische Cluster von *M. ulcerans* unterschieden werden, deren Verteilung mit dem geographischen Vorkommen korreliert (87). Die Untersuchungen genetischer Insertions-Deletions

Polymorphismen ergaben für Stämme verschiedener Kontinente ebenso die Möglichkeit einer Unterscheidung, wohingegen in lokalen klonalen Populationen keine Variationen detektiert wurden (45). Hierzu eignet sich die SNP Typisierung (**SNP**, engl. **s**ingle **n**ucleotide **p**olymorphism) als „fingerprinting" Methode klonaler Populationen, mittels welcher innerhalb einer geographischen Region in Ghana der Nachweis von zehn unterschiedlichen *M. ulcerans* Haplotypen erbracht wurde (70, 71).
Als entscheidender Virulenz-Faktor spielt das plasmidkodierte (*pMUM*) polyketidähnliche Makrolid „Mykolakton" als Exotoxin eine Schlüsselrolle: Neben einer lokalen und systemischen immunsuppressiven Wirkung wird diesem Zytotoxin ein wesentlicher nekrotisierender Effekt auf Zellen des Binde- und Fettgewebes zugeschrieben (29, 39, 41). Das Toxin ist in der Lage beim Menschen die Aktivierung der Th_1-Zellen und die Produktion von Interferon Gamma (IFN-**γ**), welches zur weiteren Aktivierung der Makrophagen führt, zu supprimieren (16, 32, 41, 68). Aus in-vitro Tests geht hervor, dass Mykolakton in zahlreichen humanen Zellreihen neben Nekrosen auch Apoptose induziert; in jüngster Zeit wurde die Induktion von Apoptose in Keratinozyten nachgewiesen (10, 21, 30, 33, 41, 77). Es existieren strukturelle Varianten des Mykolaktons, die in unterschiedlichen geographischen Regionen konserviert auftreten. Die Anzahl und Art der Kongenere legt einen Zusammenhang zur Virulenz der Stämme nahe (92). Da das Mykolakton in allen Stadien des Buruli Ulkus nachweisbar ist, jedoch unterschiedliche Bioaktivität aufweist, stellt es einen potentieller Biomarker für Therapiekontrollen dar (77).
Unter den **M**ykolakton **p**roduzierenden **M**ykobakterien (**MPM**) werden neben der humanpathogenen Spezies *M. ulcerans* vier weitere phylogenetisch eng verwandte Spezies (*M. marinum, M. liflandii*, *M. shinsuense* und *M. pseudoshottsii*) subklassifiziert, die keine klinische Relevanz beim Menschen aufweisen und lediglich Infektionen in Fischen und Fröschen der Gattung Xenopus auslösen (31, 60, 65, 116). Den vier nicht-humanpathogenen Spezies ist mit *M. ulcerans* das Vorhandensein der Insertionssequenz IS*2404*, einem mobilen genetischen Element, das die Expression von Genen beeinflusst, gemeinsam (116). Die IS*2404* ist hingegen nicht im Genom des humanpathogenen *M. marinum* kodiert (65). Die Anzahl der IS*2404* Kopien variiert geografisch unter den *M. ulcerans* Stämmen und korreliert positiv mit dem Grad der Virulenz; afrikanische Stämme weisen durchweg eine hohe Anzahl an IS*2404* Kopien (z.B. 213 Kopien im Stamm Agy99, Ghana) im Genom auf (15, 86).

2.3 Epidemiologie

Das Buruli Ulkus ist nach der Tuberkulose und Lepra weltweit die dritthäufigste mykobakterielle Infektionskrankheit bei immunkompetenten Menschen und wurde bislang aus über 30 Ländern Afrikas, Lateinamerikas, Asiens und des Westpazifiks berichtet, wobei es sich hauptsächlich um tropische sowie subtropische Klimaregionen handelt und Länder Westafrikas (v.a. die Elfenbeinküste, Ghana und Benin) den Hauptfokus darstellen (82, 99, 102). Zwischen 2006 und 2009 wurden beispielsweise aus Ghana insgesamt mehr als 3.000 Fälle gemeldet [Dr. K. Asiedu, WHO, GBUI, persönliche Kommunikation]. Aufgrund mangelnder Kapazitäten zur Laborbestätigung der klinischen Verdachtsfälle sind keine definitiven globalen Daten zu Inzidenz und Prävalenz des Buruli Ulkus bekannt. Zwischen 2004 und 2008 wurden der WHO aus 20 ausgewählten Ländern insgesamt 26.275 Fälle gemeldet, wobei die Dunkelziffer nicht gemeldeter und nicht registrierter Fälle weitaus höher liegen dürfte (97).
Auffällig ist das fokussierte Auftreten der Erkrankung innerhalb der endemischen Regionen (5, 99). Endemiegebiete liegen hauptsächlich in ländlichen Regionen an stehenden beziehungsweise

langsam fließenden Gewässern. Kinder unter 15 Jahren, die zumeist unter Armutsbedingungen leben, sind hauptsächlich von der Erkrankung betroffen (36, 82, 83, 109). Im Gegensatz zur Tuberkulose gibt es keinen signifikanten Hinweis auf eine erhöhte Inzidenz des Buruli Ulkus bei immunsupprimierten HIV-infizierten Patienten. Lediglich in Einzelfällen wurde bei AIDS-Patienten eine stärkere Dissemination der Erreger beobachtet (43, 44, 48, 90).

2.4 Transmission

Mittels Polymerase-Ketten-Reaktion (**PCR**, engl. **p**olymerase **c**hain **r**eaction) konnte *M. ulcerans* DNA (**DNA**, engl. **d**esoxyribo**n**ucleic **a**cid) aus Wasserproben und direkt aus dem Erdreich, sowie in Wasserpflanzen, Mollusken, Fischen und kleinen Nagern in West-Afrika nachgewiesen werden. Während eines spontanen Ausbruchs in einem kleinen Fischerdorf nahe Melbourne (Süd-Ost Australien) wurde *M. ulcerans* DNA in „salt marsh" Moskitos der Gattung *Aedes* detektiert. Weitere Untersuchungen an Possums der Gattung *Kusus* in australischen Endemiegebieten ergaben den Nachweis laborbestätigter BUD Fälle bei eben diesen Tieren; *M. ulcerans* DNA wurde u.a. in den Fäzes der Possums detektiert. Der kulturelle Nachweis von *M. ulcerans* aus der belebten Natur ist sehr schwierig und gelang erstmals aus Schwimmwanzen (lat. *Naucoridae*) und Riesenwanzen (lat. *Belastomatidae*) der Ordnung Hemiptera. Im Mausmodell wurde gezeigt, dass ein Biss durch infizierte Schwimmwanzen zur Ausbildung des Buruli Ulkus führt. Der exakte Transmissionsweg ist jedoch gegenwärtig ungeklärt. (22-24, 42, 54, 55, 66, 67, 94, 114)

Im Gegensatz zu den Erregern der Tuberkulose und Lepra scheint eine Übertragung von Mensch zu Mensch keine Rolle zu spielen und lediglich in sehr seltenen Einzelfällen und nur in Verbindung mit einem adäquaten Trauma i.S. einer Verletzung der Haut aufzutreten; lediglich zwei Fallberichte aus den Jahren 1987 und 2002 beschreiben einen solchen Übertragungsweg (19, 28).

2.5 Ätiologie und Pathogenese

Der primäre Krankheitsprozess geht nach unbekannter Inkubationszeit von der Subkutis aus. Die von den Bakterien sezernierten zytotoxischen und immunsuppressiven Mykolakton-Kongenere diffundieren ins infizierte und umgebene Gewebe, wobei sie eine Affinität zu den subkutanen Fettzellen besitzen und hier zu Kolliquationsnekrosen führen. Das durch die Fettgewebsnekrosen entstehende Milieu ist für die weitere Proliferation der Mykobakterien optimal (21, 72, 95).

Im initialen Stadium treten massive subkutane Nekrosen ohne Ulzerationen der Dermis auf. Im Gegensatz zu vielen anderen mykobakteriellen Infektionen findet in diesem Stadium keine Granulombildung statt. Immunologische Untersuchungen stützen die Hypothese, dass während einer Infektion mit *M. ulcerans* eine „Downregulation" der protektiven, zellulären Th_1-Immunantwort stattfindet (41, 68, 91, 115). Im Zuge zunehmender Nekrosen des subkutanen Fettgewebes kommt es zu Gefäßokklusionen, Nervenschädigungen und Destruktionen des Gewebes bis zum Durchbruch auf die Muskelfaszien. Es folgen massive Ulzerationen der Dermis mit typischen unterminierten Rändern. Spontan oder antibiotisch induziert, tritt die Heilungsphase ein, die durch eine Remission unter Granulombildung gekennzeichnet ist. Unter narbiger Regeneration der Dermis kommt es teils zur Epithelisation; eine „Restitutio ad integrum" ist jedoch nicht möglich. (34, 41, 62, 72, 74, 79, 91)

2.6 Klinisches Bild und Differentialdiagnose

Die Buruli Ulkus Erkrankung tritt durch unterschiedliche klinische Manifestationen in Erscheinung und umfasst ulzerative sowie nicht-ulzerative Stadien. Zumeist ist das erste Symptom ein schmerzloser, verschieblicher, derber Tumor unter der Haut, der als Nodulus (engl. nodule) bezeichnet wird. Frühe Manifestationsformen sind ebenso Plaques, Papeln und Ödeme. Im Verlauf treten indolente Ulzerationen der Dermis auf, die an Größe zunehmen und initial typische unterminierte Ränder aufweisen. Dieser Übergang kann sich innerhalb von Tagen teils mit begleitendem Ödem manifestieren. Der Knochen kann ebenfalls infiziert werden: Osteitiden bzw. Osteomyelitiden sind gefürchtete Komplikationen, die neben großflächigen Ulcera eine Indikation zur Amputation der betroffenen Gliedmaßen darstellen können. (101, 102)
Klinisch werden die Läsionen in drei Kategorien eingeordnet: zur Kategorie I zählen einzelne Läsionen unter 5 cm im Durchmesser, Kategorie II umfasst einzelne Läsionen zwischen 5 cm und 15 cm und in die Kategorie III werden alle Läsionen über 15 cm im Durchmesser, Osteomyelitiden, Gelenkbeteiligung sowie multiple Läsionen und Läsionen an kritischen Lokalisationen (Gesicht, Genitalregion und Damm) klassifiziert (109).
Sowohl spontane Remission der Infektion als auch späte therapeutische Interventionen sind häufig mit Kontrakturen der betroffenen Gelenke und folglich Funktionseinschränkungen vergesellschaftet (63, 78, 98).
Als Differentialdiagnose sollten sowohl infektiöse als auch nicht-infektiöse Erkrankungen in Betracht gezogen werden. Die noduläre Form kann klinisch zu einer Verwechslung mit der Onchozerkose, Staphylokokkenabszessen, Lipomen, oder Lymphadenopathie führen. Ulzerative Läsionen können Manifestationen einer kutanen Leishmaniose, einer Mykose, des tropischen Ulkus, eines Ulcus cruris venosum oder einer Neoplasie darstellen (101, 102).

2.7 Diagnostik

Nach WHO Empfehlungen sollten mind. 50% aller klinischen BUD Verdachtsfälle mittels PCR bestätigt werden, wobei generell die Laborbestätigung als Voraussetzung zur Einleitung einer antimykobakteriellen Therapie gefordert wird (99, 105, 111).
Zum Nachweis von *M. ulcerans* stellen Wundabstriche (engl. swab) und Gewebeproben (exzidiertes Gewebe, 3 mm Stanzbiopsien sowie Feinnadelaspirate) adäquates Untersuchungsmaterial dar.
Während der „swab" von unterminierten Rändern den Goldstandard für die Diagnostik ulzerativer Läsionen darstellt, werden bei nicht-ulzerativen Läsionen Gewebeproben aus dem Zentrum der Läsion entnommen, wobei das in jüngster Zeit eingeführte Feinnadelaspirat (FNA) aufgrund der minimalen Invasivität bevorzugt Anwendung finden sollte (13, 26, 37, 64, 103).
Zur Laborbestätigung eines Verdachtsfalles stehen derzeit die Mikroskopie, PCR, Kultur und Histopathologie als etablierte diagnostische Methoden zur Verfügung (8, 36). Die Entwicklung eines diagnostischen Tests zur Detektion des Mykolaktons ist gegenwärtig Gegenstand intensiver Forschung (77, 103).
In verschiedenen Studien konnten BUD Verdachtsfälle abhängig von der Art der Läsion und diagnostischen Probe, den Transportbedingungen zum Labor sowie Dauer der Erkrankung und der antibiotischen Vorbehandlung in 40-78% mittels Mikroskopie, in 34-80% mittels Kultur, in 61-72% mittels IS*2404* PCR und in >70% durch histopathologische Untersuchungen (HISTO) labordiagnostisch bestätigt werden (2, 8, 11, 36, 57, 63, 80). Die diagnostische Sensitivität wird

dabei mit 50-70% für die Mikroskopie, 45-70% für die Kultur sowie 79-85% für die IS*2404* PCR und 82% für die HISTO angegeben (1, 8, 35, 36, 84).
Die Mikroskopie eignet sich als schnelle, kostengünstige und einfach durchzuführende Methode vor allem in Endemiegebieten unter Feldbedingungen als primärer Bestätigungstest. Der mikroskopische Nachweis der AFB gelingt unter Färbung nach Ziehl-Neelsen, Kinyoun oder durch Auramin-Rhodamin (8, 25, 101). Die Beurteilung hängt jedoch wesentlich von der Qualität des Ausstriches sowie der Erfahrung des Untersuchers ab. Generell ist die Sensitivität mit ca. 40% eingeschränkt, wobei insbesondere bei Patienten aus Endemiegebieten mit neu diagnostiziertem Buruli Ulkus ohne vorherige antibiotische Therapie Sensitivitäten bis zu 70% ermittelt wurden (8, 36).
In vitro gelingt die Kultur des Bakteriums bei einem Temperaturoptimum von 29-33°C, einem Sauerstoffpartialdruck (pO_2) < 2,5 kPa sowie pH-Werten zwischen 5,4-7,4 prinzipiell auf verschiedenen Nährmedien, wobei das solide, eierhaltige Löwenstein-Jensen Nährmedium gut geeignet ist (101). Mit einer durchschnittlichen Länge von 2-3 µm wächst *M. ulcerans* in unregelmäßiger Morphologie. Der kulturelle Nachweis stellt derzeit die einzige etablierte Methode zum Viabilitätsnachweis von *M. ulcerans* dar und ist unter optimalen Bedingungen innerhalb von 9-12 Wochen möglich. Aufgrund der langen Inkubationszeit eignen sich Kulturen jedoch weder für die schnelle Labordiagnose, noch für eine konventionelle Resistenztestung, wenn zeitnahe Therapieentscheidungen getroffen werden sollen (7).
Mit einer analytischen Spezifität von 100% stellt die IS*2404* PCR den zurzeit verlässlichsten Test zur Laborbestätigung dar (8, 36). Diese Methode ist unter tropischen Bedingungen als IS*2404* „**DRB**" PCR" (engl., **d**ry **r**eagent-**b**ased PCR) valide anwendbar (80, 81).
Neben der klinischen Diagnose werden nach WHO Kriterien zwei positive Labortests gefordert um zweifelsfrei einen Buruli-Ulkus Verdachtsfall zu bestätigen (101). Aus den aktuellen Daten der eigenen Arbeitsgruppe geht allerdings hervor, dass aufgrund der hohen positiven prädiktiven Werte für die Mikroskopie (97%) und die PCR (100%) in Endemiegebieten ein positiver Test zur Bestätigung eines Verdachtsfalles ausreicht (8, 36).

2.8 Therapie

Die Behandlung des Buruli Ulkus erfolgte bis 2004 in erster Linie chirurgisch, wobei abhängig von der Art der Läsion und der Erfahrung des Operateurs Rezidivraten von 6,1 bis 32 % berichtet wurden (4, 18, 88). Untersuchungen der Schnittränder exzidierter Ulzera zeigten, dass die Erreger im makroskopisch gesund erscheinenden umgebenden Gewebe persistieren, wodurch das Rezidivrisiko erklärt wurde (12, 74). Im Falle von Noduli oder Plaques kann aufgrund der Konzentration der Erreger im Zentrum der Läsion unter Einhaltung eines ausreichenden Sicherheitsabstandes die Exzision kurativ sein (38).
Mehrere Studien wiesen im Tiermodell eine effektive Behandlung *M. ulcerans* infizierter Mäuse durch eine Kombinationstherapie mit Rifampicin (RMP) und Streptomycin (SM) über acht Wochen (RS8) nach (9, 20, 53). Der bakterizide Effekt dieser Kombinationstherapie konnte folglich auch für BUD Patienten in einer ersten klinischen Studie bestätigt werden (27). Daraufhin erstellte die WHO 2004 die ersten Empfehlungen zur antimykobakteriellen Behandlung des Buruli Ulkus mittels der Kombinationstherapie aus Rifampicin (10 mg/kg KG/d) per os und Streptomycin (15 mg/kg KG/d) intramuskulär für acht Wochen, ggf. gefolgt von chirurgischen Interventionen (105). Diese Empfehlungen wurden ab 2006 in den meisten westafrikanischen Ländern in die Praxis umgesetzt (7, 78). Weitere klinische Studien PCR

bestätigter BUD Patienten u.a. aus Benin (N = 219), Ghana (N_1 = 76, N_2 = 160) und der Demokratischen Republik Kongo (N = 91) bestätigten die Effektivität dieses Therapie Regimes (14, 47, 61, 76). Seit Einführung der antimykobakteriellen Therapie werden weitaus niedrigere Rezidivraten von unter zwei Prozent verzeichnet (99, 106).
Die Therapie Richtlinien und Falldefinitionen wurden 2007 durch die „**T**echnical **A**dvisory **G**roup" (**TAG**) der WHO angepasst. Demnach werden alle Neuerkrankungen (engl. „new case") - dabei handelt es sich um BUD Patienten, die bisher nicht antibiotisch behandelt wurden - der achtwöchigen Kombinationstherapie (RS8) zugeführt. Derzeit können hierdurch bis zu 50% der Kategorie I und II Läsionen kurativ behandelt werden. Eine Verkleinerung der Läsion gelingt nahezu immer, auch wenn die vollständige Heilung, insbesondere großflächiger Kategorie III Ulzera, nicht innerhalb von acht Wochen erreicht werden kann. Vor allem bei großflächigen Ulzera der Kategorie III kann die Heilung u.U. nach adjuvanter vierwöchiger Chemotherapie durch eine chirurgische Revision beschleunigt werden (106, 109). Essentiell bleibt die frühzeitige Erkennung der Erkrankung und chirurgische und/oder chemotherapeutische Intervention. Großflächige Exzisionen bedingen oft eine anschließende Spalthauttransplantation zur Defektdeckung. Kontrakturen der benachbarten Gelenke sollten im Idealfall frühzeitig physiotherapeutisch behandelt werden (98).
Zu beachten ist, dass mögliche Therapieversager und Rezidive frühzeitig erkannt werden, um im Einzelfall das klinische Management für ein optimales individuelles Therapieergebnis umzustellen. Bei verzögerter Wundheilung sowie der Entstehung neuer Läsionen an gleicher oder benachbarter Lokalisation der initialen Läsion innerhalb von drei Monaten nach Abschluss der Therapie (engl.: „non-healers") wird eine weiterführende konservative Wundbehandlung empfohlen. Treten neue Läsionen drei Monate nach Beendigung der initialen Therapie, die in einer vollständigen Heilung resultierte, auf, so handelt es sich um ein Rezidiv (engl.: recurrence). Zur labordiagnostischen Bestätigung des klinischen Verdachts eines Rezidivs, ist der kulturelle Nachweis des Erregers erforderlich (109). Dem Rezidiv können eine Re-Infektion oder auch die Ausbildung von Antibiotika-Resistenzen zugrunde liegen, wobei letztere insbesondere auch bei „non-healers" ursächlich sein können. In diesen Fällen wäre eine zeitnahe Resistenztestung erforderlich, um die weitere Therapie effizient zu gestalten. Wird ein zweiter Behandlungszyklus erwogen, so muss darauf geachtet werden, dass die kumulative toxische Dosis des Streptomycins (90 g bei Erwachsenen) nicht überstritten wird.
Erste erfolgsversprechende Ergebnisse zur Etablierung rein oraler Therapieregimes, bestehend aus Rifampicin und Clarithromycin (CLT) liegen bereits für frühzeitig diagnostizierte begrenzte Läsionen vor (RMP + SM für vier Wochen gefolgt von RMP + CLT für vier Wochen) und werden gegenwärtig in weiteren klinischen Studien in Westafrika evaluiert (61).

2.9 Antibiotika Resistenzen

Die signifikante Bedeutung der synergistischen Wirkung einer Kombinationstherapie des Buruli Ulkus wurde anhand der Antibiotika Rifampicin (RMP) und Streptomycin (SM) im Tiermodell verdeutlicht (20, 53). Als Ursache für das mäßige Ansprechen auf die alleinige Gabe von Rifampicin werden erworbene Resistenzen diskutiert, die sich unter einer Monotherapie etablieren können (52). Mutationen in Genen von Zielproteinen antibakterieller Substanzen stellen einen äußerst spezifischen Resistenzmechanismus dar. Betrifft eine Mutation das Gen des Zielproteins, so kann u.U. eine veränderte Struktur die Bindung des Antibiotikums verhindern.

Für RMP und SM sind Mutationen in den Genen der Zielproteine bekannt, die insbesondere bei *M. tuberculosis* und *M. leprae* für einen Großteil der Resistenzen gegen diese Antibiotika ursächlich sind (58, 113).
Phänotypische RMP-Resistenz korreliert bei *M. tuberculosis* genotypisch zu 95% mit Mutationen innerhalb der hochkonservierten 81bp „**r**ifampicin **r**esistance-**d**eterming **r**egion" (**RRDR**, Codon 507-533) des *rpoB*-Gens, welches für das Zielprotein des Rifampicins, die β-Untereinheit der DNA-abhängigen RNA-Polymerase, kodiert (3, 50). In der überwiegenden Anzahl sind Punktmutationen der Codons Ser531 und His526 involviert, wobei Codon Ser531 die höchste Mutationsfrequenz aufweist (49, 59). Unter RMP resistenten *M. leprae* Stämmen wurden analog Mutationen in der RRDR (Codon 401-427, äquivalent zu Codon 507-533 in *M. tuberculosis*) detektiert, wobei ebenso das Codon Ser425 (äquivalent zu Codon Ser531 in *M. tuberculosis*) die höchste Mutationsfrequenz aufweist (75).
Phänotypisch Streptomycin resistente *M. tuberculosis* Stämme weisen zu 81,4% Mutationen in Codon 43 des *rpsL*-Gens auf, das für das ribosomale Protein S12 kodiert (17, 40, 58). Mutationen im *rrs*-Gen wurden nur in Einzelfällen neben bisher unbekannten Mechanismen als mögliche Ursache klinischer Streptomycin Resistenz diskutiert (17).
Die globale Resistenzentwicklung der Erreger der Tuberkulose wird durch ein aktives Surveillance-Programm der WHO überwacht. Dabei wurde 2008 der weltweite Anteil der nach Proportionsmethode phänotypisch bestimmter RMP und SM resistenter Stämme des *Mycobacterium tuberculosis complex* (MTBC) auf jeweils 6,3% und 12,6% geschätzt (96). Die genotypische sequenzbasierte Resistenztestung stellt eine exakte und verlässliche Alternative dar, um phänotypische Resistenz gegenüber RMP und/oder SM vorauszusagen (89, 93).
Eine umfassende Studie aus Süd-Ost Asien detektierte bei Lepra-Patienten RMP resistente *M. leprae* Stämme in 2% der neu diagnostizierten Fälle und 8% der Rezidive (56). Analog zur Tuberkulose wurde 2009 ein globales Surveillance-System auf der Grundlage sequenzbasierter genotypischer Analysen für die Lepra implementiert (104).
Bisher wurden unter klinischen *M. ulcerans* Isolaten keine antibiotikaresistenten Stämme beschrieben. Nach experimenteller Therapie *M. ulcerans* infizierter Mäuse mit RMP in Monotherapie, gelang Marsollier und Kollegen erstmalig der Nachweis von Resistenz assoziierten Mutationen in den *rpoB*-Genen dreier phänotypisch RMP resistenter *M. ulcerans* Stämme. Die Mutationen wurden in den Codons Ser416 und His420 (Ser522 und His526 in *M. tuberculosis*) der RRDR nachgewiesen (52).
Für die labordiagnostische Bestätigung eines klinischen Rezidivs sowie für künftige Resistenztestungen bei Rezidiven und „non-healers" wird von der WHO die Abnahme von diagnostischen Proben für die Kultur empfohlen (106). Aufgrund der langen Generationszeit von *M. ulcerans* in Kultur und der geringen Sensitivität dieser Methode insbesondere bei antibiotisch vorbehandelten Patienten, sind konventionelle Resistenztestungen in Hinblick auf zeitnahe klinische Entscheidungen für die individuelle Therapie wenig geeignet. Insbesondere hierfür eignet sich die sequenzbasierte Resistenztestung diagnostischer Proben als Gesamt-DNA Extrakte besser (7).
Aus diesem Grund und vor dem Hintergrund der irregulären Applikation von RMP und SM bei BUD Patienten vor der Einführung der standardisierten Kombinationstherapie in 2006, wurde die vorliegende Pilotstudie in Ghana durchgeführt, um Basisdaten zur Prävalenz von Resistenz assoziierten Mutationen in klinischen *M. ulcerans* Isolaten zu evaluieren.

3. Zusammenfassung

Das Buruli Ulkus (BUD), eine Erkrankung der Haut und des subkutanen Fettgewebes, wird durch *Mycobacterium ulcerans* verursacht und stellt weltweit nach der Tuberkulose und Lepra die dritthäufigste mykobakterielle Infektionskrankheit beim immunkompetenten Menschen dar. Die BUD Hauptendemiegebiete liegen fokussiert in West Afrika. Die Krankheit beginnt typischerweise mit einem Nodulus, einer Papel oder Plaque aus denen im Verlauf ein schmerzloses Ulkus mit typischen unterminierten Rändern und ggf. begleitendem Ödem entstehen kann. Unbehandelt führt die Erkrankung bei geringer Mortalität zu entstellenden Narben und Kontrakturen, die teils mit erheblichen Funktionseinschränkungen betroffener Gelenke einhergehen. Neben der Mikroskopie von säurefesten Stäbchenbakterien, stehen die Kultur des Erregers, die IS*2404* PCR und die histopathologische Untersuchung diagnostischer Proben (Wundabstriche, Feinnadelaspirate, 3 mm Stanzbiopsien und exzidiertes Gewebe) zur Laborbestätigung eines BUD Verdachtsfalles zu Verfügung. Generell wird die Laborbestätigung eines BUD Verdachtsfalles gefordert, um eine antimykobakterielle Therapie einzuleiten. Die WHO empfiehlt hierzu seit 2004 eine standardisierte acht-wöchige antimykobakterielle Kombinationstherapie mit Rifampicin (RMP) und Streptomycin (SM), die seit 2006 in den meisten Ländern Westafrikas angewendet wird. Zuvor erhielten Patienten in Ghana teils unregelmäßige Therapie-Schemata adjuvant zur vorwiegend chirurgischen Behandlung. Unregelmäßige oder nicht standardisierte Applikation der Antibiotika kann die Entstehung von Resistenzen begünstigen und zur Entstehung von Rezidiven führen und dem Auftreten von Therapieversagern zugrunde liegen. Marsollier und Kollegen gelang im Mausmodell der Nachweis induzierter phänotypischer RMP Resistenzen in drei *M. ulcerans* Stämmen unter Monotherapie. Bisher lagen jedoch keine Daten zur Prävalenz antibiotikaresistenter klinischer *M. ulcerans* Stämme vor.

Die vorliegende kumulative Dissertation beinhaltet zwei Manuskripte. Die Arbeiten beschreiben eine Pilot-Studie aus Ghana, in der *M. ulcerans* Isolate von PCR bestätigten BUD Patienten genotypisiert wurden um den potentiellen Nutzen – im Sinne der technischen Anwendbarkeit und des Gewinns für das individuelle klinische Management - sequenzbasierter Ansätze in der Resistenzbestimmung klinischer Isolate zu evaluieren und erste Basisdaten zur Prävalenz Antibiotika-resistenter *M. ulcerans* Stämme in Ghana zu ermitteln.

Im ersten Teil dieser Arbeit wurden vergleichende Untersuchungen der unterschiedlichen diagnostischen Labortests in Relation zur klinischen Präsentation, Probenabnahmetechnik und zur antibiotischen Vorbehandlung evaluiert, um anhand der ermittelten Sensitivitäten optimale Bedingungen für unterschiedliche klinische Szenarios zu bestimmen.

Von 384 BUD Verdachtsfällen mit nicht-ulzerativen und ulzerativen Läsionen aus neun Studienzentren in Ghana wurden diagnostische Proben (Wundabstriche, 3 mm Stanzbiopsien und exzidiertes Gewebe) mittels Mikroskopie, IS*2404* PCR und Kultur analysiert.

Aus dieser Kohorte wurden 268 Patienten durch mindestens einen positiven Labortest bestätigt. Die Sensitivität der PCR war mit 85% insgesamt signifikant höher als die der Mikroskopie (57%) sowie der Kultur (51%). Nach Stratifizierung der Daten in unterschiedliche Behandlungsgruppen, Art der Läsion und Probeabnahmetechnik ergab die PCR bei unbehandelten Patienten für 3 mm Stanzbiopsien aus nicht-ulzerativen Läsionen und für Wundabstriche von ulzerativen Läsionen die höchste diagnostische Sensitivität von 94% bzw. 90%. Während die Dauer der Erkrankung keinen signifikanten Einfluss auf einen der diagnostischen Labortests zeigte, ergab sich eine signifikante Abnahme der Sensitivität für die PCR und Kultur unter antimykobakterieller

Therapie. Zu Zwecken der Therapiekontrolle eignet sich jedoch lediglich die Kultur, da mykobakterielle DNA auch nach dem Absterben der Erreger längere Zeit im Gewebe persistieren kann.
Im zweiten Teil dieser Arbeit wurden klinische *M. ulcerans* Isolate von insgesamt 162 PCR bestätigten BUD Patienten aus der Kohorte des ersten Teils der Arbeit mittels Sequenzierung der *rpoB*- und *rpsL*-Gene auf das Vorhandensein von Rifampicin- bzw. Streptomycin-Resistenz assoziierten Mutationen untersucht.
Hierzu wurden Gesamt-DNA-Extrakte direkt aus diagnostischen Patientenproben (Wundabstriche, 3 mm Stanzbiopsien und exzidiertes Gewebe) sowie DNA Extrakte aus positiven Kulturen dieser diagnotsichen Proben bezüglich der Effizienz der Sequenzanalysen des *rpoB*- und *rpsL*-Gens verglichen.
Bei 99,1% (*rpoB*) und 100% (*rpsL*) der Patienten wurde der *M. ulcerans* Wildtyp detektiert. In einem Isolat (0,9%) wurde eine Punktmutation im Codon Ser416 detektiert, die in einem Aminosäureaustausch resultierte. Die gleiche Mutation wurde von Marsollier und Kollegen beschrieben und eine phänotypische Rifampicin-Resistenz der mutierten Stämme konnte bestätigt werden. Die Daten zeigen eine niedrige Prävalenz an Resistenz assoziierten Mutationen unter klinischen *M. ulcerans* Isolaten aus Ghana aus den Jahren 2004-2007.
Dennoch können Mutationen auftreten und sollten insbesondere bei ausbleibendem Ansprechen auf die antimykobakterielle Therapie, prolongierten und disseminierten Erkrankungen („non-healers“) sowie bei Rezidiven untersucht werden, um insbesondere für individuelle Patienten das klinische Management, i.S. einer Umstellung der Therapie, zu verbessern.
Der Vergleich von Sequenzanalysen der Gesamt-DNA Extrakte zeigte eine signifikant geringere Effizienz als die der Kulturextrakte ($P < 0{,}01$). Aufgrund der langen Generationszeit von *M. ulcerans* in Kultur und der generell niedrigen Sensitivität dieser Methode insbesondere bei antibiotisch vorbehandelten Patienten, stellen die direkten Gesamt-DNA Extrakte das optimale Probematerial zur zeitnahen, genotypischen Resistenzbestimmung dar. Aus diesem Grunde sollten in zukünftigen Studien die molekularen Methoden durch die Anwendung spezifischerer Primer und verbesserter Extraktionsmethoden optimiert werden, um die Effizienz der direkten DNA Sequenzierung aus Gesamt-DNA Extrakten zu steigern.

4. Summary

Buruli ulcer disease (BUD), caused by *M. ulcerans* involves the skin and subcutaneous adipose tissue and constitutes the third most common mycobacterial infection in immunocompetent hosts worldwide following tuberculosis and leprosy. Major endemic areas of BUD are located focally in West Africa. The disease initially presents as nodule, papule or plaque evolving into a painless ulcer with typically undermined edges at times surrounded by huge edema. Though mortality is low, functional disabilities may occur due to severe scarring and contractures if left untreated. Microscopic detection of acid fast bacilli (AFB), culture, IS*2404* PCR and histopathological analysis of diagnostic samples (swabs, fine needle aspirates, 3 mm punch biopsies and excised tissue samples) constitute laboratory methods for the confirmation of BUD suspects. Basically, initiation of antimycobacterial therapy should be based on the laboratory confirmation of BUD suspects. The WHO recommends a standardized antimycobacterial treatment with Rifampicin (RMP) and Streptomycin (SM) for eight weeks, which was applied in clinical practice since 2006 in most West African countries. Before 2006, patients in Ghana were partially administered irregular treatment schemes concomitant to the predominantly conducted surgical excisions. Irregular or non-standardized application of antibiotics may cause the emergence of resistances that could lead to recurrences and constitute a reason for non-healers. Marsollier et al. reported three *M. ulcerans* strains with induced phenotypic RMP resistance following monotherapy of experimentally infected mice. However, no data on the prevalence of drug resistant clinical *M. ulcerans* strains were reported to date.

The present cumulative dissertation comprises two manuscripts. A pilot study determining the genotypes of *M. ulcerans* isolates of PCR confirmed BUD patients was conducted in Ghana in order to evaluate the potential use – in terms of technical application and benefit for the individual clinical management – of sequence-based approaches in resistance testing of clinical isolates and to obtain baseline data on the prevalence of antibiotic-resistant *M. ulcerans* strains in Ghana.

The first part of this dissertation deals with comparative analyses of different diagnostic laboratory tests in relation to the clinical presentation, type of sample collection and pre-treatment with antibiotics to establish optimal conditions for various clinical scenarios according to the determined sensitivities.

Diagnostic samples (swabs, 3 mm punch biopsies and excised tissue samples) from 384 BUD suspects presenting with non-ulcerative and ulcerative lesions at nine study sites in Ghana were analyzed by microscopy, IS*2404* PCR and culture. Out of this cohort, 268 patients were confirmed by at least one positive laboratory test. The overall sensitivity of PCR (85%) was significantly higher than those determined for microscopy (57%) and culture (51%). Following stratification of data by treatment groups, kind of lesion and type of diagnostic sample, PCR of 3 mm punch biopsies from untreated patients with non-ulcerative lesions and swab samples from untreated patients with ulcerative lesions resulted in the highest diagnostic sensitivities of 94% and 90%, respectively. While the duration of disease did not show a significant influence on any of the diagnostic tests, the sensitivities for PCR and culture decreased significantly under antimycobacterial treatment. For monitoring of treatment response only culture is appropriate as mycobacterial DNA of dead bacilli may persists for a longer period in tissue.

Within the second part of the study, clinical *M. ulcerans* isolates of 162 PCR confirmed BUD patients of the cohort from the first part of the study were subjected to sequencing of the *rpoB*- und *rpsL*-genes to detect mutations associated with resistance to Rifampicin or Streptomycin.

Therefore, the efficiencies of sequence analysis of *rpoB*- und *rpsL*-genes of whole-genome extracts from diagnostic samples (swabs, 3 mm punch biopsies and excised tissue samples) and DNA extracts from positive cultures of the diagnostic samples were compared.
For 99.1% (*rpoB*) und 100% (*rpsL*) of the patients the *M. ulcerans* wildtype was detected. In one isolate (0.9%) a point mutation was detected in Codon Ser416 resulting in an amino acid change. This mutation was also described by Marsollier et al. and phenotypic Rifampicin resistance of the mutated strains was confirmed. The data reveal a low prevalence of mutations associated with drug resistance among clinical isolates from Ghana from 2004-2007.
However, mutations may occur and should be especially assessed in patients who do not respond to antimycobacterial treatment, patients with prolonged and disseminated course of disease and non-healers as well as for recurrences in order to improve the clinical management (i.e. changing the therapeutic strategies) for individual patients.
The comparison of sequence analyses of whole-genome extracts revealed a significantly lower efficiency than that obtained for culture extracts ($P < 0.01$). Due to the long generation time of *M. ulcerans* in culture and the generally low sensitivity of this method especially in antibiotic pre-treated patients, whole-genome extracts constitute the optimal samples for timely, genotypic resistance testing. Therefore, future studies could improve the molecular methods by using more specific primer and using optimized extraction procedures to increase the efficiency of direct DNA sequencing of whole-genome extracts.

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6. Danksagungen

An erster Stelle möchte ich meiner langjährigen akademischen Lehrerin Frau PD Dr. Gisela Bretzel für die Aufnahme in Ihre Forschungsgruppe, die Überlassung des Themas meiner Dissertation, sowie die kontinuierliche, exzellente wissenschaftliche Betreuung, die Förderung meiner weiterführenden Ausbildung und stetige Unterstützung danken.

Herrn Prof. Dr. Löscher danke ich für die Aufnahme in die Abteilung für Infektions- und Tropenmedizin, und insbesondere danke ich für die vielen wissenschaftlichen Anregungen und Diskussionen, die stets dafür sorgen, dass ich meinen Geist anstrenge.

Herrn Prof. Dr. Nothdurft danke ich ganz besonders für ein „immer offenes Ohr", die Förderung meiner klinischen Ausbildung und die vielen nützlichen Ratschläge.

Frau Dr. Ingrid Hanus danke ich für die klinische Einarbeitung in die Tropenmedizin, ihre guten Ratschläge und das ständige Interesse am Fortgang meiner Arbeit.

Bei Frau Dr. Vera Siegmund möchte ich mich für die Einarbeitung in die Thematik und ihrem ständigen Interesse an dem Verlauf meiner Arbeit danken.

Herrn Dr. Karl-Heinz Herbinger danke ich für seine stetige Hilfsbereitschaft und Unterstützung bei allen statistischen Fragestellungen.

Herrn Dr. Jörg Nitschke danke ich für die wichtigen Beiträge „from a surgical point of view".

Frau Kerstin Helfrich, Frau Nana-Yaa Awua-Boateng und Frau Rachel Kaufmann danke ich für ihre tatkräftigen Unterstützungen im Labor.
Ich danke Frau Carolin Mengele für die Einarbeitung im Labor des Tropeninstitutes.
Insbesondere danke ich Frau Erna Fleischmann für ihre unermüdliche Hilfsbereitschaft, ihr fortwährendes Interesse an meiner Arbeit und ihre einzigartige Motivation.

Ich danke Herrn Dr. P Lichtner und Frau J. Golic für die Unterstützung beim Sequenzieren.

Ich danke Herr Dr. P. Stragier und Frau Prof. F. Portaels vom Tropeninstitut Antwerpen für die Kooperation i.R. des Burulico Sequenzierprojektes.

Bei Frau Kristina Huber möchte ich mich für die stetige gegenseitige Unterstützung bedanken.

Ich danke weiter allen Kolleginnen und Kollegen des Tropeninstitutes, die zum Gelingen dieser Arbeit beigetragen haben.

Bei Frau Heinke Bock bedanke ich mich für die orthographische Korrektur dieser Arbeit.

Meinen Eltern, denen diese Arbeit gewidmet ist, danke ich für die gesamte Unterstützung, die ich in allen Bereichen während meines Studiums erfuhr.

7. Originalarbeiten

Comparative Study of the Sensitivity of Different Diagnostic Methods for the Laboratory Diagnosis of Buruli Ulcer Disease

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Background. Several diagnostic laboratory methods are available for case confirmation of Buruli ulcer disease. This study assessed the sensitivity of various diagnostic tests in relation to clinical presentation of the disease, type of diagnostic specimen, and treatment history.

Methods. Swab samples, 3-mm punch biopsy tissue specimens, and surgically excised tissue specimens from 384 individuals with suspected Buruli ulcer disease were obtained at 9 different study sites in Ghana and were evaluated with dry reagent–based polymerase chain reaction (PCR), microscopic examination, culture, and histopathological analysis. The study subjects presented with nonulcerative and ulcerative lesions and were divided into 3 treatment groups: (1) previously untreated patients scheduled for antimycobacterial treatment, (2) patients treated with surgery alone, and (3) patients treated with surgery in combination with previous antimycobacterial treatment.

Results. Of 384 suspected cases of Buruli ulcer disease, 268 were confirmed by at least 1 positive test result. The overall sensitivity of PCR (85%) was significantly higher than that of microscopic examination (57%) and culture (51%). After data were stratified by treatment group, type of lesion, and diagnostic specimen type, analysis revealed that PCR of 3-mm punch biopsy tissue specimens (obtained from previously untreated nonulcerative lesions) and of swab samples (obtained from previously untreated ulcers) had the highest diagnostic sensitivity (94% and 90%, respectively). Although duration of the disease did not significantly influence the sensitivity of any test, previous antimycobacterial treatment was significantly associated with decreased sensitivity of PCR and culture.

Conclusions. Across all subgroups, PCR had the highest sensitivity. PCR assessment of 3-mm punch biopsy tissue specimens proved to be the best diagnostic tool for nonulcerative lesions, and PCR assessment of swab samples was the best diagnostic tool for ulcerative lesions. For monitoring of antimycobacterial treatment success within controlled trials, however, only culture is appropriate.

Buruli ulcer disease (BUD), which is caused by *Mycobacterium ulcerans,* affects the skin and subcutaneous adipose tissue. BUD occurs in >30 countries worldwide, with a focus and an increasing number of cases occurring in West Africa [1–3]. The disease initially presents as a painless nodule, papule, plaque, or edema and evolves into a painless ulcer with characteristically undermined edges. If untreated, scarring and contractures may cause serious functional disabilities [3, 4]. Previously, BUD was treated with wide surgical excision; in 2004, however, antimycobacterial treatment alone or in combination with surgery was introduced [3–8]. Currently available diagnostic laboratory tests include microscopic examination, culture, IS2404 PCR

Received 17 August 2008; accepted 14 December 2008; electronically published 10 March 2009.

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Clinical Infectious Diseases 2009; 48:1055–1064

1058-4838/2009/4808-0005$15.00
DOI: 10.1086/597398

of swab samples and tissue specimens, and histopathological analysis.

According to various studies of the laboratory confirmation of clinically suspected cases of BUD, microscopic examination detects 29%–78% of BUD cases, and culture detects 34%–79%. Antimycobacterial treatment before specimen collection was shown to reduce culture positivity ratios (i.e., the number of positive test results divided by the number of cases of clinically suspected BUD) to <5%. Histopathological analysis confirmed >90% of clinically diagnosed BUD cases and >70% of clinically suspected BUD cases [8–15].

With an analytical specificity of 100% [16–19], a sensitivity of 79%–85% [10,12], and culture positivity ratios of 61%–72% [14, 15], IS2404 PCR is considered to be the most reliable technique for the detection of *M. ulcerans* in human diagnostic samples. Laboratory assessment of nonulcerative lesions is restricted to an analysis of tissue specimens, whereas diagnostic swab samples provide a valuable alternative for assessment of ulcerative lesions. According to recent data from Ghana, microscopic examination and/or PCR of swab samples allowed confirmation of up to 70% of suspected cases of BUD [14, 20].

In 2001, the World Health Organization recommended that 2 positive laboratory test results be obtained to confirm a positive diagnosis [21]. However, laboratory confirmation of suspected cases of BUD by 1 positive test result yields ~20% more confirmed cases than does confirmation by 2 positive test results. Because of the high positive predictive values of IS2404 PCR (100%) and microscopic examination (97%), 1 positive test result is considered to be sufficient for confirmation of a diagnosis of BUD [14, 20]. A positive IS2404 PCR result is also regarded as adequate evidence to commence antimycobacterial treatment [22].

In addition to swab samples, punch biopsy tissue specimens also allow the pretreatment laboratory confirmation of suspected BUD [3, 22]. Data on the diagnostic use of punch biopsy tissue specimens, however, are still scarce. Phillips et al. [23] reported sensitivities of 42% for microscopic examination, 49% for culture, 98% for IS2404 PCR, and 82% for histopathological analysis of 4-mm and 6-mm punch biopsy tissue specimens.

In the context of a research program funded by the European Commission on diagnosis and antimycobacterial treatment of BUD, various types of diagnostic specimens were obtained from patients who presented with different clinical forms of the disease and were grouped into 3 different treatment categories. The aim of this study was to determine the sensitivities of diagnostic laboratory methods for various types of specimens, depending on the type of lesions and prior treatment history.

PATIENTS, MATERIALS, AND METHODS

Study population and inclusion criteria. The study included individuals with clinical suspicion of BUD who had nonulcerative or ulcerative lesions and were seen from January 2006 through February 2008 at 9 different study sites in Ghana. The study subjects belonged to 1 of 3 different treatment groups: (1) the drug treatment group, which included patients who were scheduled for drug treatment, had received no antimycobacterial treatment before specimen collection, had a ⩽6-month duration of disease, had lesions ⩽10 cm in diameter, and were ⩾5 years of age; (2) the surgical treatment group, which included patients who were treated with surgical excision and had received no previous antimycobacterial treatment; and (3) the surgical treatment plus antimycobacterial treatment group, which included patients who were treated with surgical excision and had received at least 7 days of previous antimycobacterial treatment.

Standardized specimen collection. In the majority of cases, the diagnostic specimens were collected during the patients' initial presentation to the hospital. For a limited number of patients, additional follow-up samples were analyzed. Swab samples were taken by circling the entire undermined edges of ulcerative lesions. Three-millimeter punch biopsy tissue specimens and surgically excised tissue specimens with a maximum size of 10×10 mm were taken from the center of nonulcerative lesions or from undermined edges of ulcerative lesions, including necrotic tissue [20, 24].

The following sets of specimens were taken: for those in the drug treatment group with nonulcerative lesions, 3 punch biopsy specimens; for those in the drug treatment group with ulcerative lesions, 2 swab samples and 3 punch biopsy specimens; for those in the surgical treatment group and surgical treatment plus antimycobacterial treatment group with nonulcerative lesions, 3 surgically excised tissue specimens; and for those in the surgical treatment group and surgical treatment plus antimycobacterial treatment group with ulcerative lesions, 2 swab samples and 3 surgically excised tissue specimens obtained during the surgical procedure (figure 1). Standardized specimen collection bags, including containers with transport and storage media and data entry forms (BU01 and laboratory data entry form [3]) were provided to the study sites. PCR specimens were collected in 700 μL of cell lysis solution (Gentra Systems), culture specimens were collected in 5 mL of polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin, and histological specimens were collected in 5 mL of 10% buffered neutral formalin [14, 20].

Diagnostic methods and laboratories. Diagnostic specimens were processed at the Kumasi Centre for Collaborative Research in Tropical Medicine in Kumasi, Ghana, by dry reagent–based IS2404 PCR, microscopic examination, and culture with use of standardized procedures [14, 19–21]. Standard IS2404 PCR was performed and slides were reread for external quality assurance by the Department for Infectious Diseases and Tropical Medicine at the University of Munich (Munich,

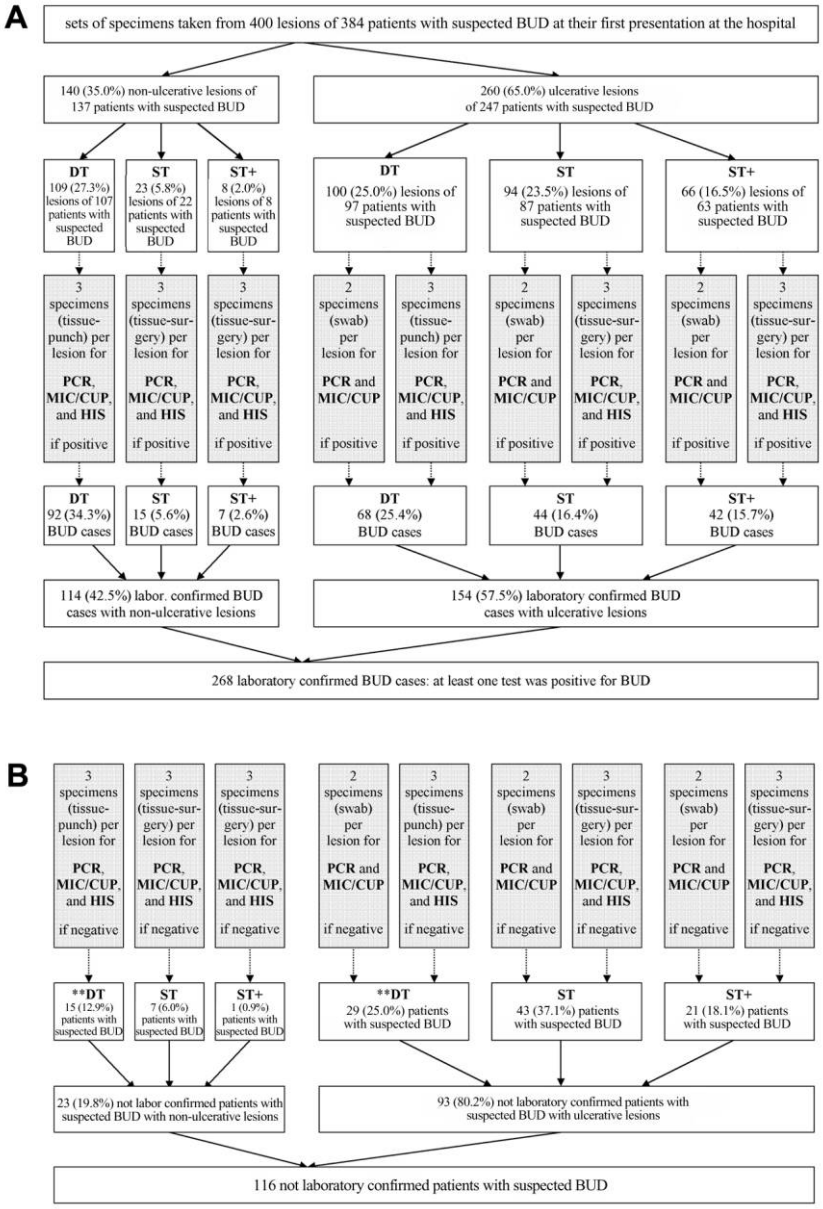

Figure 1. Flow chart of the study procedure. Specimens (swab samples [swab], punch biopsy tissue specimens [tissue-punch], and surgically excised tissue specimens [tissue-surgery]) obtained from 384 patients with suspected Buruli ulcer disease (BUD) from 3 different treatment groups (drug treatment [DT], surgical treatment without previous antimycobacterial treatment [ST], and surgical treatment with previous antimycobacterial treatment [ST+]) for BUD diagnostic testing with dry reagent–based IS2404 PCR (PCR), microscopy (MIC), culture with confirmatory IS2404 dry reagent–based PCR (CUP), and histopathologcial analysis (HIS). There were a total of 268 laboratory-confirmed cases of BUD *(A)* and 116 suspected cases of BUD without laboratory confirmation *(B)*. The statistics are based on the data obtained from each patient's first lesion (if there was >1 lesion per patient) and each patient's first visit at hospital (if there was >1 visit per patient). *Four of 16 patients with suspected BUD who had negative findings at initial presentation (3 in the ST group and 1 in the DT group) had BUD confirmed at follow-up visits. **Twenty-six of 44 patients in the DT group with suspected BUD that was not laboratory confirmed (15 patients with nonulcerative lesions and 29 patients with ulcerative lesions) received antimycobacterial treatment, because the clinical findings were suggestive of BUD. Three additional patients with suspected BUD in the DT group received treatment for onchocerciasis. The remaining 15 patients in the DT group with suspected BUD were lost to follow-up.

Germany), and histopathological examination was performed at the Bernhard Nocht Institute for Tropical Medicine (Hamburg, Germany) and the Department of Pathology at the University of Munich [11, 17, 24].

In brief, DNA was prepared using the Puregene DNA isolation kit, with minor modifications [19]. For dry reagent–based PCR, the oligonucleotides MU5 and MU6 [17] were lyophilized in reaction tubes. PuReTaqTM Ready-To-GoTM PCR Beads (Amersham Biosciences) were added and dissolved in water before adding the template DNA. The standard PCR was performed according to the protocol described by Stinear et al. [17]. Both PCR assays included negative extraction and positive, negative, and inhibition controls.

Culture specimens were decontaminated by the Petroff method, inoculated on Loewenstein-Jensen media, and incubated at 32°C for 6 months. Microscopy smears were prepared from decontaminated material and were stained with the Ziehl-Neelsen technique [22].

Cultures with growth were subjected to Ziehl-Neelsen staining and a confirmatory IS2404 PCR. If a negative PCR result was obtained, sequence analysis of the *rpoB* gene (342 base pairs), *16S–23S*ribosomal RNA (rRNA) internal transcribed spacer gene (273 base pairs), *16S* rRNA gene (924 base pairs), and 65-kDa *HSP* gene (644 base pairs) was performed for strain identification [25–28].

Definition of sensitivity for each individual test. In this study, the sensitivity of a certain test was defined as the number of positive test results divided by the number of patients with at least 1 positive result of any diagnostic test [14, 20].

Statistical analysis. Approximative tests (χ^2 tests), exact tests (Fisher's exact tests), and Student's *t* tests as parametric test were conducted using Stata software, version 9.0 (Stata). Statistically significant differences were defined as *P* values <.05 or as nonoverlapping 95% CIs of proportions. Dependent variables were diagnostic test results. Type of lesion, duration of disease, duration of antimycobacterial treatment (if conducted before specimen collection), and type of diagnostic specimen were independent variables.

Ethical clearance and informed patient consent. Ethical clearance for the study was sought through the Committee of Human Research Publication and Ethics, Kwame Nkrumah University of Science and Technology, and the Komfo Anokye Teaching Hospital, Kumasi, Ghana. Informed patient consent was used for patients who received drug treatment and for patients who underwent surgical procedures.

RESULTS

Subjects. Four hundred sets of specimens from 384 individuals with suspected BUD (drug treatment group, 204 patients; surgical treatment group, 109 patients; and surgical treatment plus antimycobacterial treatment group, 71 patients) who presented with 140 nonulcerative (35%) and 260 ulcerative (65%) lesions were collected at the first presentation at the hospital. Sixteen patients had 2 lesions. A total of 43 sets of follow-up specimens were obtained from 30 study subjects; however, only data from the patients' initial specimen collection were considered for analysis. Forty-eight percent of the suspects were 5–14 years of age (age range, 1–95 years; mean age, 21 years; median age, 14 years); 40.9% of the patients were male (figure 1).

Laboratory-confirmed BUD cases. One hundred seventy-two (44%) of the BUD cases were confirmed by at least 2 positive laboratory test results; 268 (69.8%) were confirmed by at least 1 positive laboratory test result (figure 1). One hundred fourteen (42.5%) of the patients with BUD presented with nonulcerative lesions, and 154 (57.5%) presented with ulcerative lesions; 108 (40.3%) of the patients were male, and 150 (56.6%) of 265 patients (for 3 patients, age was unknown) were 5–14 years of age (range, 2–80 years; mean age, 18 years; median age, 12 years) (figure 2). In 231 (93.1%) of 248 patients, the lesions were located on the limbs or shoulders, with the right side being affected statistically significantly more often (in 138 [59.7%] of 231 patients; $P = .035$) than the left side (93 [40.3%] of 231 patients) (table 1). The lesions of 243 patients with confirmed cases with known lesion sizes were distributed according to World Health Organization categories [3], as follows: category I (a single lesion <5 cm in diameter), 108 patients (44.4%), including 61 in the drug treatment group, 23 in the surgical treatment group, and 24 in the surgical treatment plus antimycobacterial treatment group; category II (a single lesion 5–15 cm in diameter), 127 patients (52.3%), including 93 in the drug treatment group, 15 in the surgical treatment group, and 19 in the surgical treatment plus antimycobacterial treatment group; and category III (a single lesion >15 cm in diameter, multiple lesions, or osteomyelitis), 8 patients (3.3%), including 4 in the drug treatment group, 3 in the surgical treatment group, and 1 in the surgical treatment plus antimycobacterial treatment group.

Of 268 patients with BUD with at least 1 positive laboratory test result, 229 (85.4%) had a positive swab sample and/or tissue specimen with a positive PCR result, and 152 (56.7%) had a positive swab sample and/or a tissue specimen with positive microscopy findings. One hundred forty-nine (98.0%) of the 152 specimens with positive microscopy findings had those findings confirmed by at least 1 of the 3 other tests.

Of the 115 isolates (42.9%) obtained from swab sample and/or tissue specimen cultures with positive results, 108 isolates were confirmed by IS2404 PCR (positive predictive value, 93.9%). Among the remaining 7 isolates, sequence analysis identified 2 *M. ulcerans* strains. Two further strains were identified as *Mycobacterium mucogenicum* and *Mycobacterium phocaicum*, indicating a coinfection or superinfection in 2 indi-

viduals with confirmed BUD. For 3 isolates, sequencing did not provide definitive identification, probably because of contamination with closely related species that colonize the human skin.

Among the follow-up samples, sequencing identified 2 strains as *Mycobacterium gordonae* (cultured from an ulcer swab sample with positive IS2404 PCR results) and *Mycobacterium szulgai* (isolated from an additional lesion at a different location on a patient whose initial lesion had a swab sample with positive IS2404 PCR results).

Histopathological examination confirmed results for 42 of 49 tissue specimens from individuals with otherwise laboratory-confirmed BUD (sensitivity, 85.7%) and for 17 (29.3%) of 58 specimens from individuals with suspected BUD who had negative microscopy findings and negative culture and PCR results (6 [10.3%] of these 58 specimens were obtained from lesions in the healing stages). In 4 (6.9%) of the 58 individuals with suspected BUD, histopathological features did not allow an unambiguous diagnosis, and histological findings in 37 (63.8%) were not suggestive of BUD (8 of these 37 patients received a diagnosis of onchocerciasis). Missing or poor-quality specimens did not allow histopathological analysis for the remaining 75 individuals with suspected BUD.

Of 30 individuals who were followed up over time, 16 received laboratory confirmation of BUD at their first presentation to the hospital. Of the remaining 14 subjects, 4 had BUD confirmed during subsequent follow-up visits. External quality assurance for microscopic examination and PCR showed >90% concordance of results (table 2).

Overall sensitivities of laboratory tests among all laboratory-confirmed BUD cases. The overall sensitivities were 85.4% (229 of 268 cases) for PCR, 56.7% (152 of 268 cases) for microscopic examination, and 48.0% (108 of 225 cases) for confirmatory IS2404 PCR. The sensitivity of PCR was significantly higher than that of microscopic examination and confirmatory IS2404 PCR ($P < .01$), with no statistically significant difference between microscopic examination and confirmatory IS2404 PCR ($P = .054$) (table 3).

Table 1. Location of nonulcerative and ulcerative lesions in 248 cases of laboratory-confirmed Buruli ulcer disease.

	No. (%) of lesions, by general location (n = 248)			
Specific location	Head and trunk	Right shoulder and limbs	Left shoulder and limbs	Total
Head, neck	6	…	…	6 (2.4)
Back	7	…	…	7 (2.8)
Abdomen	0	…	…	0 (0)
Buttock, hip	4	…	…	4 (1.6)
Shoulders	…	7	6	13 (5.2)
Arms				
Overall	…	65	39	104 (41.9)
Upper arm	…	24	17	41 (16.5)
Forearm	…	34	18	52 (21.0)
Wrist	…	3	1	4 (1.6)
Hand, dorsal	…	3	3	6 (2.4)
Hand, volar	…	1	0	1 (0.4)
Legs				
Overall	…	66	48	114 (46.0)
Thigh	…	20	13	33 (13.3)
Knee	…	9	7	16 (6.5)
Lower leg	…	26	18	44 (17.7)
Ankle	…	8	8	16 (6.5)
Foot, dorsal	…	3	2	5 (2.0)
Foot, plantar	…	0	0	0 (0)
Overall	17 (6.9)	138 (55.6)	93 (37.5)	248 (100)

NOTE. For 20 cases, the specific location of the lesions was not known.

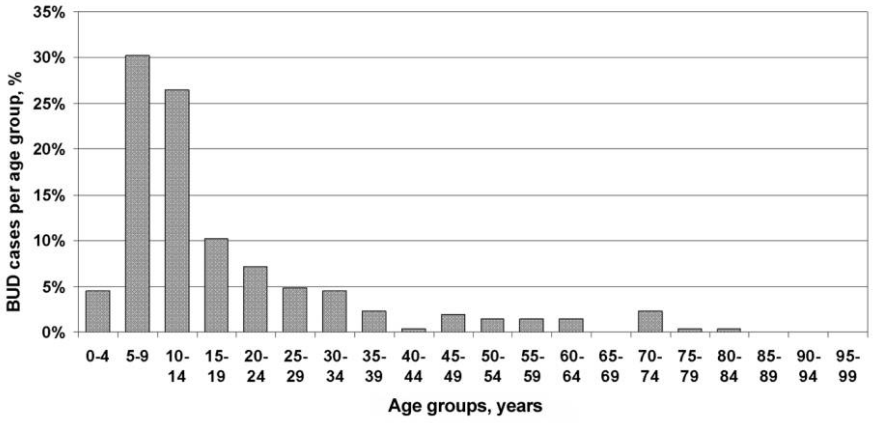

Figure 2. Age distribution of 268 patients with laboratory-confirmed Buruli ulcer disease (BUD). For 3 patients, age was unknown. Age range was 2–80 years, the mean age was 18 years, and the median age was 12 years. For the group of patients who received drug treatment, patients with suspected BUD who were <5 years of age were not included. For the surgical treatment without previous antimycobacterial treatment group and the surgical treatment with previous antimycobacterial treatment group, patients with suspected BUD were included regardless of age.

Table 2. External quality assurance for PCR and microscopic examination.

KCCR result, by method	DITM result			Concordant results	Concordance ratio, %[a]
	Positive	Negative	Total		
PCR[b]					
Positive	37	0[c]	37	37	…
Negative	7[c]	33	40	33	…
All	44	33	77	70	90.9
Microscopic examination					
Positive	56	5[d]	61	56	…
Negative	2[d]	33	35	33	…
All	58	38	96	89	92.7

NOTE. Data are no. of specimens, unless otherwise indicated. DITM, Department of Infectious Diseases and Tropical Medicine (Munich, Germany); KCCR, Kumasi Centre for Collaborative Research in Tropical Medicine (Kumasi, Ghana). Microscopic examination was performed for 96 slides at KCCR, and the slides were reexamined by staff at DITM.

[a] No. of specimens with concordant results divided by the total number of specimens tested with the same test at KCCR and DITM.

[b] PCR was performed with parallel testing of 77 DNA extracts. Dry reagent–based IS2404 PCR was performed at KCCR; standard PCR was performed at DITM.

[c] Dry reagent–based PCR performed at KCCR gave false-positive results for 0 (0%) of the specimens tested and false-negative results for 7 (9.1%) of the specimens tested.

[d] Microscopic examination performed at KCCR gave false-positive results for 5 (5.2%) of the specimens tested and false-negative results for 2 (2.1%) of the specimens tested.

Sensitivities of laboratory tests among confirmed BUD cases involving nonulcerative lesions. Among 114 confirmed nonulcerative BUD cases (drug treatment group, 92 cases; surgical treatment group, 15 cases; surgical treatment plus antimycobacterial treatment group, 7 cases), the sensitivities were 89.5% (102 of 114 specimens) for PCR, 57.0% (65 of 114 specimens) for microscopic examination, and 60.6% (57 of 94 specimens) for confirmatory IS2404 PCR analysis of tissue specimens. PCR was significantly more sensitive than microscopic examination and confirmatory IS2404 PCR ($P < .01$ for each). Stratified into treatment groups and specimens, the sensitivities for analysis of punch biopsy tissue specimens from the drug treatment group were 93.5% for PCR and 70.8% for confirmatory IS2404 PCR, which was statistically significantly higher than that for surgically excised tissue specimens in the surgical treatment group (table 3).

Sensitivities of laboratory tests among patients with confirmed BUD and ulcerative lesions. Among 154 laboratory-confirmed cases of ulcerative BUD (drug treatment group, 68 cases; surgical treatment group, 44 cases; surgical treatment plus antimycobacterial therapy group, 42 cases), the sensitivities were 69.8% (169 of 242 specimens) for PCR, 44.6% (108 of 242 specimens) for microscopic examination, and 25.0% (52 of 208 specimens) for confirmatory IS2404 PCR analysis of swab samples and tissue specimens. PCR was statistically significantly more sensitive than microscopic examination and confirmatory IS2404 PCR ($P < .01$ each). Stratified into treatment groups and specimens, the sensitivities for PCR analysis of swab specimens were 89.9% for the drug treatment group, 73.1% for the surgical treatment group, and 72.2% for the surgical treatment plus antimycobacterial therapy group. In all treatment groups, PCR sensitivity was greater for swab samples than it was for punch biopsy tissue specimens (67.8%) or surgically excised tissue specimens (surgical treatment group, 57.7%; surgical treatment plus antimycobacterial therapy group, 44.4%) (table 3).

Sensitivities of laboratory tests among patients with ulcerative lesions without previous antimycobacterial treatment depending on the duration of disease. According to duration of disease, 101 patients with laboratory-confirmed, previously untreated cases of BUD with ulcerative lesions were divided into 5 groups. In all groups, PCR of swab samples had a statistically significantly higher sensitivity (70%–91%) than did microscopic examination of swab samples (30%–67%) or confirmatory IS2404 PCR of swab samples (29%–59%). Despite a slight downward trend, no statistically significant association between test sensitivity and duration of disease for any test was found (figure 3).

Test sensitivity depending on duration of previous antimycobacterial treatment. In the 49 laboratory-confirmed cases of BUD in the surgical treatment plus antimycobacterial treatment group (7 nonulcerative case and 42 ulcerative cases), the test sensitivity was correlated with the duration of previous antimycobacterial treatment. Regardless of treatment duration, PCR was always the test with the highest sensitivity. The sensitivities of PCR and confirmatory IS2404 PCR were statistically significantly higher in the group of untreated patients than in the group of patients treated for >40 days (for PCR) or >20 days (for confirmatory IS2404 PCR). No statistically significant difference was found for microscopic examination (figure 4).

Table 3. Sensitivity of 3 different methods for the diagnosis of nonulcerative and ulcerative lesions of Buruli ulcer disease (BUD), by method of specimen collection and treatment group.

Type of lesion, treatment group (specimen type)	Sensitivity, % (no. of positive results/no. of specimens tested)			P[a]		
	Dry reagent–based IS2404 PCR	Microscopic examination	CUP	PCR vs. microscopic examination	PCR vs. CUP	Microscopic examination vs. CUP
Nonulcerative						
DT (punch biopsy tissue)	93.5 (86/92)[b]	57.6 (53/92)	70.8 (51/72)	<.01	<.01	.08
ST (surgically excised tissue)	66.7 (10/15)	40.0 (6/15)	40.0 (6/15)	.15	.15	1.00
P[c]	<.01	.21	.02			
ST+ (surgically excised tissue)	85.7 (6/7)[d]	85.7 (6/7)[e]	0 (0/7)	>.99	<.01	<.01
Overall	89.5 (102/114)[b]	57.0 (65/114)	60.6 (57/94)	<.01	<.01	.60
Ulcerative[f]						
DT (swab sample)	89.9 (53/59)[b]	67.8 (40/59)	57.4 (27/47)	<.01	<.01	.27
DT (punch biopsy tissue)	67.8 (40/59)[b]	33.9 (20/59)	23.4 (11/47)	<.01	<.01	.24
P[g]	<.01	<.01	<.01			
ST (swab sample)	73.1 (19/26)[b]	23.1 (6/26)	12.5 (3/24)	<.01	<.01	.34
ST (surgically excised tissue)	57.7 (15/26)[d]	38.5 (10/26)[e]	12.5 (3/24)	.17	<.01	.04
P[g]	.25	.24	>.99			
ST+ (swab sample)	72.2 (26/36)[d]	50.0 (18/36)[e]	3.0 (1/33)	.055	<.01	<.01
ST+ (surgically excised tissue)	44.4 (16/36)[d]	38.9 (14/36)	21.2 (7/33)	.63	.04	.11
P[g]	.02	.35	.03			
DT and ST (swab sample)	84.7 (72/85)[b]	54.1 (46/85)	42.3 (30/71)	<.01	<.01	.14
DT and ST (punch biopsy tissue and surgically excised tissue)	64.7 (55/85)[b]	35.3 (30/85)[e]	19.7 (14/71)	<.01	<.01	.03
P[h]	<.01	.01	<.01			
Overall[i]	69.8 (169/242)[b]	44.6 (108/242)[e]	25.0 (52/208)	<.01	<.01	<.01
All lesions[j]	85.4 (229/268)[b]	56.7 (152/268)	48.0 (108/225)	<.01	<.01	<.054

NOTE. Specimens were obtained for a total of 268 laboratory-confirmed cases of BUD. In 43 cases, however, no results for culture and confirmatory dry reagent–based IS2404 PCR were available. Therefore, the denominator for CUP is 225, not 268. CUP, culture and confirmatory PCR; DT, drug treatment with <7 days of previous antimycobacterial treatment; ST, surgical treatment with <7 days of previous antimycobacterial treatment; ST+, surgical treatment with ⩾7 days of previous antimycobacterial treatment.

[a] For comparison of sensitivity of tests performed on specimens taken from the same lesion.

[b] Sensitivity of PCR was statistically significantly ($P < .05$) higher than that of microscopic examination and CUP.

[c] P value comparing the sensitivity of PCR, microscopic examination, and CUP between punch biopsy tissue specimens and surgically excised tissue specimens obtained from patients with nonulcerative lesions.

[d] Sensitivity of PCR was not statistically significantly ($P > .05$) higher than that of microscopic examination and was statistically significantly ($P < .05$) higher than that of CUP.

[e] Sensitivity of microscopic examination was statistically significantly ($P < .05$) higher than that of CUP.

[f] Data for patients with BUD who had ulcerative lesions were included in the calculation for the swab samples if test results were obtained for both swab samples and tissue specimens taken from the same lesion.

[g] P value comparing the sensitivity of PCR, microscopic examination, and CUP between swab samples and tissue specimens obtained from the same ulcerative lesion.

[h] P value comparing the sensitivity of PCR, microscopic examination, and CUP between punch biopsy tissue specimens and surgically excised tissue specimens obtained from the same ulcerative lesion.

[i] Includes all results, regardless of whether 1 specimens (swab sample or tissue specimen) or 2 specimens (swab sample and tissue specimen) were obtained per lesion.

[j] Includes both nonulcerative and ulcerative lesions. For ulcerative lesions, only 1 result was included; if 2 results from the same ulcerative lesion (from a swab sample and a tissue specimen) were not concordant, then the lesion was considered to be positive if 1 of the 2 tests had a positive result.

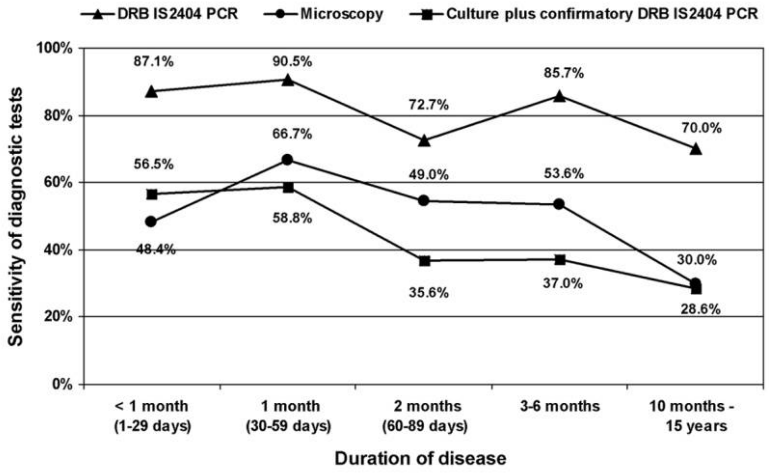

Figure 3. Sensitivity of diagnostic tests (dry reagent–based [DRB] IS2404 PCR, microscopic examination, and culture plus confirmatory DRB IS2404 PCR) by duration of disease among 112 patients with laboratory-confirmed Buruli ulcer disease and ulcerative lesions who had not received previous antibiotic treatment (68 swab specimens from patients in the drug treatment group and 44 swab specimens from patients in the surgical treatment without prior antimycobacterial therapy group). No patient reported a 7–9-month duration of disease.

DISCUSSION

This is, to our knowledge, the largest study to date to evaluate the diagnostic yield of various diagnostic tests after the introduction of antimycobacterial therapy in a West African region in which BUD is highly endemic. Diagnostic tests proved to be reliable within a range of disease durations and across a range of clinical presentations and treatment groups. The results are relevant for case definition for drug trials. Furthermore, locally conducted laboratory confirmation may improve the reliability of epidemiological data.

The majority of recent data on the laboratory confirmation of clinically suspected BUD are from the era when surgical excision was considered to be the standard treatment, when surgically excised tissue specimens accounted for the majority of specimens analyzed. Following the introduction of antimycobacterial treatment, swab samples and punch biopsy tissue specimens, which provide the possibility of pretreatment diagnosis, have become increasingly important. Surgical excision and subsequent skin grafting, however, are still used to treat patients who experience treatment failure and have lesions that do not heal completely after antimycobacterial therapy. In these cases, surgically excised tissue specimens are available for laboratory analysis.

Comparable with previous data [20], laboratory confirmation by 1 positive test result gave 26% more confirmed cases than did confirmation by at least 2 positive test results. As in previous studies, histopathological examination identified an additional ~30% of cases, mainly in patients whose disease was in the paucibacillary stage [14, 19, 20]. However, histopathological features may not provide unambiguous identification, and the availability of the method is limited [11, 14, 19, 20]. Findings obtained with follow-up samples suggest retesting of patients who have typical clinical features but initial laboratory results that are negative.

The overall sensitivities of dry reagent–based PCR (85%), microscopic examination (57%), and confirmatory IS2404 PCR (48%), as determined in our study, are comparable with data published by other groups [8–15]. Independently of treatment group, type of lesion, or diagnostic specimen, the overall sensitivity of PCR was statistically significantly ($P < .01$) higher than that of any other test, whereas there was no statistically significant difference between microscopic examination and confirmatory IS2404 PCR ($P = .054$).

The majority of mycobacterial isolates were confirmed to be *M. ulcerans* by IS2404 PCR or other methods. However, a few study patients, most of whom had received antimycobacterial treatment, harbored other mycobacteria. Because these strains were only isolated from these patients, laboratory contamination is unlikely. In the absence of other confirmatory tests or persistent mycobacterial growth after drug treatment, confirmation by molecular methods is important to identify coinfections or superinfections due to other mycobacteria.

Stratification by lesion type and treatment group gave the following major findings. The sensitivity (93.5%) of PCR performed on 3-mm punch biopsy tissue specimens obtained from previously untreated patients in the drug treatment group who had nonulcerative lesions was significantly higher than the sensitivity of any other diagnostic test. These data are in line with the 98.3% sensitivity of PCR of 4-mm and 6-mm punch biopsy tissue specimens determined by Phillips et al. [23]. Confir-

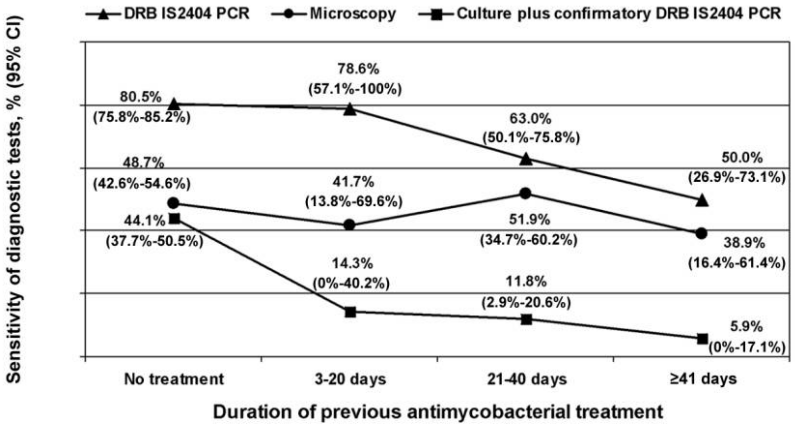

Figure 4. Sensitivity of diagnostic tests (dry reagent–based [DRB] IS2404 PCR, microscopy, and culture plus confirmatory DRB IS2404 PCR) among 49 patients with laboratory-confirmed cases of Buruli ulcer disease who received surgical treatment with previous antimycobacterial treatment (7 with preulcerative and 42 with ulcerative lesions), stratified by duration of previous antimycobacterial treatment (3–20 days, 9 patients; 21–40 days, 30 patients; ⩾40 days, 10 patients). A total of 219 patients received no treatment.

matory IS2404 PCR of punch biopsy tissue specimens also provided an excellent sensitivity of 70.8% among patients with BUD who had nonulcerative lesions. Therefore, 3-mm punch biopsy tissue specimens can be recommended for the pretreatment diagnosis of patients with nonulcerative lesions before initiation of antimycobacterial therapy. However, the small size of punch biopsy tissue specimens hampers histopathological analysis [29].

In previous studies in Ghana, PCR of swab samples confirmed 60%–70% of suspected cases of BUD with ulcerative lesions [14, 20]. The data on the sensitivity of PCR of swab samples obtained in this study also suggest considering PCR analysis of diagnostic swab samples to be the method of choice for cases that involve ulcerative lesions. Especially among the previously untreated patients in the drug treatment group with ulcerative early lesions, PCR of swab samples (sensitivity, 89.9%) proved to be superior to PCR analysis of 3-mm punch biopsy tissue specimens (sensitivity, 67.8%). In the surgical treatment group and the surgical treatment plus antimycobacterial treatment group, PCR of swab samples also had higher sensitivity than did analysis of tissue specimens. In accordance with previous observations, the lower sensitivity for tissue specimens obtained from patients who underwent surgery may be attributable to difficulties in determining the correct location for specimen collection once tissue is excised [14].

Despite a slight downward trend, no statistically significant association between duration of disease and diagnositic sensitivity was detected for swab specimens in this study. However, according to our own experience, in the course of the disease, the edges of ulcers often develop scarring, which can hinder the collection of swab samples. The duration of antimycobacterial treatment influenced the diagnostic sensitivities of PCR and confirmatory IS2404 PCR. Compared with their sensitivity in untreated patients, the sensitivities of both tests were statistically significantly lower after treatment. A statistically significant decrease in culture sensitivity was detected after 20 days of treatment. More than 40 days of treatment were required to produce the same effect for PCR. In pretreated patients, the sensitivity of PCR was still 50%, whereas the sensitivity of confirmatory IS2404 PCR decreased to 6%. In contrast with the rapid decrease in the viability of *M. ulcerans* in the first weeks after onset of treatment, PCR findings suggest extended persistence of *M. ulcerans* DNA in treated lesions.

This study describes the relative sensitivity of currently available diagnostic tests. Data on the specificity and the positive predictive values of these tests cannot be provided, because analysis of diagnostic samples from healthy individuals (including tissue specimens) would have been required to determine the number of false-positive test results. Assessment of positive and negative predictive values requires comparison with a reference test. Because of the limited availability of reference methods (e.g., histopathological examination as a reference test for PCR), the determination of positive and negative predictive values was not feasible for the entire range of tests used in this study.

According to the results of this study, IS2404 PCR was the test with the highest sensitivity overall and in all subgroups of this study cohort; therefore, it is most suitable for the early diagnosis of all clinical forms of BUD. For monitoring of antimycobacterial treatment success within controlled trials, however, only culture seems to be the appropriate tool.

Acknowledgments

Financial support. European Commission (project no. INCO-CT-2005-051476-BURULICO).

Potential conflicts of interest. All authors: no conflicts.

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Am. J. Trop. Med. Hyg., 83(5), 2010, pp. 1059–1065
doi:10.4269/ajtmh.2010.10-0263

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

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

INTRODUCTION

Buruli ulcer disease (BUD), caused by *Mycobacterium ulcerans*, is the third most common mycobacterial infection in humans after tuberculosis and leprosy, and it has been reported from more than 30 countries worldwide, with dominant endemic foci in West Africa. BUD involves the skin and the subcutaneous adipose tissue. The disease starts as a painless nodule, papule, plaque, or edema and evolves into a painless ulcer with characteristically undermined edges. If left untreated, severe disability may occur. Previously, BUD was treated by wide surgical excision, and the World Health Organization (WHO) recommended antimycobacterial treatment of 56 days with streptomycin (SM) and rifampicin (RMP), if necessary, followed by surgical excision in 2004.[1,2]

Most West African countries implemented the standardized antibiotic therapy in 2006. However, non-standardized regimens of RMP and/or SM were also used as concomitant treatment before or after surgical interventions before 2006.[3–5] Although effective and in many aspects advantageous over surgery, introduction of antimycobacterial treatment poses new challenges for the management of BUD.[1]

As is well-known from tuberculosis and leprosy, antimycobacterial treatment is prone to the development of drug resistance. Risk factors hereby encompass a lack of patients' compliance as well as irregular and inadequate treatment regimens in terms of duration, dosage, and drug combination. The WHO estimates the current global level of drug resistance of *M. tuberculosis* complex (MTBC) to RMP and SM, as determined by the proportion method, to be 6.3% and 12.6%, respectively.[6] Genotypic drug-resistance testing constitutes a reliable and expedient alternative to predict phenotypic drug resistance of *M. tuberculosis* to RMP and SM.[7,8] In 2007, WHO also emphasized the importance of systematic global drug resistance surveillance for leprosy and implemented a genotypic drug-resistance surveillance system in 2009. So far, high concordance between conventional drug-susceptibility testing (mouse footpad technique) and DNA sequencing methods has been observed. In a large-scale study on sequence-based detection of drug resistance in human leprosy, rifampicin-resistant *M. leprae* strains were detected among 2% of new cases and 8% of relapses in Southeast Asia.[9–11]

The majority of *M. tuberculosis* strains expressing phenotypic resistance to rifampicin shows mutations within the highly conserved 81-bp RMP resistance-determining region (RRDR) of the *rpoB* gene comprising codons 507–533, with codons Ser531 and His526 being involved most frequently.[12] For *M. leprae*, *rpoB* gene mutations leading to phenotypic rifampicin resistance were likewise detected within the RRDR comprising codons 401–427 (equivalent to codons 507–533 in *M. tuberculosis*), predominantly affecting codon Ser425 (equivalent to Ser531 in *M. tuberculosis*).[13] Resistance of *M. tuberculosis* to SM was most frequently associated with a single mutation in codon 43 of the *rpsL* gene and less commonly with mutations of the *rrs* gene.[14,15]

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

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

MATERIALS AND METHODS

Ethics statement. The study was approved by the Committee of Human Research Publication and Ethics,

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School of Medical Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana (CHRPE/07/01/05). For all patients who were enrolled in a drug trial, written informed consent was obtained from the study participants and/or their legal representatives if aged under 18 years. For patients who underwent routine surgical treatment (with or without concomitant drug therapy), written consent or in the case of the participants who were illiterate, verbal consent, according to the requirements of the ethics committee, for all treatment-related procedures including the collection of diagnostic specimens was obtained in the treatment centers and documented in the medical records of the respective patients.

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

Between 2004 and 2007, 80 patients (49.4%) were treated by surgical excision with or without concomitant antimycobacterial treatment. At the time of collection of clinical samples, 29 of those patients (36.3%) had not received any antimycobacterial drugs (ST). Thirty-two patients (40.0%) had received either a combination of RMP and SM ($N = 30$) or RMP alone ($N = 2$; ST+). Fourteen patients were treated for ≤ 7 days (short-term; ST+S), and two of those patients received RMP only. Twelve patients received 8–28 days of drug treatment (intermediate; ST+IN), and six were treated for 29–42 days (long term; ST+L). Nineteen patients (23.8%) had received antimycobacterial treatment; however, detailed information on the drugs applied and duration of treatment was not available (ST-NK). The mean duration of treatment before surgical excision and collection of clinical samples was 13.1 days (Table 1).

Between 2005 and 2007, according to the clinical records, 82 patients (50.6%) were treated with antimycobacterial drugs alone (DT). Of these, 44 patients were enrolled in a randomized controlled drug trial.[17] By the time of collection of clinical samples, 81 (98.8%) of these patients had not received antibiotics; one recurrent case, however, had received a full course of RMP and SM before the trial (Table 1).

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

TABLE 1
Sequencing results of 162 BUD patients in different treatment groups

					Results per patient‖					
					rpoB gene results**			*rpsL* gene results††		
Treatment group*	Patients†	Culture extracts‡	Swab extracts§	Tissue extracts¶	WT‡‡	MUT§§	NR¶¶	WT	MUT	NR
DT	82									
		50	37	40	82			82		
					56 (68.3)	0	26 (31.7)	53 (64.6)	0	29 (35.4)
ST	29									
		14	14	22	29			7		
					19 (65.5)	0	10 (34.5)	3 (42.9)	0	4 (57.1)
ST+S	14									
		1	9	8	14			13		
					7 (50.0)	0	7 (50.0)	5 (38.5)	0	8 (61.5)
ST+IN	12									
		1	4	10	12			11		
					4 (33.3)	0	8 (66.7)	4 (36.4)	0	7 (63.6)
ST+L	6									
		5	2	5	6			6		
					5 (83.3)	0	1 (16.7)	5 (83.3)	0	1 (16.7)
ST+NK	19									
		16	2	3	19			6		
					17 (89.5)	1 (5.3)	1 (5.3)	4 (66.7)	0	2 (33.3)
Total‖‖	162	87	68	88	162 (100%)			125 (100%)		
			Definite results		109 (67.3%)			74 (59.2%)		
					108 (99.1%)	1 (0.9%)		74 (59.2%)	0	
			No results				53 (32.7%)			51 (40.8%)

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

* Patients were divided into different treatment groups as follows. DT = drug treatment [patients received a full course of rifampicin (RMP) and streptomycin (SM) for 56 days after specimen collection; one recurrent case, however, had received a full term of antimycobacterial treatment at the time of specimen collection]; ST = surgical treatment (patients were treated by surgical excision only); ST+S = surgical treatment plus short-term antimycobacterial treatment before specimen collection with RMP and SM for ≤ 7 days (two patients received RMP only); ST+IN = surgical treatment plus intermediate-term antimycobacterial treatment before specimen collection with RMP and SM for 8–28 days; ST+L = surgical treatment plus long-term antimycobacterial treatment before specimen collection with RMP and SM for > 28 days; ST+NK = patients were surgically treated and had received antibiotics before specimen collection, but exact treatment data was missing in clinical records.

† Number of patients per treatment group.

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

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

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

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

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

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

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

§§ MUT = mutation. One isolate showed a point MUT at Ser522 of the *rpoB* gene. Species identification by sequence analysis of genes for *16S rRNA*, *rpsL*, *hsp65*, and the *ITS* allowed the distinct allocation to *M. ulcerans* strain Agy99 (accession number CP000325) by 100% nucleotide concordance.

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

‖‖ From 24 patients, a swab and a tissue extract were sequenced; additionally, 12 culture extracts were analyzed from these patients. For 45 patients, a swab or tissue extract was available with a corresponding culture extract inoculated from the respective clinical specimen.

RMP and SM for 21 days. Analysis of these specimens was done separately and is not provided in the attached tables.

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

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

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

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

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

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

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

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

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

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

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

Statistical analysis. For all statistical analyses, approximate tests (χ^2 tests) and exact tests (Fisher's exact tests) were conducted using Stata software (version 9.0; Stata Corp., College Station, TX). The results of statistical analyses were presented by means of *P* values, whereby significant differences were defined as *P* values below 0.05. *P* values did not serve only for hypothesis testing but as base for discussion. The study was cross-sectional, and no specific selection or randomization of study participants was performed.

RESULTS

Laboratory confirmation. From all 162 patients (100%), the *IS2404* DRB-PCR result was positive. Of those, positive culture results (confirmed by standard *IS2404* PCR) were obtained from 87 patients (53; 7%).

***RpoB* sequencing results per patient.** Definite sequencing results of the *rpoB* gene (obtained from culture isolates and/or whole-genome extracts from swab and/or tissue samples) were retrieved from 109 of 162 laboratory-confirmed cases (67.3%). The *rpoB* WT sequence of *M. ulcerans* was detected in 108 patients (99.1%; Table 1). The isolate of one patient (0.9%; treatment group S+NK) showed a mutation at codon Ser522

TABLE 2
Comparison of *rpoB* and *rpsL* gene-sequencing results of whole-genome extracts and culture extracts

Sequenced genes	Culture extracts*			Whole-genome extracts†			*P* value‡
	Swab extracts	Tissue extracts	Total	Swab extracts	Tissue extracts	Total	
rpoB§							
Total analyzed	39	57	96	64	92	156	
Definite sequences¶	35 (89.7%)	53 (93.0%)	88 (91.7%)	25 (39.1%)	30 (32.6%)	55 (35.3%)	< 0.01
Non-analyzable‖	4 (10.3%)	4 (7.0%)	8 (8.3%)	39 (60.9%)	62 (67.4%)	101 (64.7%)	
Negative**	3 (75%)	3 (75%)	6 (75%)	23 (59%)	41 (66.1%)	64 (63.4%)	
Mixed††	1 (25%)	1 (25%)	2 (25%)	16 (41%)	21 (33.9%)	37 (36.6%)	
rpsL‡‡							
Total analyzed	26	41	67	50	69	119	
Definite sequences¶	23 (88.5%)	33 (80.5%)	56 (83.6%)	16 (32.0%)	27 (39.1%)	43 (36.1%)	< 0.01
Non-analyzable‖	3 (11.5%)	8 (19.5%)	11 (16.4%)	34 (68.0%)	42 (60.9%)	76 (63.9%)	
Negative**	2 (66.7%)	7 (87.5%)	9 (81.8%)	21 (61.8%)	31 (73.8%)	52 (68.4%)	
Mixed††	1 (33.3%)	1 (12.5%)	2 (18.2%)	13 (38.2%)	11 (26.2%)	24 (31.6%)	

Table 2 shows *rpoB*- and *rpsL*-sequencing results from whole-genome extracts and culture extracts of 162 *IS2404* PCR-confirmed BUD patients.
* DNA was extracted from mycobacterial cultures that were inoculated from swab or tissue specimens.
† DNA was extracted from tissue or swab specimens as whole-genome extracts.
‡ *P* value is the comparison of efficiencies of definite sequences from the total of whole-genome extracts and culture extracts.
§ *rpoB* is a 324-bp region of the mycobacterial *rpoB* gene comprising the rifampicin resistance-determining region (RRDR).
¶ Number of extracts with definite sequences.
‖ Number of extracts with non-analyzable sequences (the result is a mixed or non-amplifiable sequence in PCR).
** Number of extracts with non-amplifiable sequences (negative).
†† Mixed sequence is the number of extracts that could not be analyzed, because amplified sequences were contaminated.
‡‡ *RpsL* is the complete sequence of the gene encoding the ribosomal protein S12 (375 bp).

(*M. tuberculosis* numbering) within the RRDR, leading to a transversion of thymine to guanine (TCC to GCC) and resulting in an amino acid change from serine to alanine. Species identification allowed the distinct allocation of the strain to *M. ulcerans* by 100% nucleotide concordance with the *16S rRNA*, *rpsL*, *hsp65* genes and the *ITS* of *M. ulcerans* strain Agy99.

For 53 patients (32.7%), no result could be obtained (NR). Among these, five strains showed highly deviated *rpoB* sequences (> 3% aberrance) from the *M. ulcerans* WT. Species identification, however, did not allow the allocation to a distinct mycobacterial species; therefore, a contamination with closely related species could not be excluded for these cases.

TABLE 3
Comparison of corresponding whole-genome extracts and culture extracts

Sequenced genes	Corresponding specimens*		*P* value§
	Culture extracts†	Whole-genome extracts‡	
rpoB¶			
Total analyzed	45	45	
Definite sequences‖	44 (97.8%)	19 (42.2%)	< 0.01
Non-analyzable**	1 (2.2%)	26 (57.8%)	
Negative§	0	12 (46.2%)	
Mixed††	1 (100%)	14 (53.8%)	
rpsL‡‡			
Total analyzed	45	45	
Definite sequences‖	41 (91.1%)	16 (35.6%)	< 0.01
Non-analyzable**	4 (8.9%)	29 (64.4%)	
Negative§	4 (100%)	19 (65.5%)	
Mixed††	0	10 (34.5%)	

Table 3 shows *rpoB*- and *rpsL*-sequencing results of corresponding whole-genome and culture extracts.
* From 45 patients, culture extracts (inoculated from swab or tissue specimens) were analyzed, and results are compared with the corresponding whole-genome extract.
† DNA was extracted from cultured strains that were inoculated from swab or tissue specimens (for ulcerative lesions) or solely from tissue specimens of non-ulcerative lesions.
‡ DNA was extracted from tissue or swab specimens as whole-genome extracts from ulcerative lesions and solely from tissue specimens of non-ulcerative lesions. Per patient, either a swab or a tissue specimen was analyzed corresponding to the specimen of which a mycobacterial culture was inoculated and a positive confirmatory *IS2404* PCR result was obtained.
¶ *rpoB* is a 324-bp sequence of the mycobacterial *rpoB* gene comprising the rifampicin resistance-determining region (RRDR).
‖ Number of extracts with definite sequences.
** Number of extracts with non-analyzable sequences (the result is a mixed or non-amplifiable sequence in PCR).
§ Number of extracts with non-amplifiable sequences (negative).
†† Mixed sequence is the number of extracts that could not be analyzed, because amplified sequences were contaminated.
‡‡ *RpsL* is the sequence of the gene for ribosomal protein S12 (complete sequence = 375 bp).

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

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

Among the whole-genome extracts, 64.7% of the *rpoB* sequences (63.4% negative and 36.6% mixed) and 63.9% of the *rpsL* sequences (68.4% negative and 31.6% mixed) were non-analyzable. Among the culture extracts, 8.3% of the *rpoB* sequences (75% negative and 25% mixed) and 16.4% of the *rpsL* sequences (81.8% negative and 18.2% mixed) were non-analyzable (Table 2).

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

Follow-up patients. Among follow-up samples of five patients who had received full-term antibiotic treatment, WT *rpoB* and *rpsL* sequences were detected in isolates from three patients, whereas sequences from two patients' isolates were not amplifiable; thus, no results were retrieved (NR). For two patients who had received 21 days of antimycobacterial treatment, *rpoB* and *rpsL* WT sequences were detected for

one patient and could not be amplified from specimens of the second patient (NR).

DISCUSSION

Conventional *in vitro* resistance testing according to the proportion method constitutes the most widespread technique within the WHO global surveillance system of antimycobacterial drug resistance of tuberculosis.[6] Because of the inability to cultivate *M. leprae*, sequence-based detection of drug resistance has successfully been applied for leprosy.[10] According to WHO recommendations, *M. leprae* isolates with an amino acid change in a drug resistance-determining region that have been confirmed by mouse footpad testing to confer phenotypic drug resistance are scored as resistant.[11] Sequence-based resistance testing was also proven to be a rapid and reliable alternative to conventional resistance testing of *M. tuberculosis*.[7,8]

M. ulcerans strains expressing phenotypic and genotypic resistance were generated under RMP monotherapy in a mouse model, but systematic drug resistance surveillance in clinical *M. ulcerans* strains has not yet been conducted.[16]

To obtain baseline data on resistance to RMP and SM in Ghana, we screened clinical *M. ulcerans* isolates obtained from BUD patients treated by surgery and/or antimycobacterial drugs. At the time of the study, conventional susceptibility testing for *M. ulcerans* was not established in Ghana; therefore, we applied a sequence-based approach for the detection of mutations associated with drug resistance.

Our findings showed no mutations among patients without previous antimycobacterial treatment. One strain isolated from a patient treated by surgery and concomitant antibiotic therapy as early as 2004 (information on drug combination, dosage, and duration of treatment could not be retrieved from the files) expressed a mutation at codon Ser522 of the *rpoB* gene. The respective mutation was also described by Marsollier et al.[16] after RMP monotherapy of experimentally infected mice, and phenotypic resistance was confirmed.[16] These findings suggest that antimycobacterial treatment, especially if administered as monotherapy or in an irregular, nonstandardized fashion, may also lead to *rpoB* mutations of human *M. ulcerans* isolates. To establish phenotypic correlates of the mutation detected in the respective strain, conventional susceptibility testing was attempted. However, we did not succeed in obtaining subcultures from the original isolate. Mutations of the *rpsL* gene were not detected. This may be related to the fact that streptomycin is applied intramuscularly and has presumably not been administered in monotherapy.

According to current WHO recommendations, all new BUD cases are subjected to drug treatment. Patients who develop a new BUD lesion after complete healing of the initial lesion (recurrences) and BUD patients who missed a total of 14 days since the start of treatment (defaulters) may receive a second course of antimycobacterial therapy with regard of the SM lifetime dose (90 g in adults).[27] Since the introduction of antimycobacterial treatment in 2006, more than 3,000 BUD cases have been reported in Ghana, and presumably, the majority of these cases have been subjected to drug treatment (Asiedu K, personal communication). Whereas human-to-human transmission plays a crucial role for the spread of resistant MTBC and *M. leprae* strains, according to current knowledge, *M. ulcerans* is acquired from the environment.[1,28–30] With only a few reported cases of infections contracted from humans, the risk of transmitting resistant strains among populations afflicted with BUD may be considered minimal.[31,32]

The rate of drug resistance detected in our study among clinical *M. ulcerans* isolates obtained between 2004 and 2007 was low (0.9%). Nevertheless, the emergence of drug-resistant strains is possible and will, in the first place, affect the treatment outcome of individual patients under antimycobacterial treatment.[16,33,34] Therefore, monitoring of drug resistance will facilitate individual clinical management decisions, especially in recurrences, defaulters, and patients with non-healing lesions.

With respect to the long generation time of *M. ulcerans* and the limited sensitivity of the method, especially in pre-treated patients, cultures alone are not ideal for genotypic drug resistance testing.[5] Especially for clinical management decisions, whole-genome extracts constitute a better diagnostic target to obtain rapid results. In our study, analysis of cultured isolates yielded definite sequencing results for *rpoB* and *rpsL* genes in > 80%, whereas the number of definite results obtained from whole-genome extracts was significantly lower (< 40% for *rpoB* and *rpsL* genes). The percentage of non-analyzable sequences among whole-genome extracts was > 60%. Among these, the respective sequences of 63.4% of the *rpoB* and 68.4% of the *rpsL* genes were non-amplifiable. In general, the presence of only a low amount of mycobacterial DNA in clinical samples often hampers diagnostic procedures and may also constitute a source of error in the present study.[35] Amplification of mixed sequences from related species existing as commensals on the human skin represents another challenge in sequencing of whole-genome extracts.

According to the results of this study, the authors consider it essential to apply refined techniques in further studies. The design of more specific primers can improve the efficiency of sequencing of whole-genome extracts. Optimization of extraction procedures (e.g., mechanical homogenization by zirconium beads followed by enzymatic lysis) can augment the yield of *M. ulcerans* DNA recovered from swab and tissue samples.[36] Immunomagnetic separation, developed and successfully applied in environmental studies by Marsollier and others,[37] may be a promising tool to concentrate and purify *M. ulcerans* from clinical samples; however, this technique is currently restricted to BUD reference laboratories or collaborative research programs.

From 12% of the study subjects, documentation of previous antimycobacterial treatment could not be retrieved from the clinical records. These partially incomplete sets of data, therefore, constitute a weakness of this study. With respect to correct interpretation of results, further studies on drug resistance in *M. ulcerans* should aim at obtaining complete and detailed patient-related information. WHO provides a form (BU01) for the recording of clinical, epidemiological, and treatment data, which, in general, is available in all endemic countries. A consequent use of the BU01 form will facilitate the collection of relevant information.[1]

Received May 9, 2010. Accepted for publication July 30, 2010.

Acknowledgments: Primer sequences (rpsL-F and rpsL-R) were kindly provided by F. Portaels and P. Stragier (Institute for Tropical Medicine, Antwerp, Belgium). The authors thank David Schenavsky for proofreading of the manuscript. The article contains parts of the doctoral dissertation of Marcus Beissner. There is no conflict of interest among authors.

Financial support: This project was supported by the European Commission (Project INCO-CT-2005-015476-BURULICO).

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