Aus der Abteilung für Infektions- und Tropenmedizin

Leiter: Prof. Dr. med. Thomas Löscher

Medizinische Poliklinik Innenstadt

Kommisarischer Leiter: Professor Dr. med. Martin Reincke

Klinikum der Universität München

Untersuchungen zu Diagnostik und Therapie des Buruli Ulkus mittels tropenadaptierter Labormethoden in endemischen Regionen: Etablierung eines diagnostischen Netzwerkes in Ghana

vorgelegt von

Dr. med. Gisela Bretzel

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Für meine Eltern

Inhaltsverzeichnis

Abkürzungsverzeichnis

Abb.	Abbildung
AITM	Abteilung für Infektions- und Tropenmedizin
BNITM	Bernhard Nocht Institut für Tropenmedizin
bp	Base pairs (Basenpaare)
BUD	Buruli Ulcer Disease
cm	Zentimeter
d	diem (Tag)
DAHW	Deutsche Lepra- und Tuberkulosehilfe e.V.
DNA	Desoxyribonucelic acid (Desoxyribonukleinsäure)
DRB-PCR	Dry reagent based PCR (Trockenreagenz-PCR)
DT	Drug Treatment (antimykobakterielle Behandlung)
E. coli	Escherichia coli
EQA	External Quality Assurance (externe Qualitätssicherung)
FNA	Feinnadelaspirat
g	Gramm
GBUI	Global Buruli Ulcer Initiative
HIV	Human immunodeficiency virus (Hi-Virus)
hsp	Heat Shock Protein (Hitzeschock Protein)
IS	Insertionssequenz
ITS	Internal transcribed spacer
kb	Kilobasen
KCCR	Kumasi Centre for Collaborative Research in Tropical Medicine
kg	Kilogramm
M.	Mycobacterium
Mb	Megabasen
mg	Milligramm
mm	Millimeter
PCR	Polymerase chain reaction (Polymerase-Kettenreaktion)
RNA	Ribonucleic acid (Ribonukleinsäure)
ROM	Reduced range of motion (Bewegungseinschränkung)
rpoB	RNA Polymerase Beta Untereinheit
rRNA	Ribosomale RNA
S	Svedberg
SOP	Standard Operating Procedure (Standardarbeitsanweisung)
SPR	Slide positivity rate (Positivitätsrate mikroskopischer Präparate)
ST	Surgical Treatment (chirurgische Behandlung)
ST+	Surgical Treatment plus antimykobakterielle Therapie
TAG	Technical Advisory Group on Buruli Ulcer
u.a.	Unter anderem
VNTR	Variable number tandem repeats
WHA	World Health Assembly
WHO	World Health Organisation (Weltgesundheitsorganisation)
z.B.	Zum Beispiel

Einleitung

Historischer Überblick

Die Erstbeschreibung ausgedehnter, zur klinischen Diagnose eines Buruli Ulkus („Buruli Ulcer Disease“, BUD) passender Hautulzera stammt von dem britischen, in Uganda tätigen Arzt Sir Albert Cook aus dem Jahr 1897. Peter MacCallum und Kollegen beschrieben im Jahr 1948 sechs weitere Fälle aus der Region um Bairnsdale nahe Melbourne, Australien. Ihnen gelang die Erstisolierung des Erregers, *Mycobacterium ulcerans*. Aufgrund der Herkunft der ersten australischen Patienten ist die Erkrankung im südlichen Australien bis heute auch unter dem Namen „Bairnsdale ulcer“ bekannt. In den sechziger Jahren des 20. Jahrhunderts wurde ein gehäuftes Auftreten des Buruli Ulkus im Buruli County in Uganda (heute: Nakasongola District) beobachtet. Die derzeit meist gebrauchte Bezeichnung „Buruli Ulkus“ hat hier ihren Ursprung. Zu Beginn der achtziger Jahre des letzten Jahrhunderts wurden zunehmend neue Fälle aus weiteren Ländern, mit Schwerpunkt in West Afrika beschrieben. Die Weltgesundheitsorganisation (WHO) gründete als Reaktion auf die stetig steigenden Fallzahlen 1998 die Global Buruli Ulcer Initiative (GBUI). Die World Health Assembly (WHA) verabschiedete schließlich im Jahr 2004 eine Resolution mit dem Ziel der Verbesserung von Überwachung und Kontrollmaßnahmen, sowie einer Intensivierung der Forschung (50, 51)

Erreger

Mycobacterium ulcerans, ein grampositives, säurefestes, nicht-chromogenes, langsam wachsendes Stäbchen (Gruppe III der Runyon Klassifizierung) aus der Familie der *Mycobacteriaceae*, Gattung *Mycobacterium*, zählt zu den atypischen Mykobakterien (MOTT: mycobacteria other than tuberculosis, synonym NTM: non tuberculous mycobacteria). *M. ulcerans* kann bei Temperaturen zwischen 29 und 33 °C auf Löwenstein-Jensen Nährböden kultiviert werden (47). Das 5.8 Mb große *M. ulcerans* Genom, bestehend aus zwei zirkulären Replikons, einem 5632 kb Chromosom und einem Virulenzplasmid, weist eine Sequenzhomologie von über 98% mit *Mycobacterium marinum* auf. Eine Abspaltung von *M. marinum* durch lateralen Gentransfer ist daher anzunehmen. Phylogenetischen Analysen zufolge entstanden im weiteren evolutionsbiolgischen Verlauf zwei Abstammungslinien. Die „ancestral lineage“ (Isolate aus Asien [China, Japan], Südamerika, Mexiko) weist einen hohen genetischen Übereinstimmungsgrad mit *M. marinum* auf, die phylogenetische Evolution der „classical lineage“ (Isolate aus Afrika, Australien, Süd-Ost Asien) dagegen führte zu

umfangreicher Reorganisation und Reduktion des Genoms und ermöglichte *M. ulcerans* eine Anpassung an neue ökologische Nischen (23, 41). Mittels molekularbiologischer Typisierungsmethoden, beispielsweise der Analyse von „variable number tandem repeats“ (VNTR), können gegenwärtig 11 *M. ulcerans* Genotypen unterschieden werden, die sich gemäß der geographischen Herkunft der untersuchten Isolate in derzeit vier Cluster aufteilen lassen (Asien, Süd-Ost Asien, Westafrika, Ostafrika) (42).

Das von *M. ulcerans* produzierte, plasmidkodierte, in unterschiedlichen strukturellen Varianten (Mykolakton A/B, C, D, E, F) nachgewiesene Exotoxin Mykolakton führt zu massiven Gewebszerstörungen. Neben seiner direkten toxischen Aktivität zeigt es eine immunmodulatorische Wirkung im Sinne einer Suppression der primären T-Zellantwort und der Rekrutierung von Entzündungszellen. Dies erklärt die Abwesenheit von Schmerzen und Entzündung trotz ausgedehnter Läsionen (8).

Epidemiologie

Das Buruli Ulkus ist gegenwärtig die dritt-, in einigen westafrikanischen Ländern bereits die zweithäufigste mykobakterielle Erkrankung und ist in über 30 Ländern (Afrika, Asien, Süd- und Mittelamerika, West-Pazifik) endemisch. Aufgrund des Mangels an Laborkapazität zur Bestätigung klinisch diagnostizierter Verdachtsfälle in Endemiegebieten liegen allerdings keine präzisen globalen Inzidenz- und Prävalenzdaten vor. In Hochendemiegebieten, wie z.B. dem Amansie West District, Ghana, betrug die Prävalenz im Jahre 1999 150.8 pro 100.000 Einwohner (2). Zwischen 2002 und 2006 wurden nach Angaben der „Technical Advisory Group on Buruli Ulcer“ (TAG) der WHO 25.465 Fälle aus 16 Ländern gemeldet. Diese Angaben beziehen sich jedoch nur auf die während des „WHO annual meeting on Buruli ulcer“ vorgestellten Fallzahlen aus ausgewählten Ländern und geben somit nicht die wahre globale Prävalenz wieder. Das Buruli Ulkus tritt hauptsächlich in abgelegenen ländlichen Gegenden auf, deren Bevölkerung unter Armutsbedingungen lebt. Hauptsächlich betroffen sind Kinder unter 15 Jahre. Epidemiologische Studien weisen auf eine Assoziation der Erkrankung mit langsam fließenden Gewässern, Teichen, Seen, und Sümpfen hin (38, 50, 51, 54). Ob eine HIV Infektion als Risikofaktor für eine Erkrankung zu werten ist, ist derzeit noch unklar. Es wurden allerdings bei HIV Patienten gravierende Verläufe mit multiplen Läsionen beobachtet (20, 45, 46).

Transmission

M. ulcerans DNA konnte mittels Polymerase-Ketten-Reaktion (PCR) in Wasser und Bodenproben von Gewässern, Wasserpflanzen, Wasserorganismen (Schnecken, Fische), in den Speicheldrüsen von Wasserinsekten der Gattung *Naucoridae* und *Belostomatidae* (Ordnung Hemiptera) sowie in „salt marsh mosquitoes“ (Gattung *Aedes* u.a.) in Süd-Ost-Australien nachgewiesen werden. Die erstmalige Kultivierung des Erregers gelang aus Wasserinsekten der Ordnung Hemiptera aus einer endemischen Region in Benin. Der genaue Transmissionsweg ist jedoch bislang ungeklärt. Es gibt derzeit keine Hinweise für eine Übertragung von Mensch zu Mensch (18, 32).

Klinisches Bild und Differentialdiagnose

Das Buruli Ulkus ist eine meist im Bereich der Extremitäten auftretende Erkrankung der Haut und des subkutanen Fettgewebes. Typisch ist die Schmerzlosigkeit auch ausgedehnter Läsionen. Ein Übergreifen der Infektion auf Knochen (Osteomyelitis) ist möglich. Ein Teil der betroffenen Patienten berichtet über vorausgehende Traumen am Entstehungsort der Erkrankung. Das klinische Bild der Erkrankung umfaßt nicht-ulzerative und ulzerative Formen. Das nicht-ulzerative Stadium manifestiert sich als schmerzlose noduläre oder papuläre Läsion, Plaque, und/oder Ödem (Abb. 1a-c). Der Übergang dieser Formen in ebenfalls schmerzlose, oft großflächige Ulzerationen mit charakteristisch weit unterminierten Rändern mit oder ohne begleitendes Ödem kann sich innerhalb von Tagen vollziehen (Abb. 1d). Die Läsionen werden derzeit in drei Kategorien eingeteilt: Kategorie I: einzelne Läsion, Durchmesser <5cm; Kategorie II: einzelne Läsion, Durchmesser 5-15 cm; Kategorie III: einzelne Läsion, Durchmesser >15 cm, sowie multiple Läsionen, Läsionen an kritischer Lokalisation, und Osteomyelitis (50). Unbehandelt sistiert die Erkrankung meist im Laufe von Monaten. Selbstheilungsprozesse können zu Narbenbildung und umfangreichen Kontrakturen mit Funktionseinschränkungen der betroffenen Gelenke führen (Abb. 1e). Die im Hinblick auf therapeutische Entscheidungen bedeutsame Differentialdiagnose umfasst ein breites Spektrum von infektiösen und nicht-infektiösen Erkrankungen. Noduläre Formen müssen beispielsweise von Onchozerkomen (Abb. 1f), Abszessen, Lipomen oder vergrößerten Lymphknoten, ulzerative Formen von tropischen Ulzera, kutaner Leishmaniose oder Tuberkulose, Lepra, Mykosen, oder Neoplasmen unterschieden werden (47).

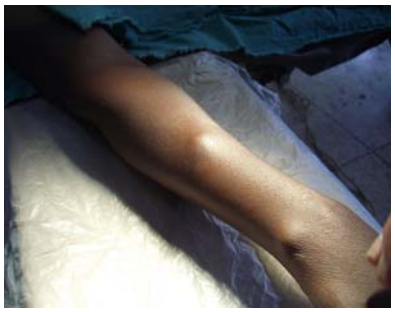
Abb. 1a

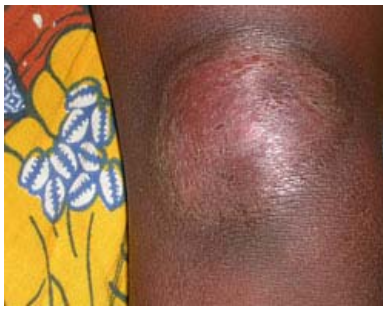
Abb. 1b

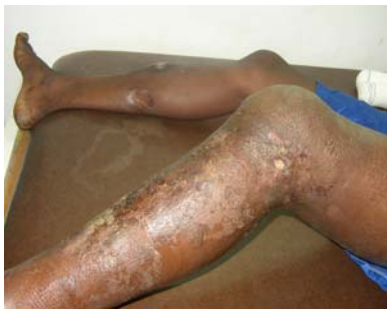
Abb. 1c

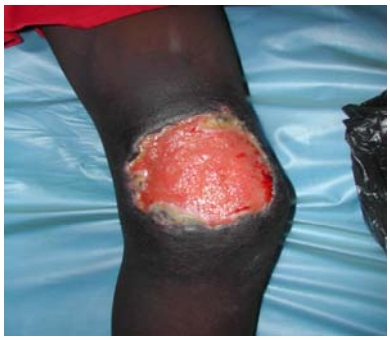
Abb. 1d

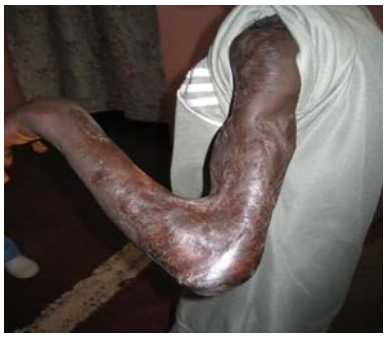
Abb. 1e

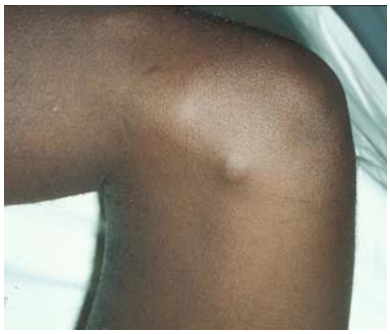
Abb. 1f

Abb. 1. Erkrankungsformen und Differentialdiagnose (1a: noduläre Form, 1b: Plaque [Quelle: WHO, http://www.who.int/buruli/photos], 1c: Ödem, 1d: Ulkus, 1e: Kontraktur, 1f: Onchozerkom [Quelle Prof. Dr. D. Büttner, Bernhard Nocht Institut für Tropenmedizin, Hamburg])

Labordiagnose

Zum bakteriologischen Nachweis von *Mycobacterium ulcerans* eignen sich Abstriche (Abb. 2a) und Gewebeproben (Punch Biopsien oder operativ entnommenes Exzisionsmaterial, Abb. 2c und 2d). Als diagnostische Methoden stehen die Mikroskopie Ziehl-Neelsen gefärbter Präparate (Abb. 2e), Kultur auf Löwenstein-Jensen-Medien (Abb. 2f), die *IS2404* PCR (Amplifikation des in über 200 Kopien vorhandenen Insertionselementes *IS2404*) sowie die Histopathologie zur Verfügung (47)**.** Nach bisher publizierten Daten zur Laborbestätigung klinisch diagnostizierter Buruli-Verdachtsfälle können mittels Mikroskopie 29-78%, mittels Kultur 34-79%, mittels Histopathologie >70%, mittels *IS2404* PCR 61-72% der Verdachtsfälle bestätigt werden. Die diagnostische Sensitivität wird für Histopathologie mit >90%, für die *IS2404* PCR mit 79-85% angegeben (3, 14, 15, 26, 31, 36, 39, 55). Die Eignung von Feinnadelaspiraten (Abb. 2b) zur Laborbestätigung vor allem nicht-ulzerativer Läsionen mittels Mikroskopie und PCR befindet sich derzeit unter Evaluierung. Phillips et al. ermittelten in einer Studie zu 4 mm und 6 mm Punch-Biopsien diagnostische Sensitivitäten von 42% für Mikroskopie, 49% für Kultur, 98% für *IS2404* PCR, sowie 82% für die histologische Untersuchung (30).

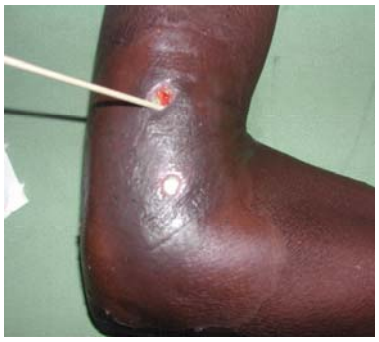

Abb. 2a

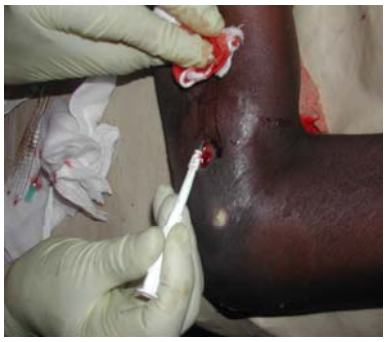

Abb. 2c

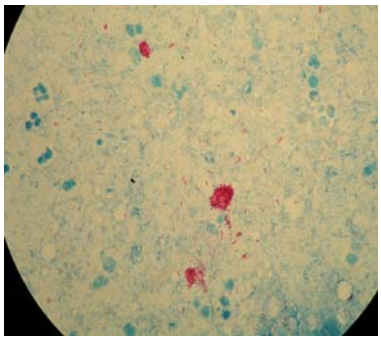

Abb. 2e

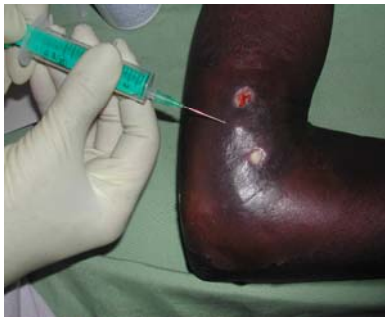

Abb. 2b

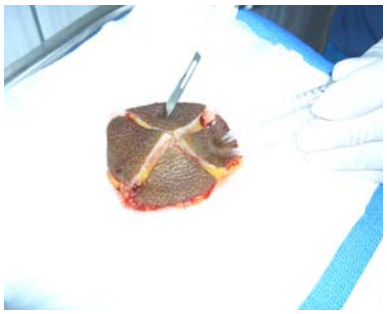

Abb. 2d

Abb. 2f

Abb. 2. Diagnostische Untersuchungsmaterialien und Laboruntersuchungen (2a Abstrich, 2b Feinnadelaspirat, 2c Punch-Biopsie, 2d operativ entnommenes Gewebe, 2 e Mikroskopisches Abstrichpräparat, 2f *M. ulcerans* Kultur auf Jöwenstein-Jensen)

Unterschiedliche Faktoren wie beispielsweise Alter der Läsion/Erkrankungsdauer, sowie Qualität der Probenabnahme und Probentransport können die diagnostischen Sensitivitäten entscheidend beeinflussen. Generell sollten folgende Aspekte beachtet werden: Die Probenabnahme von nicht-ulzerativen Läsionen sollte aus dem Zentrum der Läsion, von ulzerativen Läsionen dagegen unter dem unterminierten Rand, im Grenzbereich zwischen nekrotischem und makroskopisch gesundem Gewebe erfolgen. Abstriche ulzerativer Läsionen werden durch kreisförmiges Umfahren der Läsion tief unter dem unterminierten Rand gewonnen. Da die PCR-Untersuchung von Abstrichen in den meisten Fällen bereits zur Sicherung der Diagnose führt, ist eine routinemäßige Gewebeabnahme bei ulzerativen Fällen nicht erforderlich, sondern sollte nur bei negativem Abstrich und begründetem klinischen Verdacht durchgeführt werden (5). Für den Transport der Proben ins Labor empfiehlt sich die Verwendung geeigneter Transportmedien (11). Bislang gültige WHO-Empfehlungen fordern zwei positive Laborteste als Kriterium für eine positive Labordiagnose (47). Neuere eigene Untersuchungen konnten jedoch zeigen, dass im Hinblick auf die hohe Spezifität der *IS2404* PCR ein positives PCR-Ergebnis hinreichende diagnostische Sicherheit gibt (5). *Mycobacterium liflandii* gilt derzeit als die einzige kreuzreagierende, mittels *IS2404* PCR aus Umweltproben amplifizierbare, allerdings nicht humanpathogene Spezies (27). Für den

Einsatz in endemischen Gebieten steht eine an tropische Bedingungen adaptierte Trockenreagenz-*IS2404*-PCR zur Verfügung (36, 37, 51).

Therapie

Bis vor kurzem bestand die Therapie der Wahl aus weiträumiger chirurgischer Exzision der Läsion, meist gefolgt von Hauttransplantation. In Abhängigkeit von chirurgischer Technik und Erfahrung des Operateurs wurden Rezidivraten von 6.1% bis 32% beobachtet (9). Untersuchungen der Ränder chirurgischer Exzisionspräparate konnten bei ulzerativen Läsionen eine Präsenz des Erregers in makroskopisch gesundem Gewebe nachweisen. Der makroskopische Aspekt des die Läsion umgebenden Gewebes allein garantiert demnach keine Exzision im Gesunden, die Gefahr von Rezidiven ist gegeben (6, 33, 34). Für noduläre Läsionen und Plaques konnten eigene Untersuchungen jedoch zeigen, dass aufgrund der Konzentration der Erreger im Zentrum der Läsionen die chirurgische Exzision unter Wahrung eines ausreichenden Sicherheitsabstandes eine kurative Therapie ermöglicht (17). Seit 2004 empfiehlt die WHO eine achtwöchige antimykobakterielle Kombinationstherapie mit Rifampicin (10 mg/kg/d) und Streptomycin (15 mg/kg/d) (52). Die Umsetzung der WHO-Empfehlungen erfolgte in den endemischen Regionen West Afrikas im Jahr 2006. Bisher durchgeführte klinische Studien belegen die Wirksamkeit dieses Therapieschemas. Läsionen der Kategorie I und II können in vielen Fällen durch alleinige medikamentöse Behandlung therapiert werden. Nach derzeitigem Kenntnisstand erfolgt bei bis zu 50% der antimykobakteriell behandelten Patienten eine Heilung allein durch medikamentöse Therapie. Kann keine vollständige Heilung erreicht werden, bewirkt die medikamentöse Behandlung dennoch eine Verkleinerung der Läsionen.

Die Rezidivraten antimykobakteriell behandelter Patienten liegen mit unter 2% deutlich unter den nur rein chirurgisch therapierten Fällen. Rein orale Medikamentenkombinationen befinden sich derzeit unter Evaluierung. Vor Beginn einer antimykobakteriellen Therapie ist die Laborbestätigung der klinischen Verdachtsdiagnose anzustreben

Die antibiotische Behandlung kann mit nachfolgender chirurgischer Exzision und Hauttransplantation kombiniert werden. Nach gegenwärtigem Kenntnisstand sollte die Indikationsstellung für einen chirurgischen Eingriff hauptsächlich im Hinblick auf Beschleunigung der Heilung von großen Ulzera der Kategorie III, nach einer mindestens vierwöchigen antibiotischen Therapie erfolgen. Weitere Indikationen sind Osteomyelitis, bestehender Wunsch des Patienten nach chirurgischer Behandlung, sowie mögliche Kontraindikationen für medikamentöse Therapie (7, 19, 50, 52-54).

Erkennung und Therapie im Frühstadium der Erkrankung sind essentiell für eine komplikationslose Heilung. Die Prävention funktioneller Bewegungseinschränkungen und Behinderungen als Spätkomplikationen von Erkrankung und Therapie mittels physiotherapeutischer Maßnahmen gewinnt zunehmend an Bedeutung (49, 50).

Im Zuge der Einführung antimykobakterieller Therapie revidierte die TAG der WHO die bislang geltenden Definitionen und Behandlungsrichtlinien für Neuerkrankungen („new case"), nicht heilende Läsionen („non-healers" oder „ongoing cases") und Rezidive („recurrent cases"). Als Neuerkrankung gilt derzeit jeder BUD-Patient, der vor der Diagnosestellung nicht mit Antibiotika behandelt wurde. Demzufolge werden auch Patienten, die im Vorfeld einer traditionellen oder chirurgischen Therapie unterzogen wurden, als Neuerkrankungen eingestuft.

Bei unzureichender oder fehlender Wundheilung nach achtwöchiger Antibiotikagabe wird derzeit eine konservative Weiterbehandlung durch Wundreinigung und Verbände empfohlen, da die Möglichkeit einer verzögerten Wundheilung in Betracht gezogen werden sollte. Auch neue Läsionen, die innerhalb von drei Monaten nach Beendigung antimykobakterieller Therapie in der Region der ursprünglichen Läsion auftreten, werden derzeit definitionsgemäß als „non-healers" eingestuft und konservativ behandelt.

Neue Läsionen, die mehr als drei Monate nach beendeter antibiotischer Therapie (mit oder ohne nachfolgende chirurgische Behandlung) mit abgeheilter initialer Läsion in der Region der ursprünglichen Läsion auftreten, werden als Rezidive eingestuft. Eine erneute Kombinationstherapie mit Rifampicin und Streptomycin ist möglich, die kumulative toxische Dosis von Streptomycin (90 Dosen, bzw. 90 g in Erwachsenen) darf jedoch nicht überschritten werden. Alternativ kann Rifampicin bei Kindern mit Clarithromycin (12.5 mg/kg/d) bzw. bei Erwachsenen mit Minocyclin (400 mg/d) kombiniert werden, entsprechende Daten aus klinischen Studien stehen jedoch noch aus.

Rezidive ohne vorherige antimykobakterielle Therapie werden wie Neuerkrankungen behandelt.

Treten Läsionen an anderen Körperstellen als die initiale Läsion auf, gelten die Therapierichtlinien für Rezidivpatienten. Eine Unterscheidung zwischen Reinfektion und Rezidiv, beispielsweise durch molekularbiologische Methoden, ist gegenwärtig nicht möglich.

Zum Bestätigung der klinischen Verdachtsdiagnose eines Rezidivs ist der kulturelle Nachweis von *M. ulcerans* erforderlich. Aufgrund möglicher Persistenz mykobakterieller DNA oder säurefester Stäbchen unter Therapie sind PCR und Mikroskopie hierfür ungeeignet (53, 54).

Zielsetzung der vorliegenden Arbeit

Gemäß aktueller WHO Empfehlungen sollte die Laborbestätigung mindestens 50% aller klinisch diagnostizierten BUD-Verdachtsfälle sowohl im Hinblick auf epidemiologische Fragestellungen wie der Erhebung gesicherter Inzidenz- und Prävalenzdaten, als auch vor Beginn einer antimykobakteriellen Therapie erfolgen. (50, 53, 54). Die Labordiagnostik des Buruli Ulkus in endemischen Regionen wird jedoch durch mehrere Faktoren erschwert. Zum einen stehen vor Ort meist nur wenig sensitive Methoden (wie beispielsweise Mikroskopie) zur Verfügung, während hoch sensitive Methoden wie PCR und Histopathologie in der Regel auf zum Teil im Ausland befindliche Referenzlabore beschränkt sind. Weiterhin verhindern nicht nur die relativ hohen Kosten molekularbiologischer Methoden aufgrund limitierter Budgets der Gesundheitssektoren betroffener Regionen den Einsatz dieser Techniken. Die Einführung dieser Methoden ist insbesondere aufgrund fehlender technischer Voraussetzungen unter tropischen Bedingungen, mangelhafter Logistik und Ausbildung technischen Personals, sowie des Fehlens von Mechanismen zur Qualitätskontrolle derartiger Labormethoden vor Ort oftmals sehr problematisch.
Vor diesem Hintergrund war das übergeordnete Ziel meiner Arbeit die Etablierung eines Netzwerkes zur Labordiagnostik des Buruli Ulkus in Ghana. Hierfür mussten folgende Voraussetzungen geschaffen und folgende Themenbereiche bearbeitet werden:

Entwicklung und Etablierung einer an tropische Bedingungen adaptierten diagnostischen PCR in Ghana

Zu diesem Zweck sollte, im Rahmen eines von der Volkswagenstiftung geförderten Forschungsvorhabens („A dry reagent based PCR as a novel tool for the laboratory confirmation of clinically diagnosed *M. ulcerans* disease“ Projektlaufzeit 2004 – 2007) zunächst eine Trockenreagenz-basierte, an tropische Bedingungen (z.B. mangelhafte Kühlketten bei Transport und Lagerung von Reagenzien) adaptierte PCR zum Nachweis von *Mycobacterium ulcerans* entwickelt, validiert, und im Kumasi Centre for Collaborative Research in Tropical Medicine, Kumasi, Ghana (KCCR) etabliert werden.

Etablierung eines diagnostischen Netzwerkes in Ghana

Zum Aufbau eines diagnostischen Netzwerkes zwischen anfangs zwei ghanaischen Hospitälern, dem KCCR, sowie zwei deutschen Referenzlaboren (BNITM, Abteilung für Infektions- und Tropenmedizin der Universität München, AITM) war zunächst die Organisation der Logistik für den Transport von Reagenzien, Labormaterialien und

diagnostischen Proben zwischen den teilnehmenden Partnern erforderlich. Weiterhin sollten standardisierte Kriterien für Probenabnahme, Probenverarbeitung, Labormethoden, sowie standardisierte Vorgehensweisen zur internen und externen Qualitätskontrolle festgelegt und durch entsprechende Trainingsmaßnahmen vor Ort eingeführt werden.

Validierung der Methode unter Feldbedingungen

Im weiteren Projektverlauf, sowie in einem im sechsten Rahmenprogramm der Europäischen Kommission geförderten Folgeprojekt („BURULICO. Multidisciplinary research for improvement of control in Africa“, Projektlaufzeit 2005 - 2009) sollte obengenannte Methode unter Feldbedingungen, im Vergleich mit anderen diagnostischen Methoden, und hinsichtlich ihrer Eignung für bestimmte diagnostische Fragestellungen validiert werden.

Entwicklung eines Ansatzes zur Stufendiagnostik des Buruli Ulkus

Die erhobenen Daten zur diagnostischen Sensitivität verschiedener diagnostischer Methoden bei verschiedenen Erkrankungsformen sollten – auch unter Berücksichtigung von Kostenfaktoren – in einen neuartigen Ansatz zur Stufendiagnostik des Buruli Ulkus umgesetzt werden.

Bestimmung der Sensitivität verfügbarer diagnostischer Methoden für unterschiedliche Untersuchungsmaterialien abhängig von Erkrankungsformen und Art der Therapie

Während bis 2006 eine Laborbestätigung von BUD-Verdachtsfällen in der Regel anhand der Untersuchung von Gewebepräparaten chirurgisch behandelter Patienten erfolgte, gewannen mit der Einführung der antimykobakteriellen Therapie andere Untersuchungsmaterialien wie diagnostische Abstriche und Punch-Biopsien zur Bestätigung der Verdachtsdiagnose vor Behandlungsbeginn zunehmend an Bedeutung. In einer vergleichenden Studie sollte die diagnostische Sensitivität verfügbarer diagnostischer Methoden an unterschiedlichen diagnostischen Materialien von Patienten mit unterschiedlichen Erkrankungsformen aus unterschiedlichen Behandlungsgruppen ermittelt werden.

Untersuchungen zur Möglichkeit einer kurativen Exzision und Standardisierung der Exzisionsgröße

Weiterer Schwerpunkt meiner Arbeit war die Untersuchung chirurgischer Exzisionspräparate zum Nachweis der Ausbreitung einer *M. ulcerans*-Infektion innerhalb verschiedener Läsionstypen. Insbesondere sollte die Möglichkeit einer kurativen Exzision, sowie eine mögliche Standardisierung der Exzisionsgröße evaluiert werden.

Untersuchungen zum Behandlungserfolg der BUD-Chirurgie ohne bzw. mit begleitender antimykobakterieller Therapie

Zur Evaluierung des Behandlungserfolges chirurgischer Exzision mit oder ohne begleitender antimykobakterieller Therapie führten wir eine Follow-up Studie an einer Kohorte laborbestätigter BUD-Patienten aus zwei Behandlungszentren in Ghana durch. Gegenstand unserer Untersuchung waren die Häufigkeit postoperativer Rezidive, sowie das Auftreten sowohl objektiv messbarer als auch subjektiv empfundener funktioneller Einschränkungen der Beweglichkeit („reduced range of motion“, ROM) als Folge therapeutischer Maßnahmen.

Die Ergebnisse der im Rahmen dieser Arbeit entstandenen Publikationen werden im Folgenden zusammengefasst.

Diskussion der eigenen Arbeiten

Dry reagent-based PCR as a novel tool for laboratory confirmation of clinically diagnosed Mycobacterium ulcerans-associated disease in areas in the tropics where M. ulcerans is endemic.
Siegmund V, Adjei O, Racz P, Berberich C, Klutse E, van Vloten F, Kruppa T, Fleischer B, ***Bretzel G****. J Clin Microbiol 2005;43(1):271-6.*

Zur Vermeidung der als Spätfolgen des Buruli Ulkus auftretenden, durch Narbenbildung und Kontrakturen verursachten teils schweren Behinderungen ist eine zuverlässige Diagnose und Therapie früher Stadien der Erkrankung erforderlich. Aufgrund der vielfältigen klinischen Erscheinungsformen nicht-ulzerativer und ulzerativer Stadien der Buruli-Erkrankung und der daraus resultierenden möglichen Differentialdiagnosen erfordert die klinische Verdachts-diagnose eine Bestätigung mittels geeigneter Laboruntersuchungen. Unter den zur Verfügung stehenden Methoden (Mikroskopie, Kultur, Histopathologie und *IS2404* PCR) bietet die PCR die höchste diagnostische Sensitivität. Die Verfügbarkeit molekular-biologischer Methoden in Endemiegebieten ist jedoch aufgrund eines Mangels an adäquater Laborkapazität, finanziellen Ressourcen und ausgebildetem Personal limitiert. Die Anwendung solcher Untersuchungen wird darüber hinaus durch technische Schwierigkeiten, beispielsweise die Schädigung von Reagenzien während Transport und Lagerung durch Stromausfälle und daraus resultierende Unterbrechung von Kühlketten, erheblich erschwert. Vor diesem Hintergrund wurde in meiner Arbeitsgruppe am Bernhard Nocht Institut für Tropenmedizin (BNITM) eine Trockenreagenz-PCR („Dry reagent based PCR, im folgenden „DRB-PCR“ genannt) zum Nachweis von *M. ulcerans* entwickelt. Die Methode basiert auf der von Stinear et al. etablierten diagnostischen Standard-*IS2404* PCR (40) und ist aufgrund lyophilisierter, temperaturstabiler Reagenzien für die Anwendung unter tropischen Bedingungen geeignet. Nach Etablierung und Optimierung der Reaktionsbedingungen wurde die DRB-PCR in mehreren Validierungsschritten einem Vergleich mit der Standard-PCR unterzogen.
Zur technischen Validierung wurde zunächst die 492 bp Zielregion der etablierten Standard-*IS2404* PCR in *E. coli* kloniert und serielle Verdünnungen der gewonnenen Plasmid-DNA im Vergleich mittels *IS2404* Standard- und DRB-PCR getestet. Die Nachweisgrenze beider Methoden lag bei 1.5 Genomäquivalenten pro Reaktion, somit war von einer vergleichbaren analytischen Sensitivität auszugehen.

Sensitivität und Spezifität der DRB-PCR lagen nach Testung von 39 *M. ulcerans* Isolaten sowie 15 anderen nicht-*M. ulcerans* Mykobakterienspezies (u.a. *M. marinum*) bei jeweils 100% und entsprachen somit ebenfalls der Standardmethode.

Die anschließende vergleichende PCR-Analyse diagnostischer Proben von 19 Patienten aus Ghana mittels beider Methoden in einer ersten Testreihe ergab übereinstimmende Ergebnisse für 94.7% bzw. 75% der untersuchten Abstriche bzw. operativ entnommener Gewebeproben.

Nach Etablierung der Methode am Kumasi Centre for Collaborative Research in Tropical Medicine (KCCR), Ghana, wurden weitere, jetzt nach standardisierten Kriterien von 30 Patienten entnommene diagnostische Proben parallel am KCCR (DRB-PCR) und BNITM (Standard-PCR) getestet. Die Übereinstimmungsraten beider Methoden lagen in dieser zweiten Testreihe bei 95.5% für Abstriche und 96.7% für operativ entnommene Gewebeproben. Somit konnte die Zuverlässigkeit der DRB-PCR als gesichert betrachtet und die Methode für die Routineanwendung freigegeben werden.

Die in beiden Testreihen erzielten Anteile der Abstrich- und Gewebeproben mit einem positiven PCR-Ergebnis (im Folgenden als diagnostische Sensitivität bezeichnet) sind in Tabelle 1 dargestellt.

Testreihe / **Probe**	Testreihe 1 diagnostische Sensitivität in %	Testreihe 2 diagnostische Sensitivität in %
Abstrich	**31.6**	**27.3**
Gewebe	**18.8**	**36.7**

Tabelle 1: Anteil positiv getesteter Abstrich– und Gewebeproben mit positivem PCR-Ergebnis als diagnostische Sensitivität in %

Die histopathologische Untersuchung simultan abgenommener Gewebeproben der PCR-negativen Patienten lieferte in der Mehrzahl der Fälle Erklärungen für die in Tabelle 1 dargestellte geringe diagnostische Sensitivität beider Testreihen. So enthielt das Untersuchungmaterial in 40% der PCR-negativen Fälle nur Epidermis und Dermis, war also ungeeignet zum Nachweis der in subkutanem Fettgewebe befindlichen Erreger. Für 22% der untersuchten Patienten lieferte die histopathologische Analyse eine Differentialdiagnose infektiöser oder nicht-infektiöser Genese, bei 30% der Fälle handelte es sich um Buruli-Läsionen im Spät- oder Heilungsstadium ohne nachweisbare Erreger.

Mit der DRB-PCR steht somit eine verlässliche, der Standard-PCR ebenbürtige, an tropische Bedingungen adaptierte Methode zum Nachweis von *M. ulcerans* zur Verfügung.

Lyophilisierte Reagenzien und Primer sind nicht nur unempfindlich gegen Temperaturschwankungen und hohe Luftfeuchtigkeit. Der vor-lyophilisierte Reaktionsmix senkt aufgrund einer Minimierung der Pipettierschritte zusätzlich das Kontaminationsrisiko und erlaubt auch molekularbiologisch nicht vorgebildetem Laborpersonal ein schnelles Erlernen der Methode. Wie in der vorliegenden Validierungsstudie gezeigt, korreliert die diagnostische Sensitivität des Testes jedoch in hohem Maß mit der Qualität der diagnostischen Proben sowie dem Alter der Läsion. Voraussetzung für eine optimale Sensitivität der Methode sind sowohl die korrekte Probenabnahme (inclusive subkutanem Fettgewebe) als auch die Untersuchung aktiver, früher Läsionen, da bei einer Erkrankungsdauer von mehr als sechs Monaten in der Regel nur noch wenige oder keine Bakterien in der Läsion vorhanden sind.

External quality assurance for the laboratory diagnosis of Buruli ulcer disease in Ghana.
***Bretzel G**, Siegmund V, Nitschke J, Herbinger KH, Thompson R, Fleischmann E, Fleischer B, Adjei O. Trop Med Int Health 2006;11(11):1688-93.*

Im Rahmen eines von der Volkswagenstiftung geförderten Forschungsprojektes wurde in Ghana ein diagnostisches Netzwerk zur Labordiagnose des Buruli Ulkus etabliert. Nach standardisierten Kriterien gewonnene diagnostische Abstriche und Gewebeproben aus zwei, auf die Behandlung der Buruli-Erkrankung spezialisierten Hospitälern wurden in einem lokalen Referenzlabor (im folgenden „Testlabor" genannt) mittels Ziehl-Neelsen Mikroskopie, Kultur und *IS2404*-Trockenreagenz-PCR („dry reagent based PCR", im folgenden „DRB-PCR" genannt) untersucht. Zur Qualitätssicherung der diagnostischen Ergebnisse wurden Mikroskopie und PCR während eines Untersuchungszeitraumes von zwei Jahren einer Überprüfung durch ein externes Referenzlabor in Deutschland (im Folgenden „controller" genannt) unterzogen („external quality assurance", im Folgenden „EQA" genannt). Da die im Testlabor angewandten Laboruntersuchungen erst ein Jahr vor Beginn der EQA eingeführt worden waren, wurde nicht nur eine Stichprobe ausgewählt, sondern alle Präparate bzw. Proben einer EQA unterzogen.
Die Qualitätskontrolle der mikroskopischen Präparate wurde wie folgt durchgeführt: Die Präparate wurden zunächst routinemäßig vom Testlabor gelesen. Im Rahmen von regelmäßigen, vom controller durchgeführten Laborsupervisionen im Testlabor, wurden die Präparate vom controller zunächst verblindet gegengelesen. Ergaben sich bei der anschließenden Auswertung Diskrepanzen zwischen von Testlabor und controller erzielten Resultaten, wurden die betreffenden Präparate erneut vom controller begutachtet, wobei das erste und zweite Lesen der Präparate von unterschiedlichen Personen durchgeführt, und das zweite controller-Ergebnis als Endergebnis gewertet wurde. Zu Trainingszwecken wurden alle Präparate mit diskrepanten Ergebnissen erneut vom Testlabor gelesen und fragliche Befunde mit dem controller diskutiert.
Die Qualitätskontrolle der PCR wurde wie folgt durchgeführt: Parallel und von der gleichen Lokalisation der Läsion abgenommene Gewebeproben wurden von Testlabor (DRB-PCR) und controller (konventionelle Standard-PCR) verblindet getestet. Proben mit abweichenden Ergebnissen wurden vom controller in einem zweiten Testlauf erneut getestet, wobei das zweite controller-Ergebnis als Endergebnis gewertet wurde. Die Untersuchung von Proben mit diskrepanten Ergebnissen wurde im Rahmen der regelmäßigen Laborsupervisionen in Anwesenheit des controllers im Testlabor zu Trainingszwecken wiederholt.

Aus den erhaltenen Daten wurden unter anderem folgende Parameter bestimmt: Anteil positiver Ergebnisse des Testlabors (für Mikroskopie „slide positivity rate“ im folgenden „SPR“ genannt), Sensitivität des Testlabors im Vergleich zum controller, falsch negative und falsch positive Ergebnisse des Testlabors bezogen auf das controller-Ergebnis, sowie Übereinstimmungsraten zwischen Testlabor und controller. Die Ergebnisse aus erstem und zweitem Untersuchungsgang sind in Tabelle 1 dargestellt.

Test/Untersuchungsgang	Übereinstimmungsrate (%)	Falsch negativ (%)	Falsch positiv (%)
Mikroskopie 1	82.9	27.1	10.1
Mikroskopie 2	97.9	4.2	0.6
PCR 1	87.9	8.2	19.1
PCR 2	96.2	4.7	2.1

Tabelle 1. Mikroskopie- und PCR- Ergebnisse aus erstem und zweitem Untersuchungsgang (Übereinstimmungsrate zwischen controller und Testlabor, Anteil falsch negativer und falsch positiver Ergebnisse).

Die Mikroskopieergebnisse aus dem ersten Untersuchungsgang des Testlabors ergaben eine SPR von 35.9%. Nach dem wiederholten Lesen diskrepanter Präparate im Testlabor im zweiten Untersuchungsgang verbesserten sich sowohl die Übereinstimmungsrate zwischen beiden Laboratorien, als auch Sensitivität (von 72.9% im ersten Untersuchungsgang auf 95,8%) und Spezifität (von 89.9% im ersten Untersuchungsgang auf 99.4%).

Die PCR-Ergebnisse aus dem ersten Untersuchungsgang des Testlabors ergaben eine Positivitätsrate von 66%. Nach der wiederholten Analyse diskrepanter Proben im Testlabor im zweiten Untersuchungsgang verbesserten sich sowohl die Übereinstimmungsrate zwischen beiden Laboratorien, als auch Sensitivität (von 91.8% im ersten Untersuchungsgang auf 95.3%) und Spezifität (von 80.9% im ersten Untersuchungsgang auf 97.9%).

Ursachen für fehlerhafte Ergebnisse lagen beispielsweise in der Untersuchung einer zu geringen Anzahl von Gesichtsfeldern (falsch negative Mikroskopieergebnisse) oder in der Überinterpretation der Geldokumentation (falsch positive PCR-Ergebnisse).

Die in dieser Studie vorgestellte externe Qualitätssicherung und die damit verbundenen Trainingsmaßnahmen konnten somit die Qualität der Diagnostik im Trainingslabor deutlich verbessern und werden im künftigen Routinebetrieb nach dem gleichen System kontinuierlich weitergeführt. Die Kosten für PCR-Untersuchungen sowie die Arbeitsbelastung des controllers machen jedoch eine Reduktion der Stichprobengröße erforderlich.

A stepwise approach to the laboratory diagnosis of Buruli ulcer disease.
***Bretzel G**, Siegmund V, Nitschke J, Herbinger KH, Thompson W, Klutse E, Crofts K, Massavon W, Etuaful S, Thompson R, Asamoah-Opare K, Racz P, Vloten F, van Berberich C, Kruppa T, Ampadu E, Fleischer B, Adjei O. Trop Med Int Health 2007;12(1):89-96.*

Zur Labordiagnostik des Buruli Ulkus (bzw. der Laborbestätigung klinischer Verdachtsfälle) eignet sich die Untersuchung von Abstrichen und Gewebeproben mittels Mikroskopie, Kultur und *IS2404* PCR sowie die histopathologische Analyse von Gewebeproben. Seit 2001 gültige WHO-Empfehlungen zur Diagnostik des Buruli Ulkus (47) fordern das Vorliegen zweier, mittels verschiedener Laboruntersuchungen gewonnener, positiver Testergebnisse für eine positive Diagnose.
Verschiedene Faktoren erschweren jedoch die praktische Umsetzung dieser Vorgaben. Da die vorhandenen Untersuchungsmethoden über unterschiedliche diagnostische Sensitivitäten verfügen (Mikroskopie <40%, PCR >90%), ist die Wahrscheinlichkeit zweier positiver Laborergebnisse für eine Kombination hochsensitiver Untersuchungsmethoden (z.B. die Kombination von PCR und Histopathologie) am höchsten. Diese Methoden sind in Endemiegebieten jedoch in der Regel nur eingeschränkt (beispielsweise in nationalen Referenzzentren) oder nicht verfügbar. Weiterhin erfordert die Untersuchung diagnostischer Proben in Referenzzentren den zeitnahen und regelmäßigen Probentransport vom peripheren Krankenhaus zum diagnostischen Labor. Augrund des Fehlens entsprechender Infrastruktur sind diese logistischen Voraussetzungen jedoch meist nicht gegeben, die Untersuchung diagnostischer Proben im Referenzzentrum erfolgt somit mit großer zeitlicher Verzögerung. Zur Primärdiagnostik steht in einigen Krankenhauslaboratorien auf Distriktebene nur die mikroskopische Untersuchung von Abstrichpräparaten zur Verfügung. In Anbetracht der geringen Sensitivität der Methode sowie der verzögerten Verfügbarkeit der Testergebnisse des Referenzlabors, gründet sich die Therapieentscheidung somit meist nicht auf eine positive Labordiagnose durch zwei positive Testergebnisse, sondern auf den klinischen Befund.
Um die benötigten positiven Testergebnisse zu erhalten, erfolgt – bei Vorhandensein entsprechender Laborkapazität – in der Regel die simultane Untersuchung von Abstrichen und Gewebeproben mit allen verfügbaren Labormethoden. Im Hinblick auf die nicht unerheblichen Kosten für beispielsweise molekularbiologische Untersuchungen ist diese Praxis der simultanen Probenanalyse durch die limitierten Budgets der Gesundheitssektoren betroffener Länder nicht finanzierbar.

Die Fragestellung der vorliegenden Arbeit war die Etablierung eines auf sukzessiver Anwendung und Kombination verfügbarer Labormethoden basierenden modifizierten Ansatzes zur Labordiagnostik des Buruli Ulkus. Hierbei fanden insbesondere Praktikabilität, Kosteneffizienz und zeitnahe Verfügbarkeit von Laborergebnissen Berücksichtigung.

Im Rahmen eines von der Volkswagenstiftung geförderten Forschungsprojektes wurden diagnostische Proben von 161 klinisch diagnostizierten Buruli Verdachtsfällen (nicht-ulzerative Läsionen: n=67, ulzerative Läsionen n=94) mit frühen Läsionen (Erkrankungsdauer unter sechs Monaten) aus vier Behandlungszentren in Ghana nach standardisierten Kriterien entnommen. Abstriche wurden durch kreisförmiges Umfahren unter dem gesamten unterminierten Rand der Läsion, Gewebepräparate aus chirugisch exzidiertem Gewebe aus dem Zentrum der Läsion (nicht-ulzerative Läsionen) bzw. aus dem unter dem unterminierten Rand befindlichen Grenzbereich zwischen nekrotischem und nicht nekrotischem Gewebe (ulzerative Läsion) entnommen. Die Proben wurden am „Kumasi Centre for Collaborative Research in Tropical Medicine“ (KCCR), Kumasi, Ghana, mittels standardisierter Testmethoden (Mikroskopie und Trockenreagenz („DRB“-) *IS2404* PCR) untersucht. Die histopathologische Analyse zur Ermittlung von Differentialdiagnosen sowie die externe Qualitätssicherung für Mikroskopie und DRB-PCR wurden vom Bernhard-Nocht Institut für Tropenmedizin (BNITM), Hamburg, sowie der Abteilung für Infektions- und Tropenmedizin der Ludwig Maximilians Universität München (AITM) nach standardisierten Kriterien durchgeführt (4, 14, 37).

Anhand der Testergebnisse wurden für die in dieser Studie verwendeten Untersuchungsmaterialien und Testmethoden die diagnostischen Sensitivitäten (definiert als Anteil positiver Testergebnisse bezogen auf die Gesamtzahl klinisch diagnostizierter Buruli Verdachtsfälle) für nicht-ulzerative und ulzerative Läsionen ermittelt. Ausgehend von der mikroskopischen Untersuchung als einfachstem Test wurde weiterhin der zusätzliche Gewinn an diagnostischer Information bei Kombination mit weiteren Testmethoden bestimmt. Die Testkosten der verwendeten Untersuchungsmethoden wurden sowohl für sukzessive als auch simultane Anwendung anhand der in der Studie verwendeten Reagenzien und Labormaterialien für jeweils 100 nicht-ulzerative und ulzerative Läsionen berechnet. Insgesamt konnten 85 (52.8%) der 161 Patienten gemäß WHO Richtlinien mittels der am KCCR verfügbaren Testmethoden durch mindestes zwei positive Testergebnisse bestätigt werden. Der Gesamtanteil positiver Labordiagnosen mit mindestens einem positiven Testergebnis lag bei 70.8%. Die Spezifität von Mikroskopie und PCR betrug hierbei 96.6% bzw. 100% (hierbei wurde die Bestätigung des Testergebnisses durch eine zweite lokal

verfügbare Untersuchungsmethode sowie durch den histopathologischen Befund zugrunde gelegt). Die histopathologische Analyse von 47 Proben mit negativem Testergebnis aller in Ghana durchgeführter Untersuchungen ergab 13 zusätzliche Buruli-Diagnosen, Differentialdiagnosen (meist bakterielle oder parasitäre Erkrankungen) für 24 weitere Patienten, sowie den Ausschluß einer Buruli-Erkrankung in 34 Fällen. Für die Laboruntersuchung nicht-ulzerativer Läsionen wurden für die in Ghana angewandten Methoden folgende Sensitivitäten ermittelt: Mikroskopie (Gewebe) 40.3%, PCR (Gewebe) 62.7%. Die Sensitivitäten der Untersuchungsmethoden bei ulzerativen Läsionen lagen bei 29.8% (Mikroskopie Abstrich), 66.0% (PCR Abstrich), 42.6% (Mikroskpie Gewebe), und 57.4% (PCR Gewebe). Die für die Kombination der genannten Testmethoden ermittelten Werte (zusätzlicher Gewinn an diagnostischer Sensitivität bei sukzessiver Anwendung der einzelnen Testmethoden, sowie die durch Kombination aller Methoden erhaltene Gesamtsensitivität) sind für nicht-ulzerative und ulzerative Läsionen in Tabelle 1 und 2 dargestellt.

Test	Gewebe Mikroskopie	Kombination mit:	Gewebe PCR	Gesamt
Sensitivität (%)	40.3 %	Zusätzlicher Sensitivitätsgewinn:	25.4	65.7

Tabelle 1. Diagnostische Sensitivität Gewebe-Mikroskopie, zusätzlicher Sensitivitätsgewinn bei Kombination mit Gewebe-PCR für nicht-ulzerative Läsionen

Durch mikroskopische Gewebeuntersuchungen konnten 40.3% der Buruli-Verdachtsfälle mit nicht-ulzerativen Läsionen bestätigt werden, die zusätzliche Untersuchung mikroskopisch negativer Gewebepräparate mittels PCR ergab weitere 25.4% positive Labordiagnosen. Insgesamt konnten 65.7% der untersuchten Fälle durch die Kombination beider Methoden bestätigt werden.

Test	Abstrich Mikroskopie	Kombination mit:	Abstrich PCR	Gewebe Mikroskopie	Gewebe PCR	Gesamt
Sensitivität (%)	29.8	Zusätzlicher Sensitivitätsgewinn:	38.3	4.3	2.1	74.5

Tabelle 2: Diagnostische Sensitivität Abstrich-Mikroskopie, zusätzlicher Sensitivitätsgewinn bei Kombination mit Abstrich-PCR, Gewebe-Mikroskopie, und Gewebe-PCR

Durch mikroskopische Abstrichuntersuchungen konnten 29.8% der Buruli-Verdachtsfälle mit ulzerativen Läsionen bestätigt werden, die zusätzliche Untersuchung mikroskopisch negativer Abstrichpräparate mittels PCR ergab weitere 38.3% positive Labordiagnosen. Durch

mikroskopische Untersuchung von Gewebepräparaten wurden weitere 4.3%, durch Gewebe-PCR weitere 2.1% der Verdachtsfälle bestätigt. Insgesamt konnten 74.5% der untersuchten Fälle durch die Kombination von Abstrich- und Gewebeuntersuchung mittels Mikroskopie und PCR bestätigt werden.
Der Kostenanalyse ergab eine signifikante Reduktion der Testkosten bei sukzessiver Anwendung (nicht-ulzerativ: 1125 €, ulzerativ: 1250 €) im Vergleich zu simultaner Anwendung (nicht-ulzerativ: 1725 €, ulzerativ: 3250 €) der genannten Labormethoden.

Die in dieser Studie erhobenen Daten erlauben folgende Schlussfolgerungen: Wird statt der bisher geforderten zwei positiven Laborergebnisse einer positiven Diagnose nur ein positives Laborresultat als Kriterium zugrunde gelegt, erhöht sich der Anteil positiver Diagnosen um rund 20%. Im Hinblick auf die hohe Spezifität von Mikroskopie und PCR (in dieser Studie 96.6% bzw. 100%) ist ein zweiter Bestätigungstest nicht erforderlich. Die bisher geltenden WHO-Empfehlungen sollten somit zugunsten eines höheren Anteils positiver Labordiagnosen revidiert werden.
Die Labordiagnostik des Buruli Ulkus sollte im Sinne einer Stufendiagnostik wie folgt durchgeführt werden: Mikroskopische Untersuchung von Gewebepräparaten nicht-ulzerativer Läsionen erlaubt unseren Daten zufolge einen Erregernachweis in etwa 40% der klinisch diagnostizierten Verdachtsfälle. Die anschließende PCR-Untersuchung mikroskopisch negativer Proben ermöglicht weitere etwa 25% positive Diagnosen. In beiden Untersuchungen negative Gewebeproben sollten einer histopathologischen Analyse unterzogen werden. Da die Verarbeitung von Gewebepräparaten für die Mikroskopie unter einer Sicherheitswerkbank erfolgen sollte, ist diese Untersuchung in der Regel nicht im Labor des peripheren Krankenhauses durchführbar.
Aus ulzerativen Läsionen entnommene Abstriche sollten zunächst mikroskopisch (zu erwartende Sensitivität etwa 30%), mikroskopisch negative Abstriche dann mittels PCR (zusätzliche Sensitivität etwa 40%) untersucht werden. Fallen beide Abstrichuntersuchungen negativ aus (in etwa 30% der Fälle), können Gewebepräparate, analog zum oben beschriebenen Vorgehen bei nicht-ulzerativen Läsionen mittels Mikroskopie und PCR untersucht werden. Da hierbei jedoch nur etwa 6% zusätzliche positive Diagnosen erzielt werden können, ist vor allem aus Kostengründen von einer Gewebeuntersuchung mittels PCR abzuraten. Bei negativem Ergebnis der Abtrichuntersuchung sollten Gewebepräparate wenn möglich histologisch untersucht werden.

Im Vergleich zur simultanen Untersuchung diagnostischer Proben ermöglicht die Stufendiagnostik des Buruli Ulkus eine erhebliche Reduktion der Testkosten um bis zu 35% für nicht-ulzerative und bis zu 60% für ulzerative Läsionen.

Dry reagent-based polymerase chain reaction compared with other laboratory methods available for the diagnosis of Buruli ulcer disease.
Siegmund V, Adjei O, Nitschke J, Thompson W, Klutse E, Herbinger KH, Thompson R, van Vloten F, Racz P, Fleischer B, Loescher T, ***Bretzel G****. Clin Infect Dis 2007;45(1):68-75.*

Zur Labordiagnostik des Buruli Ulkus stehen Mikroskopie, *IS2404* PCR und Kultur von Abstrichen und Gewebeproben sowie die histopathologische Untersuchung von Gewebeproben zur Verfügung. Die Sensitivität von Mikroskopie und Kultur ist relativ gering, PCR und Histopathologie dagegen erreichen - mit Ausnahme der Untersuchung bakteriologisch negativer, in Abheilung befindlicher Spätstadien - hohe diagnostische Sensitivitäten, sind jedoch in Endemiegebieten selten verfügbar. Insbesondere die Anwendung molekularbiologischer Untersuchungstechniken ist in tropischen Regionen mit großen Schwierigkeiten verbunden. Transport und Lagerung von Reagenzien erfordern Kühlketten, die aufgrund häufiger Stromausfälle nicht gewährleistet werden können. Konventionelle PCRs müssen zudem zur Vermeidung von Kontaminationen mit äußerster Sorgfalt durchgeführt werden, entsprechend geschultes Personal ist vor Ort nur selten verfügbar. Um die Durchführung einer PCR-Diagnostik des Buruli Ulkus in einem Endemiegebiet zu ermöglichen, wurde im Jahr 2003 am Kumasi Centre for Collaborative Research in Tropical Medicine (KCCR) eine Trockenreagenz-basierte („dry reagent based-“ : „DRB“-), an tropische Bedingungen adaptierte *IS2404* PCR eingeführt (37). Die Methode wurde 2004 – 2006 in Ghana unter Feldbedingungen getestet und mit oben genannten diagnostischen Methoden, sowie der konventionellen Standard *IS2404* PCR nach Stinear (40) verglichen.
Im Rahmen dieser Studie wurden diagnostische Proben von 218 klinisch diagnostizierten Buruli Patienten mit frühen (definiert als Krankheitsdauer unter sechs Monaten) nicht-ulzerativen (n=98) oder ulzerativen Läsionen (n=132) aus vier ghanaischen Behandlungszentren („A“, „AG“, „D“, „G“) untersucht. Die Probenabnahme erfolgte im Rahmen der zum Zeitpunkt der Studie üblichen kurativen chirurgischen Exzision nach standardisierten Kriterien (5). Mikroskopie, DRB-PCR und Kultur wurden am KCCR, Histopathologie am Bernhard Nocht Institut für Tropenmedizin, Hamburg (BNITM), die Standard *IS2404* PCR an der Abteilung für Infektions- und Tropenmedizin der Universität München (AITM) nach standardisierten Kriterien durchgeführt (4, 5, 14, 37, 40, 47). Zur Bestimmung der Übereinstimmungsrate zwischen DRB-PCR und konventioneller Standard-PCR wurden aus jeder Läsion jeweils zwei Abstriche und/oder Gewebeproben vom selben

Ort entnommen und am KCCR (DRB-PCR) und AITM (Standard-PCR) parallel getestet. Weiterhin wurde für jede der in dieser Studie verwendeten diagnostischen Methoden der Anteil der mit der jeweiligen Untersuchungsmethode erzielten positiven Ergebnisse bezogen auf die Gesamtzahl der mit der jeweiligen Methode untersuchten diagnostischen Proben ermittelt (im Folgenden Positivitätsrate genannt). Die Übereinstimmungsrate von DRB-PCR und konventioneller Standard PCR, sowie die Positivitätsraten aller Untersuchungsmethoden wurden sowohl für die Gesamtzahl aller untersuchten Proben, als auch aufgeschlüsselt nach Herkunft der Proben aus den jeweiligen Behandlungszentren analysiert.

Die Gesamt-Übereinstimmungrate zwischen DRB-PCR und konventioneller Standard PCR betrug 91.7% für Abstriche, und 95.0% für Gewebeproben. Aufgeschlüsselt nach Probenherkunft ergaben sich vergleichbare Übereinstimmungsraten für Behandlungszentrum A und D. Für Abstriche aus Krankenhaus AG und G, sowie Gewebeproben aus Zentrum AG wurden deutlich geringere Übereinstimmungsraten ermittelt (Tabelle 1). Tabelle 2 vergleicht die Gesamt-Positivitätsraten aller angewandten Untersuchungen.

Behandlungszentrum / **Übereinstimmungsrate nach Probenart (%)**	A	AG	D	G
Abstrich	93.4	83.3	97.2	76.5
Gewebe	97.3	83.3	93.7	93.9

Tabelle 1: Übereinstimmungsraten zwischen DRB-PCR und konventioneller Standard PCR für Abstriche und Gewebe aufgeschlüsselt nach Probenherkunft aus Behandlungszentrum A, AG, D, und G.

Test / **Gesamt-Positivitäts-rate nach Probenart (%)**	DRB-PCR	Standard PCR	Mikroskopie	Kultur	Histopathologie
Abstrich	60.6	67.4	31.1	0.0	-
Gewebe	60.6	61.5	42.7	4.1	77.8

Tabelle 2: Gesamt-Positivitätsraten DRB-PCR, Standard-PCR, Mikroskopie, Kultur und Histopathologie nach Probenart in %

Aufgeschlüsselt nach der Herkunft der Proben aus den verschiedenen Behandlungszentren ergaben sich wiederum unterschiedliche Positivitätsraten. So wurden beispielsweise für die DRB-PCR Untersuchung von Abstrichen vergleichbar hohe Werte für Zentrum A, AG, und D ermittelt, während ein deutlich geringerer Anteil von Gewebeproben aus Zentrum D positiv getestet wurde (Tabelle 3).

Behandlungszentrum / Positivitätsrate pro Probenart und Test (%)	A	AG	D	G
Abstrich DRB-PCR	67.2	66.7	63.9	47.7
Gewebe DRB-PCR	71.8	83.3	41.3	51.5
Abstrich Standard PCR	73.8	66.7	66.7	70.6
Gewebe Standard PCR	70.9	75.0	47.6	51.5
Abstrich Mikroskopie	41.0	33.3	27.8	17.6
Gewebe Mikroskopie	56.4	33.3	30.2	24.2
Abstrich Kultur	0.0	0.0	0.0	0.0
Gewebe Kultur	5.5	8.3	3.2	0.0
Gewebe Histopathologie	84.7	87.5	72.2	60.0

Tabelle 3: Positivitätsraten aller angewandten Untersuchungsmethoden aufgeschlüsselt nach Art der diagnostischen Probe und Probenherkunft aus Behandlungszentrum A, AG, D, und G.

Die histopathologische Untersuchung von 167 Gewebeproben konnte in 130 Fällen die klinische BUD-Verdachtsdiagnose bestätigen, in 37 Fällen ausschließen. Für 27 dieser Patienten konnte eine Differentialdiagnose (meist bakterielle oder parasitäre Erkrankungen) gestellt werden.

Die während einer zweijährigen Erprobungsphase unter tropischen Bedingungen erhobenen Erfahrungswerte und Daten zur Leistungsfähigkeit der DRB-PCR im Vergleich mit anderen diagnostischen Methoden lassen folgende Schlussfolgerungen zu:

Trotz etwas höherer Testkosten bietet die Anwendung der Trockenreagenz-PCR in tropischen Regionen entscheidende Vorteile gegenüber der konventionellen PCR. Lyophilisierte Reagenzien sind weitgehend unempfindlich gegen Schwankungen von Umgebungstemperatur (auch wiederholtes Auftauen und Einfrieren aufgrund von Stromausfällen) und Luftfeuchtigkeit. Dies ermöglicht Versand und Lagerung von Reagenzien unter tropischen klimatischen Bedingungen. Die reduzierte Anzahl an Arbeitsschritten verringert sowohl Durchführungszeit als auch Kontaminationsrisiko. Aufgrund der einfachen Handhabung war auch Laborpersonal ohne vorherige PCR-Kenntnisse innerhalb weniger Tage in der Lage, den Test korrekt durchzuführen.

Die in dieser Studie ermittelten Gesamt-Übereinstimmungsraten zwischen DRB-PCR und konventioneller Standard-PCR entsprachen den bei der Erstetablierung in Ghana erhobenen Daten und bestätigen die Zuverlässigkeit der Methode (37). Abweichungen von bis zu 8% sind durch die Verwendung unterschiedlicher Proben in beiden Laboratorien zu erklären. Obwohl vom gleichen Ort der Läsion abgenommen, können benachbarte Gewebeproben einen unterschiedlichen Baktereingehalt aufweisen. Dies gilt ebenso für die Abnahme mehrerer Abstriche von einer Läsion. Enthalten Untersuchungsmaterialien generell nur

wenige Erreger/DNA, sind zudem variable Ergebnisse bei wiederholter Testung nicht ungewöhnlich (4, 37). Die deutlich höheren Übereinstimmungsraten der aus Behandlungszentrum A und D erhaltenen Proben im Vergleich zu Zentrum AG und G (Tabelle 1) lassen sich mit der Qualität der Probenabnahmetechnik begründen. Chirurgen beider Zentren waren aktiv an der Entwicklung der Standardisierung der Probengewinnung beteiligt, in beiden Krankenhäusern wurden regelmäßig Trainingsmaßnahmen durchgeführt, zudem war KCCR und/oder AITM Laborpersonal bei einem Großteil der chirurgischen Eingriffe und Probenabnahmen anwesend. Aufgrund größerer geographischer Distanz sowie nur unregelmäßig stattfindenden Operationen waren vergleichbare Trainingsmaßnahmen und Assistenz durch Laborpersonal in den anderen Zentren nicht möglich. Die Qualität der Labordiagnostik wird somit entscheidend von der Qualität der Proben, diese wiederum vom Ausbildungsstatus des Krankenhauspersonals beeinflusst.

Zwischen Gesamt-Positivitätsrate von DRB-PCR und Standard-PCR konnte kein signifikanter Unterschied festgestellt werden (Tabelle 2). Die Positivitätsrate der DRB-PCR lag signifikant höher als die Positivitätsraten der anderen am KCCR verfügbaren Testmethoden (Tabelle 3). Die auffallend niedere Positivitätsrate der Kulturen geht auf die in Ghana zum Studienzeitpunkt praktizierte präoperative Verabreichung antimykobakterieller Medikamente zurück. Im Vergleich zu den bei der Einführung der Methode erhobenen Daten (37) ließ sich eine Steigerung der Positivitätsrate der DRB-PCR um 30% nachweisen. Dies ist hauptsächlich der Beachtung der Einschlußkriterien der Studienpatienten (Krankheitsdauer unter sechs Monaten, somit meist aktive, noch bakterienhaltige Läsionen) sowie den standardisierten Probenabnahmekriterien zuzuschreiben. Die variablen Positivitätsraten einzelner Teste aufgeschlüsselt nach Probenherkunft (Tabelle 3) lassen sich auf die unterschiedliche Qualität der Probenabnahme zurückführen. Insbesondere bei der Abnahme von Gewebeproben spielt die Lokalisation des Entnahmeortes eine entscheidende Rolle, die Bestimmung des Entnahmeortes an exzidiertem Gewebe erfordert große Sorgfalt. Während beispielsweise Abstriche aus Behandlungszentren A und D vergleichbare DRB-PCR-Positivitätsraten ergaben, wurden deutlich weniger Gewebeproben aus Krankenhaus D positiv getestet, was auf unsachgemäß durchgeführte Probenabnahme schließen lässt.

Auch die Qualität der klinischen Diagnose beeinflusst die Positivitätsrate eines diagnostischen Tests. In unserem Patientenkollektiv wurden beispielsweise nur 48% der klinisch als noduläre Formen der Buruli-Erkrankung eingestuften Läsionen aus Behandlungszentrum D mittels DRB-PCR bestätigt (Daten nicht dargestellt). Nach histopathologischer Analyse wurden 18.5% der PCR-negativen Knoten als Onchozerkome identifiziert. Dagegen wurden 75% der

nodulären Formen aus Krankenhaus A, das sich nicht in einer endemischen Region für Onchozerkose befindet, mittels DRB-PCR bestätigt.

In der hier vorgestellten Studie konnten annähernd 80% aller klinischen Verdachtsfälle durch histopathologische Untersuchung bestätigt werden. Die Mehrzahl der Fälle mit positiver Histologie und scheinbar falsch negativem PCR-Ergebnis konnte aufgrund histopathologischer Kriterien den meist bakteriologisch negativen Spät- oder Heilungsstadien zugeordnet werden. Demzufolge eignet sich die PCR nur zur Diagnostik früher, aktiver, bakterienhaltiger Fälle. Falsch positive PCR-Ergebnisse bei der *IS2404* PCR-Diagnostik des Buruli Ulkus aus klinischen Isolaten traten nicht auf.

Die DRB-PCR kann aufgrund der hier vorgestellten Daten als verlässliches Instrument zur Diagnostik des Buruli Ulkus unter tropischen Bedingungen gelten. Um eine gute Qualität der Diagnostik zu garantieren, sind Laboratorien jedoch angehalten, kontinuierliche Trainingsmaßnahmen im Bereich der Probenabnahme in den kooperierenden Behandlungs-zentren durchzuführen.

Comparative study on the sensitivity of different diagnostic methods for the laboratory diagnosis of Buruli Ulcer Disease.
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****. Clin Infect Dis 2009, in press*

Bislang verfügbare Daten zur diagnostischen Sensitivität verschiedener Labormethoden beruhten hauptsächlich auf der Analyse diagnostischer Proben aus chirurgischem Exzisionsmaterial von chirurgisch behandelten Patienten. Mit der Einführung der antimykobakteriellen Therapie gewannen andere Untersuchungsmaterialien, wie diagnostische Abstriche und Punch-Biopsien, zur Bestätigung der Verdachtsdiagnose vor Behandlungsbeginn zunehmend an Bedeutung. Gewebeproben aus chirurgischem Exizisionsmaterial finden derzeit hauptsächlich bei chirurgisch nachbehandelten Patienten deren Läsionen unter antibiotischer Therapie nicht abheilen („non-healers“) Verwendung,
Im Rahmen des von der Europäischen Kommission geförderten Forschungsprojektes BURULICO, ermittelten wir die diagnostische Sensitivität verschiedener Labormethoden anhand der Untersuchung diagnostischer Materialien (Abstriche, 3 mm Punch-Biopsien, chirurgisch exzidiertes Gewebe) von insgesamt 384 BUD-Verdachtsfällen mit nicht-ulzerativen und ulzerativen Läsionen aus drei Behandlungsgruppen. Die Behandlungsgruppen waren definiert wie folgt: 1. „Drug treatment“ (DT): zur antimykobakteriellen Therapie vorgesehene Patienten, bis zum Zeitpunkt der Probenentnahme keine Gabe von Antibiotika. 2. „Surgical treatment“ (ST): chirurgische Exzision, Probenentnahme während des chirurgischen Eingriffes ohne vorhergehende Gabe von Antibiotika. 3. „Surgical treatment“ in Kombination mit antimykobakterieller Therapie (ST+): chirurgische Exzision mit mindestens siebentägiger antibiotischer Vorbehandlung. Probenentnahme und Laboruntersuchungen (*IS2404*-DRB-PCR, Mikroskopie, Kultur, Histologie) inklusive externer Qualitätskontrolle erfolgten mittels standardisierter Methoden (4, 5, 14, 40, 47). Bewachsene Mykobakterien-Kulturen wurden zur Bestätigung einer *IS2404* PCR unterzogen. Bei negativer *IS2404* PCR erfolgte zur weiteren Speziesdifferenzierung die Sequenzanalyse des *rpoB*-, *16S-23S rRNA* internal transcribed spacer (*ITS*-), *16s rRNA*-, und des 65 kDA *hsp*-Gens (24, 25, 35, 43). Die Sensitivität eines diagnostischen Testes wurde definiert als die Anzahl der mit diesem Test erzielten positiven Ergebnisse bezogen auf die Anzahl der Patienten, die in allen durchgeführten diagnostischen Testen in mindestens einem dieser Teste positiv getestet

wurden. Zur Analyse der diagnostischen Sensitivität wurden ausschließlich die Ergebnisse der Erstuntersuchung herangezogen, die von einigen Patienten vorhandenen follow-up–Proben wurden nicht berücksichtigt.

Von den 384 BUD-Verdachtsfällen konnten 147 (43.8%) durch Laboruntersuchungen mit mindestens zwei, 268 (69.7%) mit mindestens einem positiven Testergebnis als BUD-Patienten (DT: 160, ST: 59, ST+: 49; 114 nicht-ulzerative, 154 ulzerative Formen) bestätigt werden. Hierbei war die PCR in 229 Fällen (85.4%) positiv, 152 (56.7%) bzw. 115 (37.5%) Patienten hatten positive Mikroskopieergebnisse bzw. Kulturen. Einhundertundacht der positiven Kulturen konnten mittels *IS2404* PCR als *M. ulcerans* bestätigt werden. Sequenzanalyse der verbleibenden sieben Isolate ergab zwei weitere *M. ulcerans* Stämme, sowie *M. mucogenicum*, und *M. phocaicum*. Aus follow-up Proben von zwei weiteren Patienten wurden *M. gordonae* und *M. szulgai* isoliert, was auf eine Co- oder Superinfektion von BUD-Läsionen mit anderen Mykobakterienspezies hinweist. Die histopathologische Untersuchung der Gewebeproben von 58 in allen übrigen Laboruntersuchungen negativen Verdachtsfällen ergab 17 zusätzliche BUD-Diagnosen.

Tabelle 1 zeigt die Sensitivitäten der untersuchten diagnostischen Teste an Gewebeproben von BUD-Patienten mit nicht-ulzerativen Läsionen, stratifiziert nach Behandlungsgruppen.

Behandlungs-gruppe	**Diagnostische Probe**	**DRB-*IS2404* PCR**	**Mikroskopie**	***IS2404*-PCR bestätigte Kultur**
DT	Punch-Biopsie	**93.5%** (86/92)	57.6% (53/92)	**70.8%** (51/72)
ST	Exzidiertes Gewebe	66.7% (10/15)	40.0% (6/15)	40.0% (6/15)
ST+	Ezidiertes Gewebe	85.7% (6/7)	85.7% (6/7)	0.0% (0/7)
Gesamt	Alle Proben	89.5% (102/118	57.0% (65/114)	60.6% (57/94)

Tabelle 1: Sensitivitäten von PCR, Mikroskopie, und *IS2404*-PCR bestätigter-Kultur in Gewebeproben von BUD-Patienten mit nicht-ulzerativen Läsionen, stratifiziert nach Behandlungsgruppen

Die Sensitivität der PCR lag insgesamt signifikant höher als die Sensitivitäten von Mikroskopie und Kultur. Die Untersuchung von Punch-Biopsien in der Gruppe der DT-Patienten ergab signifikant höhere Sensitivitäten von PCR und Kultur als die Untersuchung chirurgisch exzidierter Gewebeproben von ST und ST+ Patienten.

Tabelle 2 zeigt die Sensitivitäten der untersuchten diagnostischen Teste an Abstrich- und Gewebeproben von BUD-Patienten mit ulzerativen Läsionen, stratifiziert nach Behandlungsgruppen.

Behandlungs-gruppe	Diagnostische Probe	DRB-*IS2404* PCR	Mikroskopie	*IS2404*-PCR bestätigte Kultur
DT	Abstrich	**89.9%** (53/59)	67.8% (40.59)	57.4% (27/47)
	Punch-Biopsie	67.8% (40/59)	33.9% (20/59)	23.4% (11/47)
ST	Abstrich	**73.1%** (19/26)	23.1% (6/26)	12.5% (3/24)
	Exzidiertes Gewebe	57.7% (15/26)	38.5% (10/26)	12.5% (3/24)
ST+	Abstrich	**72.2%** (26/36)	50.0% (18/36)	3.0% (1/33)
	Ezidiertes Gewebe	64.7% (55/85)	38.9% (14/36)	21.2% (7/33)
Gesamt	Abstrich	**84.7%** (72/85)	54.1% (46/85)	42.4% (30/71)
	Gewebe	64.7% (55/85)	35.3% (30/85)	19.7% (14/71)
	Alle Proben	69.8% (169/242)	44.6% (108/242)	25.0% (52/208)

Tabelle 2: Sensitivitäten von PCR, Mikroskopie, und *IS2404*-PCR bestätigter-Kultur an Abstrich- und Gewebeproben von BUD-Patienten mit ulzerativen Läsionen, stratifiziert nach Behandlungsgruppen.

Die Sensitivität der PCR lag insgesamt signifikant höher als die Sensitivitäten von Mikroskopie und Kultur. Die PCR-Untersuchung von Abstrichen ergab in allen Behandlungsgruppen signifikant höhere Sensitivitäten als die Untersuchung von Gewebeproben.

Wir konnten in dieser Studie keinen Zusammenhang zwischen Erkrankungsdauer und Testsensitivität nachweisen. Die Dauer antimykobakterieller Therapie vor Probenentnahme korreliert jedoch eindeutig mit der Sensitivität von Kultur und PCR. Antimykobakterielle Therapie von mehr als 20 bzw. 40 Tagen führte in unserem untersuchten Kollektiv zu einem signifikanten Abfall der diagnostischen Sensitivität von Kultur und PCR.

In der bislang größten, vergleichenden Studie zur Sensitivität gegenwärtig verfügbarer diagnostischer Methoden nach Einführung antimykobakterieller Therapie in West Afrika ergab sich für die *IS2404* PCR in allen untersuchten Untergruppen unserer Studienkohorte die höchste Sensitivität.

Für die Diagnostik von Patienten aus unterschiedlichen Behandlungsgruppen mit unterschiedlichen Läsionstypen können aus dieser Studie folgende Schlussfolgerungen gezogen werden: Aufgrund der hohen Sensitivität von PCR und Kultur können 3 mm Punch-Biopsien uneingeschränkt zur Diagnostik nicht vorbehandelter, nicht-ulzerativer Läsionen empfohlen werden. Die PCR-Untersuchung von 4 und 6 mm Punch-Biopsien (30) bietet keinerlei diagnostischen Vorteile. Die PCR-Untersuchung von Abstrichen aus ulzerativen Läsionen ergab in allen Behandlungsgruppen, insbesondere aber bei nicht vorbehandelten Patienten mit frühen Läsionen, signifikant höhere Sensitivitäten als die Untersuchung von Gewebeproben. Die Untersuchung von Abstrichen mittels PCR kann somit als Methode der

Wahl zur Diagnostik ulzerativer Läsionen gelten. Vernarbungsprozesse im Krankheitsverlauf können allerdings die Abnahme von Abstrichen erschweren und die Untersuchung von Gewebeproben erforderlich machen.

Die Dauer antimykobakterieller Therapie vor Probenabnahme beeinflusst die Sensitivitäten von Kultur und PCR. Während die diagnostische Sensitivität der Kultur in den ersten Behandlungswochen bis auf 6% zurückging, war *M. ulcerans* DNA jedoch auch nach mehr als sechswöchiger Behandlung in 50% der vorbehandelten Patienten nachweisbar. Aufgrund der Persistenz mykobakterieller DNA eignet sich somit nur die Kultur zur Therapiekontrolle.

In Übereinstimmung mit unseren bisherigen Daten zeigte diese Studie weiterhin, dass über 20% mehr Verdachtsdiagnosen bestätigt werden können, wenn nur ein positives Testergebnis einer positiven Diagnose zugrunde gelegt wird (5). Wie in unseren früheren Untersuchungen ergab die histopathologische Analyse von in allen anderen Laboruntersuchungen negativen Gewebeproben auch in diesem Patientenkollektiv 30% an zusätzlichen BUD-Diagnosen (5, 36, 37). Die Positivitätsraten (Anzahl positiver Testergebnisse eines Testes bezogen auf die Gesamtzahl der untersuchten BUD-Verdachtsfälle) sowie die Gesamt-Sensitivitäten der in dieser Studie untersuchten diagnostischen Methoden waren anderen bisher durchgeführten Untersuchungen vergleichbar (3, 14, 15, 26, 31, 36, 39, 55). die Sensitivität der PCR lag insgesamt signifikant höher als die Sensitivitäten von Mikroskopie und *IS2404*-PCR-bestätigter Kultur. Bei der Mehrzahl aller bewachsenen Kulturen handelte es sich um *M. ulcerans*. Von einigen Studienpatienten wurden jedoch während oder nach antimykobakterieller Therapie auch andere Mykobakterienspezies isoliert. Bei wiederholtem kulturellem Mykobakterien-Nachweis von antibiotisch therapierten Patienten sollte in jedem Fall die Identifikation der Kulturen mittels molekularer Methoden angestrebt werden, um Co- oder Superinfektionen mit anderen Mykobakterienspezies nachzuweisen.

Post-surgical assessment of excised tissue from patients with Buruli ulcer disease: progression of infection in macroscopically healthy tissue.
***Bretzel G**, Siegmund V, Racz P, van Vloten F, Ngos F, Thompson W, Biason P, Adjei O, Fleischer B, Nitschke J . Trop Med Int Health 2005;10(11):1199-206.*

Bis zur offiziellen Empfehlung der antimykobakteriellen Therapie des Buruli Ulkus im Jahr 2004 durch die WHO (52) galt die weiträumige chirurgische Exzision als Therapie der Wahl (48). Therapieerfolg und Rezidivraten variierten je nach angewandter Operationstechnik (1, 9, 22, 44). Chirurgisches Vorgehen und Exzisionsgröße wurden allein von Augenmaß und Erfahrung des Operateurs bestimmt. Der makroskopische Aspekt ist jedoch nicht beweisend für eine Exzision im Gesunden. Zur Untersuchung der Ausbreitung der Infektion innerhalb der Läsion und über die Exzisionsränder hinaus wurde in dieser Studie exzidiertes Gewebe von laborbestätigten BUD-Patienten mittels verschiedener Labormethoden untersucht.
Zwanzig Patienten aus Kamerun (n = 10) und Ghana (n = 10) mit klinisch diagnostizierten ulzerativen Buruli-Läsionen wurden in zwei Behandlungszentren (Hôpital de District, District de Santé d'Akonolinga, Akonolinga, Kamerun; Agogo Presbyterian Hospital, Agogo, Ghana) unter Allgemeinanästhesie operiert. Hierbei wurde die Läsion im makroskopisch gesunden Gewebe exzidiert, wobei nekrotisches und subkutanes Fettgewebe vollständig und bis zur Faszie entfernt wurden. Zur Laborbestätigung der klinischen Diagnose mittels *IS2404* PCR, Kultur, und Histopatholgie wurden aus dem exzidierten Gewebe diagnostische Proben aus dem unter dem unterminierten Rand befindlichen Grenzbereich zwischen nekrotischem und optisch gesundem Gewebe entnommen. Zur Bestimmung der Ausbreitung der Infektion innerhalb der Läsion wurden nach genauer Vermessung des exzidierten Gewebes aus dem zwischen Ulkusrand und äußerem Exzisionsrand liegenden Gewebe drei parallele (maximal 50 x 15 mm große) Gewebestreifen entnommen. Diese wurden wiederum in (maximal 10 x 5 mm große) Segmente unterteilt (im folgenden „Gradienten" genannt). Zur Untersuchung der Exzisionsränder mittels PCR wurde der gesamte verbleibende Exzisionsrand in (maximal 10 x 5 mm große) Segmente unterteilt. Aus anatomischen Gründen konnten nur 16 komplette Exzisionsränder untersucht werden. Die Laboruntersuchung aller genannten Proben wurde nach standardisierten Methoden im Kumasi Centre for Research in Tropical Medicine, Kumasi, Ghana (KCCR), sowie im Bernhard Nocht Institut für Tropenmedizin, Hamburg (BNITM) durchgeführt. Zur Quantifizierung der Bakterienlast wurden histopathologische Befunde in die Kategorien „negative" „mild", „moderate", und „marked" unterteilt (14, 40, 47).

Die Untersuchung der insgesamt 83 Gradientensegmente konnte in allen 20 Läsionen *M. ulcerans* DNA und/oder für eine Infektion charakteristische histopathologische Veränderungen im gesamten untersuchten Gewebe vom inneren Ulkusrand bis zum äußeren Exzisionsrand nachweisen. Lediglich für ein Gradientensegment (viertes von fünf Segmenten) einer Läsion konnte kein positiver Nachweis erbracht werden - allerdings waren für das äußerste Segment (Segment fünf) wiederum beide Nachweismethoden positiv.

Nach Untersuchung der Exzisionsränder wurden die 16 Läsionen nach dem Anteil PCR positiver Randsegmente in drei Gruppen eingeteilt: 100% der Randsegmente positiv (n=10), >50% der Randsegmente positiv (n =1), <50% der Randsegmente positiv (n=5).

Die in diversen Studien berichteten Unterschiede in Rezidiv- und Wundheilungsraten wurden auf angewandte Anästhesie- und Operationstechniken, sowie die Erfahrung des Operateurs zurückgeführt (1, 9, 22, 44). Wird nicht unter Allgemein- sondern unter Lokalanästhesie mit primärem Wundverschluss operiert, könnte dies die Entstehung von Rezidiven begünstigen. Unter Lokalanästhesie kann nicht weiträumig exzidiert werden, die primäre Wundnaht gilt aus chirurgischer Sicht bei lokalen Infektionen als kontraindiziert, die Injektion von Lokalanästhetika begünstigt zudem möglicherweise die Diffusion der Bakterien in die Peripherie der Läsion.

Obwohl unsere Studienpatienten unter Allgemeinanästhesie operiert, und weite Exzisionen im makroskopisch gesunden Gewebe durchgeführt wurden, konnten wir in allen Läsionen eine Ausbreitung der Infektion bis zur Peripherie des exzidierten Gewebes einschließlich der Exzisionsränder nachweisen. Die Bestimmung der Exzisionsgröße allein aufgrund des makroskopisch gesunden Aspektes des die Läsion umgebenden Gewebes kann somit eine Progression der Infektion in peripheres subkutanes Fettgewebe nicht sicher ausschließen.

Innerhalb eines Beobachtungszeitraumes von etwa zwei Monaten während des postoperativen stationären Aufenthaltes trat bei keinem der Studienpatienten ein Rezidiv auf. Die klinische Relevanz des Nachweises von *M. ulcerans* in Exzisionsrändern bezüglich des Auftretens von Rezidiven ist jedoch nur durch Langzeitbeobachtung definitiv zu klären. Potentielle Risikopatienten könnten durch PCR-Untersuchung von Gewebeproben aus Exzisionsrändern identifiziert werden. Patienten mit positiven Befunden sollten in jedem Fall während eines Beobachtungszeitraumes von mindestens einem Jahr regelmäßig auf das Auftreten von Rezidiven untersucht werden. Die Kombination mit antimykobakterieller Therapie erscheint zur Verhinderung möglicher Rezidive ratsam.

Excision of pre-ulcerative forms of Buruli Ulcer Disease: a curative treatment?
Herbinger KH, Brieske D, Nitschke J, Siegmund V, Thompson W, Klutse E, Awua-Boteng N Y, Bruhl E, Kunaa L, Schunk M, Adjei O, Loescher T, ***Bretzel G****. Infection 2008. Dec. 3 [Epub ahead of print]*

Seit 2004 gilt die antimykobakterielle Kombinationstherapie mit Rifampicin und Streptomycin, je nach klinischem Verlauf alleine oder in Kombination mit chirurgischer Exzision, als Therapie der Wahl für nicht-ulzerative und ulzerative Formen der Buruli-Erkrankung (52). Aufgrund der weiträumigen Ausbreitung der Bakterien in makroskopisch gesundes, die Läsion umgebendes Gewebe bei ulzerativen Formen kann die chirurgische Exzision nicht mit hinreichender Sicherheit die Entfernung des gesamten infizierten Gewebes gewährleisten (6, 33). Selbst wenige verbleibende Bakterien stellen ein Rezidivrisiko dar (34), somit ist eine antimykobakterielle Therapie ulzerativer Läsionen in jedem Fall sinnvoll. In nicht-ulzerativen Erkrankungsformen dagegen befinden sich die Erreger im Zentrum der Läsion (33). Eine Exzision mit ausreichend breiten Exzisionsrändern sollte daher die vollständige Entfernung des erregerhaltigen Gewebes ermöglichen. Erfahrungsgemäß führt - im Gegensatz zur chirurgischen Therapie ulzerativer Läsionen – die chirurgische Entfernung nicht-ulzerativer Läsionen nur sehr selten zu funktionalen Einschränkungen betroffener Gelenke und Extremitäten. Weiterhin ist die stationäre Verweildauer der Patienten nach Exzision nicht-ulzerativer Läsionen wesentlich kürzer (durchschnittliche Dauer 33,4 Tage; eigene unveröffentlichte Daten) als nach Chirurgie der ulzerativen Formen (durchschnittliche Dauer 84 Tage; eigene unveröffentlichte Daten). Da eine chirurgische Exzision nicht-ulzerativer Läsionen vor diesem Hintergrund durchaus sinnvoll erscheint, wurde in dieser Studie untersucht, ob und unter welchen Bedingungen eine alleinige operative kurative Therapie möglich ist.

Aufgrund der Einführung der antimykobakteriellen Kombinationstherapie konnten für die Studie nur elf chirurgische Patienten mit nicht antibiotisch vorbehandelten nodulären Formen (n=7) oder Plaques (n=4) rekrutiert werden. Die Exzisionen wurden in zwei ghanaischen Behandlungszentren (Agogo Presbyterian Hospital; Dunkwa Governmental Hospital) unter vollständiger Entfernung des subkutanen Fettgewebes bis zur Faszie durchgeführt. Vor der Exzision wurde die geplante Exzisionslinie markiert, und sowohl vor als auch nach der Exzision die medio-lateralen und proximo-distalen Durchmesser der Läsion sowie des gesamten exzidierten Areals vermessen. Die Distanz zwischen makroskopisch sichtbarem äußeren Rand der Läsion und dem äußeren Exzisionsrand wurde als „chirurgische Distanz“

bezeichnet. Diagnostische Proben wurden aus dem Zentrum der jeweiligen Läsion entnommen und nach standardisierten Kriterien mittels Mikroskopie, Kultur, *IS2404* PCR und Histopathologie untersucht Der gesamte Exzisionsrand der Läsionen wurde abgetrennt und in Einzelsegmente (maximalen Größe 10 x 10 mm) unterteilt. Die „chirurgische Distanz“ wurde für jedes Einzelsegment bestimmt. Sämtliche Randsegmente wurden mittels *IS2404* PCR getestet (4, 36, 37, 40). Die Assoziation zwischen abhängigen Variablen (PCR-Ergebnisse) und unabhängigien Variablen (Behandlungszentrum, Alter und Geschlecht, Typ und Lokalisation der Läsion, Krankheitsdauer, Läsions- und Exzisionsgröße, „chirurgische Distanz“) wurde mittels logistischer Regression ermittelt.

Die Patienten blieben nach der Operation bis zur Wundheilung stationär im Behandlungszentrum und wurden 12 – 18 Monate nach dem Eingriff auf das Vorliegen von Rezidiven hin nachuntersucht.

Positive PCR-Ergebnisse waren signifikant mit der „chirurgischen Distanz“ assoziiert ($p < 0.0001$). Bei einer „chirurgische Distanz“ von mehr als 9 mm betrug das Risiko für im Gewebe verbliebene Mykobakterien weniger als 10%, bei einer „chirurgischen Distanz“ von mehr als 13 mm weniger als 5%, bei mehr als 25 mm ging das Risiko gegen Null. Stratifiziert nach Läsionstyp betrug die „chirurgische Distanz“ 26.8 mm für noduläre Läsionen, 22.9 mm für Plaques. Für Patienten mit nodulären Läsionen bestand ein größeres Risiko für die Detektion PCR positiver Randsegemente ($p = 0.025$). Für die anderen unabhängigen Variablen konnte keine signifikante Assoziation nachgewiesen werden.

Während für ulzerative Läsionen in jedem Fall die Indikation zur antimykobakteriellen Therapie gegeben ist, sollten bei der Wahl der Behandlung nicht-ulzerativer Läsionen die möglichen Vorteile der Chirurgie erwogen werden. So kann die operative Entfernung kleiner, nicht-ulzerativer Läsionen in vielen Fällen ambulant durchgeführt werden. Erfordern größere Läsionen einen stationären Aufenthalt, beträgt die mittlere stationäre Verweildauer etwa einen Monat (eigene unveröffentlichte Daten). Die chirurgische Exzision bietet somit für den Patienten eine weniger zeitaufwändige Behandlungsoption als eine bis zu achtwöchige antimykobakerielle Therapie, an die sich bei unzureichender Heilung ein chirugischer Eingriff anschließen kann. Funktionseinschränkungen von Gelenken und/oder Extremitäten sind bei primären operativen Eingriffen selten (eigene unveröffentlichte Daten). Die Diagnostik nicht-ulzerativer, antimykobakteriell behandelter Läsionen erfolgt derzeit mittels Punch-Biopsien und Feinnadelaspiraten. Wird aufgrund des oftmals uncharakteristischen klinischen Erscheinungsbildes nodulärer Formen und Plaques eine differentialdiagnostische

histopathologische Untersuchung erforderlich, steht nur mit chirurgisch exzidiertem Gewebe geeignetes Material zur Verfügung.

Aufgrund der in dieser Studie erhobenen Daten ist davon auszugehen, dass nicht-ulzerative Läsionen unter Wahrung eines ausreichenden Sicherheitsabstandes („chirurgische Distanz“) durch Entfernung des im Läsionszentrum befindlichen infizierten Gewebes kurativ exzidiert werden können. Sollte dies aus anatomischen Gründen nicht möglich sein, wäre die Kombination der primären chirurgischen Exzision mit einer sekundären Antibiose als Alternative denkbar.

The outcome of patients after surgical treatment with or without antimycobacterial treatment in Ghana.
Schunk M, Thompson W, Klutse E, Nitschke J, Asamoah-Opare K, Thompson R, Fleischmann R, Siegmund V, Herbinger KH, Adjei O, Fleischer B, Loescher T, ***Bretzel G****. Am J Trop Med Hyg 2009, in press.*

Die seit 2004 geltenden WHO-Leitlinien zur antimykobakteriellen Kombinationstherapie der Buruli-Erkrankung mit Rifampicin und Streptomycin, je nach klinischem Verlauf alleine oder in Kombination mit chirurgischer Intervention, empfehlen die Durchführung von Follow-up Studien zur Evaluierung des Behandlungserfolges (52). Bereits vor der Einführung der antimykobakteriellen Kombinationstherapie nach WHO-Richtlinen in endemischen Regionen im Jahr 2006 wurde ein Teil der BUD-Patienten zusätzlich zur chirurgischen Exzision antibiotisch behandelt. Allerdings waren vor 2006 sowohl Zeitpunkt als auch Dauer der Antibiotikagabe nicht standardisiert und variierten in den einzelnen Behandlungszentren erheblich. Um die Häufigkeit postoperativer Rezidive, sowie das Auftreten sowohl objektiv messbarer als auch subjektiv empfundener funktioneller Einschränkungen der Beweglichkeit („range of motion“, ROM) als Folge therapeutischer Massnahmen zu untersuchen, führten wir an einer Kohorte von 126 laborbestätigten BUD-Patienten aus Ghana eine Follow-up Studie durch.
Die Patienten waren vor 2006 in zwei ghanaischen Hospitälern einer chirurgischen Behandlung, teils mit, teils ohne begleitende antimykobakterielle Kombinationstherapie, unterzogen worden. Bei der Mehrzahl der Patienten wurde nach weiträumiger Exzision der Läsion eine Spalthauttranplantation durchgeführt, ein Teil der Exzisionswunden wurde durch primäre Wundnaht versorgt, oder zur Sekundärheilung offen gelassen. Das Studienteam (bestehend aus Mitarbeitern meiner Arbeitsgruppe der Abteilung für Infektions- und Tropenmedizin der Universität München [AITM] und des Kumasi Centre for Collaborative Research in Tropical Medicine, Ghana [KCCR]) suchte die Patienten vier bis 29 Monate nach der initialen chirurgischen Behandlung in ihren Heimatdörfern auf und führte mit den Studienteilnehmern teilstandardisierte Interviews zum Krankheitsverlauf vor und nach Durchführung der therapeutischen Maßnahmen durch. Die Befragung zielte insbesondere auf das Auftreten von Rezidiven, sowie Spätkomplikationen im Sinne von Bewegungseinschränkungen. Die Operationsnarben aller Studienteilnehmer wurden hinsichtlich des Wundheilungsstatus untersucht, bei Gelenkbeteiligung wurde mittels goniometrischer Untersuchung die passive Gelenkbeweglichkeit dokumentiert.

Abweichungen von international standardisierten ROM-Richtwerten wurden als Bewegungseinschränkung gewertet. Darüber hinaus wurde die subjektive Einschätzung der Patienten bezüglich funktioneller Einschränkungen erfragt. Von zum Zeitpunkt der Untersuchung vorhandenen akuten, BUD-verdächtigen Läsionen wurden diagnostische Proben zur Laboruntersuchung am KCCR entnommen (36, 47). Gemäß den zum Studienzeitpunkt gültigen WHO-Definitionen (48), wurde das Auftreten einer weiteren, klinisch mit BUD vereinbaren Läsion innerhalb eines Jahres nach chirurgischer Exzision -mit oder ohne begleitende antibiotische Behandlung- als Rezidiv gewertet.

Neunundsiebzig (61%) Patienten der Studienkohorte konnten in ihren Heimatdörfern aufgefunden, befragt und untersucht werden. Die Mehrzahl der Patienten (82%) war zusätzlich zur chirurgischen Exzision antibiotisch behandelt worden. Hierbei variierten jedoch Behandlungszeitpunkt und Behandlungsdauer erheblich, einige Patienten hatten lediglich ein Medikament erhalten. In sieben Fällen (9%) konnten zum Studienzeitpunkt bzw. retrospektiv für den Zeitraum zwischen chirurgischer Exzision und Durchführung der Follow-up Studie aktuelle (n=1) oder Interims-Rezidive (n=6) dokumentiert werden. Von insgesamt vier der sieben Rezidivpatienten waren diagnostische Proben untersucht worden, drei Fälle konnten durch mindestens einen positiven Labortest bestätigt werden. Die Primärläsionen aller Patienten mit Interims-Rezidiven waren zusätzlich zur chirurgischen Exzision antibiotisch behandelt worden, die Rezidiv-Läsionen wurden einer rein chirurgischen Therapie unterzogen. Alle Operationswunden waren zum Untersuchungszeitpunkt komplikationslos abgeheilt. Für keinen der sieben Rezidivpatienten konnten spezifische Risikofaktoren (untersuchte Parameter: Größe der Primärläsion, Dauer der antimykobakteriellen Therpie, Erkrankungsdauer vor chirurgischer Intervention, Behandlungszentrum) identifiziert werden.

Bei 21 Studienpatienten (27%) wurde aufgrund reduzierter ROM-Werte eine funktionelle Einschränkung der Gelenkbeweglichkeit dokumentiert. Anamnestisch war bei neun dieser Patienten bereits vor der Exzision eine durch die Läsion selbst verursachte Beweglichkeitseinschränkung bekannt, in 12 Fällen handelte es sich um Spätkomplikationen nach therapeutischen Maßnahmen. Elf der 21 Patienten (52%) fühlten sich auch subjektiv in den Aktivitäten ihres täglichen Lebens eingeschränkt. Die Analyse möglicher Risikofaktoren ergab für drei unabhängige Variablen (Ausdehnung der Läsion über ein oder mehrere Gelenke, Läsionstyp [nicht noduläre Formen], Technik des Wundverschlusses [Spalthauttransplantate] eine signifikante Assoziation mit reduzierten ROM-Werten. Subjektiv fühlten sich 17 Studienpatienten (22%) durch funktionelle Einschränkungen in den

Aktivitäten ihres täglichen Lebens behindert, in sechs dieser Fälle konnten jedoch goniometrisch keine reduzierten ROM-Werte festgestellt werden.

Nach aktueller Datenlage treten bei weniger als zwei Prozent der nach WHO-Standards antibiotisch therapierten Patienten Rezidive auf (50). In älteren Arbeiten wurden jedoch Rezidivraten von 17% bzw. 20% für rein chirurgisch therapierte Patienten (1, 22), sowie 35% für eine Patientenkohorte aus Ghana, die vor 2001 in den gleichen Hospitälern wie unsere Studienpatienten chirurgisch (in 29% mit begleitender Rifampicin-Monotherapie) behandelt worden war, dokumentiert (44). Nur eine Studie aus Benin ermittelte an chirurgisch therapierten Patienten (davon 15% mit begleitender antimykobakterieller Therapie) eine unseren Ergebnissen vergleichbare Rezidivrate von sechs Prozent (9). Unseren Daten zufolge beeinflussten weder Größe oder Art der Läsion, noch die Erkrankungsdauer vor Therapiebeginn die Wahrscheinlichkeit des Auftretens von Rezidiven. Die relativ niedrige Rezidivrate unserer Studienkohorte kann vielmehr durch die Verbesserung chirurgischer Technik aufgrund kontinuierlicher Trainingsmaßnahmen in Ghana erklärt werden. Weiterhin lag der Anteil antimykobakteriell behandelter Patienten mit 82% in unserem Patientenkollektiv deutlich höher als in anderen Studien. Trotz fehlender Standardisierung in unserer Studienkohorte ist von einem positiven Effekt der begleitenden antibiotischen Therapie hinsichtlich einer Senkung der Rezidivrate auszugehen.

Die Häufigkeit von funktionellen Einschränkungen und Behinderungen, die als Spätkomplikation der Erkrankung selbst sowie therapeutischer Maßnahmen auftreten, wurde bislang kaum untersucht. Zwei Studien aus Ghana und der Demokratischen Republik Kongo dokumentierten für 49% bzw. 37% der Patienten Bewegungseinschränkungen nach Therapie. Die in unserer Studie goniometrisch ermittelten 27% entsprechen den von der WHO geschätzten 20-25% (12, 29, 49). Die objektiv messbaren funktionellen Einschränkungen deckten sich jedoch nicht mit dem vom Patienten subjektiv empfundenen Grad der Behinderung. Der auch in unserer Studie relativ hohe Anteil von Patienten mit objektiv messbaren funktionellen Einschränkungen sowie subjektiv empfundenen Behinderungen zeigt die Notwendigkeit präventiver Maßnahmen. Die Evaluierung modifizierter chirurgischer Techniken sowie der verstärkte Einsatz physiotherapeutische Maßnahmen könnten wirkungsvoll zur Senkung therapieinduzierter Behinderungen beitragen.

Zusammenfassung und Bewertung der Forschungsergebnisse

Diagnostik

Der erste Schritt zur Etablierung eines Netzwerkes zur Labordiagnostik des Buruli Ulkus in Ghana bestand in der Entwicklung und Validierung einer durch die Verwendung lyophilisierter Reagenzien an tropische Bedingungen adaptierten diagnostischen *IS2404* PCR („DRB-PCR“) in meiner Arbeitsgruppe am Bernhard Nocht Institut für Tropenmedizin (BNITM) (37). Die Methode basiert auf der von Stinear et al. entwickelten *IS2404* PCR (40)**.** Analytische Sensitivität und Spezifität der DRB-PCR entsprechen der Standardmethode, die vergleichende Testung diagnostischer Proben mit beiden Methoden erbrachte ebenfalls vergleichbare Ergebnisse. Die DRB-PCR wurde 2003 von mir am Kumasi Centre for Collaborative Research in Tropical Medicine, Kumasi, Ghana (KCCR) etabliert und aufgrund unserer Validierungsergebnisse für die Routineanwendung in Ghana freigegeben. Der relativ geringe Anteil (unter 40%) positiv getesteter diagnostischer Proben aus unseren initialen Validierungstestreihen konnte auf zwei wesentliche Faktoren, nämlich unsachgemäße Probenabnahme sowie einen hohen Anteil von Läsionen im Spät- bzw. Heilungsstadium (gemäß histologischem Befund) zurückgeführt werden (37).

Eine Verbesserung der diagnostischen Sensitivität der Labordiagnostik des Buruli Ulkus war nur durch umfangreiche Trainingsmaßnahmen in allen beteiligten Institutionen zu erreichen. Von mir und Mitarbeitern meiner Arbeitsgruppen am BNITM (bis September 2004) und (ab Oktober 2004) der Abteilung für Infektions- und Tropenmedizin des Klinikums der Universität München (AITM) wurden daher zunächst standardisierte Kriterien zur Abnahme diagnostischer Proben erarbeitet und die Mitarbeiter der chirurgischen Abteilungen der (anfangs drei, mittlerweile neun) kooperierenden Behandlungszentren in Ghana vor Ort entsprechend geschult. Ein wichtiges Instrument für die standardisierte Probenabnahme war ein von uns entwickeltes Probenabnahmeset, welches alle benötigten Probengefäße mit entsprechenden Transportmedien sowie von uns konzipierte Labor- und Dokumentationsformulare enthielt, und den Behandlungszentren zur Verfügung gestellt wurde (4, 5). Zur Verbesserung der Früherkennung der Erkrankung in ländlichen Regionen und Gemeinden führten wir außerdem in Zusammenarbeit mit dem KCCR entsprechende Trainingsworkshops für „village health workers“ durch.

Um eine gleichbleibende Qualität der Labordiagnostik im KCCR zu gewährleisten, führten wir ebenfalls seit 2004 mindestens zweimal jährlich vor Ort Trainingsmaßnahmen für das ghanaische Laborpersonal durch. Zur Standardisierung aller Prozesse erstellten wir

Standardarbeitsanweisungen (SOPs) für die im KCCR durchgeführten Labormethoden. Die SOPs wurden 2007 unter Einbeziehung präanalytischer (Probenabnahme) und postanalytischer Prozesse (Datenmanagement) in einem Laborhandbuch zusammengefasst („Laboratory Manual, BURULICO, WP3, Version 1, November 2007"). Um die Datenerfassung und –auswertung zu standardisieren, entwickelten wir eine auf die Erfordernisse unseres Projektes zugeschnittene Datenbank (Microsoft Access), die einen Export der Daten in Excel Tabellen zur weiteren statistischen Auswertung erlaubt.

Zur externen Qualitätskontrolle von Mikroskopie und DRB-PCR wurde ein standardisiertes System etabliert. Mikroskopische Präparate wurden von mir und Mitarbeitern meiner Arbeitgruppe gegengelesen, PCR-Proben am AITM parallel getestet. Präparate/Proben mit abweichenden Ergebnissen wurden erneut durch AITM-Mitarbeiter untersucht, wobei das Ergebnis dieser Untersuchung als Endergebnis gewertet wurde. Zu Trainingszwecken wurden Präparate/Proben mit abweichenden Ergebnissen von KCCR-Personal unter Supervision von AITM-Mitarbeitern erneut analysiert. Für 287 mikroskopische Präparate sowie 265 PCR Proben, die von 2004 bis 2006 untersucht wurden, liegen folgende Ergebnisse vor: Die Übereinstimmungsrate für Mikroskopie lag zunächst bei 83% (falsch negativ: 27.1%, falsch positiv: 10.1%), durch erneutes Lesen unter Supervision wurden 98% Übereinstimmung (falsch negativ: 4.2%, falsch positiv: 0.6%) erreicht. Die PCR-Übereinstimmungsrate verbesserte sich von 88% (falsch negativ: 8.2%, falsch positiv: 19.1%) auf 96% (falsch negativ: 4.7%, falsch positiv: 2.1%). Externe Qualitätssicherung und die damit verbundenen Trainingsmaßnahmen konnten somit die Qualität der Diagnostik im ghanaischen Partnerlabor deutlich verbessern und werden seither kontinuierlich weitergeführt (4).

Zum Zeitpunkt der Etablierung unseres diagnostischen Netzwerkes waren durch die WHO zwei positive Laborresultate als Kriterium für eine positive Labordiagnose vorgegeben (47). Daher wurden diagnostische Abstriche und Gewebeproben meist simultan mittels aller verfügbaren diagnostischen Methoden (Mikroskopie, Kultur, PCR und Histopathologie) untersucht. Um diese sowohl kosten- als auch arbeitsintensive Vorgehensweise zu revidieren, analysierten wir retrospektiv die labordiagnostischen Ergebnisse von 161 klinisch diagnostizierten Buruli-Verdachtsfällen aus vier ghanaischen Behandlungszentren. Wurde einer positiven Labordiagnose nur ein positives Laborergebnis zugrunde gelegt, liessen sich etwa 20% mehr Verdachtsfälle bestätigen als im Falle einer auf zwei positiven Resultaten beruhenden Diagnose. Wir konnten weiterhin in unserem untersuchten Kollektiv für Mikroskopie und PCR Spezifitäten von 98% bzw. 100% nachweisen und halten somit ein positives Laborergebnis für ausreichend. Die mikroskopische Untersuchung von

Gewebeproben aus nicht-ulzerativen Läsionen erbrachte 40%, die anschließende PCR-Untersuchung mikroskopisch negativer Proben weitere 25% positiver Ergebnisse. Die mikroskopische Untersuchung diagnostischer Abstriche aus ulzerativen Läsionen konnte 30%, die anschließende PCR-Untersuchung mikroskopisch negativer Abstriche weitere 40% der Verdachtsfälle bestätigen. Die sukzessive Anwendung von Mikroskopie und PCR erlaubte somit im untersuchten Kollektiv die Laborbestätigung von bis zu 70% der klinischen Verdachtsfälle. Da durch sukzessive Anwendung diagnostischer Labormethoden auch die Testkosten um bis zu 60% reduziert werden können, sollten die verfügbaren Laboruntersuchungen im Sinne einer Stufendiagnostik kombiniert werden, Unsere Daten zeigen weiterhin, dass zur primären Diagnostik ulzerativer Läsionen Abstriche untersucht werden sollten. Die Entnahme von Gewebeproben ist nur bei negativem Abstrich erforderlich und sinnvoll (5).

Nach zweijähriger Routineanwendung in Ghana von 2004 bis 2006 führten wir eine vergleichende Analyse von DRB-PCR und anderen diagnostischen Labormethoden durch, um die Leistung der Methode unter Feldbedingungen zu evaluieren. Die Übereinstimmungsraten zwischen DRB-PCR und Standard-PCR lagen, vergleichbar zu unseren initialen Validierungstestreihen (37), bei 92% bzw. 95% für Abstriche und Gewebeproben. Der Anteil mittels DRB-PCR insgesamt positiv getesteter Proben betrug für Abstriche und Gewebeproben jeweils 61%, ein signifikanter Unterschied zur Standard-PCR (Abstriche 67%, Gewebeproben 62%) war nicht nachzuweisen. Der insgesamt deutlich höhere Anteil DRB-PCR positiver Proben im Vergleich zur initialen Validierungsstudie (37) ist hierbei auf die Einführung standardisierter Probenabnahmetechniken, unsere Schulungsmaßnahmen, sowie ein verbessertes „case finding“ und (dadurch bedingt) die Untersuchung von Patienten mit meist frühen (aktiven) Läsionen zurückzuführen. Analyse der Daten nach Probenursprung ergab jedoch vor allem für Gewebeproben Unterschiede um bis zu 30% zwischen einzelnen Behandlungszentren, was auf einen kontinuierlichen Schulungsbedarf in der Probenabnahmetechnik schließen lässt. Der Anteil DRB-PCR positiver Proben war insgesamt signifikant höher als der Anteil mittels Mikroskopie und Kultur erzielter positiver Laborresultate. Wie bereits in unserer Pilotstudie (37) konnten bakteriologisch negative Heilungs-, bzw. Spätstadien nur durch histopathologische Untersuchung identifiziert werden. Die DRB-PCR kann somit - eine gute Qualität der Probenabnahme vorausgesetzt - auch unter Feldbedingungen als verlässliches diagnostisches Instrument eingestuft werden, eignet sich aber nur für die Labordiagnostik früher, bakterienhaltiger Läsionen (36).

Während bis zum Jahr 2006 die Laborbestätigung klinisch diagnostizierter, chirurgisch behandelter BUD-Verdachtsfälle für unsere Arbeit im Vordergrund stand, gewann mit der Einführung der antimykobakteriellen Therapie in Ghana die Bestätigung der Verdachtsdiagnose durch Untersuchung von Abstrichen und Punch-Biopsien vor Behandlungsbeginn zunehmend an Bedeutung. Im Rahmen des EU-geförderten Forschungsprojektes BURULICO führten wir eine vergleichende Studie zur Sensitivität diagnostischer Teste an unterschiedlichen Untersuchungsmaterialien in Abhängigkeit von Erkrankungsform und Behandlungsart durch. Von rein chirurgischen Patienten, bzw. chirurgischen Patienten mit antibiotischer Vorbehandlung wurden, wie schon in den vorangehenden Studien, Gewebeproben aus chirurgischem Exzisionsmaterial und/oder Abstriche zur Untersuchung entnommen. Von zur antimykobakteriellen Therapie vorgesehenen, zum Zeitpunkt der Probenentnahme nicht antibiotisch vorbehandelten Patienten wurden neben Abstrichen erstmals 3 mm Punch-Biopsien zur Labordiagnostik verwendet. Insgesamt war die *IS2404*-DRB-PCR, unabhängig von Art des untersuchten Probenmaterials, Läsionstyp und Behandlungsgruppe, der Test mit der höchsten Sensitivität (85.4%), gefolgt von Mikroskopie (56.7%), und *IS2404*-PCR bestätigter Kultur (48.0%). In der Gruppe nicht antibiotisch vorbehandelter Patienten mit nicht-ulzerativen Läsionen ergab die Untersuchung von 3 mm Punch-Biopsien signifikant höhere Sensitivitäten (DRB-PCR: 93.5%, *IS2404*-PCR bestätigte Kultur: 70.8%) als die Untersuchung von Gewebepräparaten chirurgisch behandelter Patienten (DRB-PCR: 66.7%, *IS2404*-PCR bestätigte Kultur: 40.0%). 3 mm Punch-Biopsien eignen sich somit uneingeschränkt zur Diagnostik nicht-ulzerativer Läsionen. Mit einer Gesamtsensitivität von 84.7% war die DRB-PCR diagnostischer Abstriche aus ulzerativen Läsionen in allen Behandlungsgruppen der DRB-PCR von Gewebeproben (Gesamtsensitivität aller Gewebeproben 64.7%) deutlich überlegen und kann somit als Methode der Wahl zur Diagnostik ulzerativer Läsionen gelten. Unsere Daten belegten weiterhin den Zusammenhang zwischen der Dauer antimykobakterieller Therapie vor Probenabnahme und der Abnahme der Sensitivitäten von Kultur und PCR. Die Isolierung unterschiedlicher Mykobakterienspezies von einigen Patienten unseres Studienkollektives während oder nach antimykobakterieller Therapie weist auf die Möglichkeit von Co- oder Superinfektionen von BUD-Läsionen hin.

Mit der in meiner Arbeitsgruppe entwickelten, auf lyophylisierten Reagenzien basierenden DRB-*IS2404* PCR steht erstmals eine an tropische klimatische Bedingungen adaptierte molekulare Methode zur Labordiagnostik des Buruli Ulkus zur Verfügung. Aufgrund unserer

Validierungsergebnisse ist die Methode in Sensitivität und Spezifität der diagnostischen Standard-*IS2404* PCR nach Stinear vergleichbar (37, 40). Die DRB-PCR wird seit 2004 erfolgreich zur Routinediagnostik des Buruli Ulkus in Ghana angewandt (36) und seit 2007 von der WHO zur Anwendung unter Feldbedingungen empfohlen (51). Der in einer vergleichenden Studie in Ghana erzielte Gesamtanteil DRB-PCR positiver Ergebnisse (61%) lag niedriger als in anderen Publikationen (15, 26, 30, 39). Eine Analyse unserer Daten nach Ursprung der Proben zeigte jedoch, dass dies nicht auf die Methode an sich, sondern vielmehr auf die Qualität der Probenabnahme in unterschiedlichen Behandlungszentren zurückzuführen ist. Werden diagnostische Proben von geschultem chirurgischem Personal entnommen, liegt der Anteil PCR-positiver Proben um bis zu 20% über dem Durchschnittswert dieser Studie (36). Analog zu anderen Studien (26, 30, 31, 39) konnten mittels DRB-PCR signifikant mehr Buruli-Verdachtsfälle bestätigt werden als mit anderen lokal verfügbaren Untersuchungen.

Im Rahmen unseres Netzwerkes zur Labordiagnostik des Buruli Ulkus publizierten wir die bislang einzigen Daten zur externen Qualitätssicherung von Mikroskopie und PCR in einem diagnostischen Labor eines Buruli-Endemiegebietes. Unsere Daten belegen eine seit 2004 gleichbleibende gute Qualität der lokalen Labordiagnostik, beweisen andererseits aber auch die Notwendigkeit kontinuierlicher Qualitätssicherungsmaßnahmen (4).

Wir konnten anhand unserer Daten weiterhin zeigen, dass aufgrund der hohen Spezifität von Mikroskopie und PCR in klinisch diagnostizierten Buruli-Verdachtsfällen beide Methoden keinen weiteren Bestätigungstest benötigen. Wird einer positiven Labordiagnose nur ein positives Laborergebnis zugrunde gelegt, lassen sich darüber hinaus unseren Daten zufolge mindestens 20% mehr Verdachtsfälle bestätigen als im Falle einer auf zwei positiven Resultaten beruhenden Diagnose. Somit sollte das bisher gültige Kriterium der von Seiten der WHO vorgegebenen zwei positiven Laborteste (47) revidiert werden (5, 16).

Während die in anderen Publikationen erhobenen Daten hauptsächlich auf der Untersuchung exzidierten Gewebes beruhen und verschiedene Läsionstypen sowie weitere diagnostische Proben bei der Analyse keine Berücksichtigung fanden (14, 15, 26, 31, 39, 55), konnten wir erstmals die Positivitätsraten für verschiedene Labormethoden, diagnostische Proben und Läsionstypen aufzeigen. Wir konnten auch den ersten Nachweis erbringen, dass die Untersuchung diagnostischer Abstriche als nicht invasives Verfahren die Methode der Wahl für die Labordiagnostik ulzerativer Läsionen darstellt, und auf diese Weise bis zu 70% der Verdachtsfälle bestätigt werden können. Die invasive Entnahme von Gewebeproben ist nur bei negativem Abstrich erforderlich. Weiterhin können durch Kombination von Mikroskopie

und PCR im Sinne einer Stufendiagnostik sowohl Arbeitsaufwand als auch Laborkosten beträchtlich gesenkt werden (5).

In der Mehrzahl der bisher publizierten Arbeiten zur Labordiagnostik des Buruli Ulkus wurden nicht Sensitivitätsraten im eigentlichen Sinn, sondern Positivitätsraten für die untersuchten Teste, d.h. die Anzahl positiver Testergebnisse eines Testes bezogen auf die Gesamtzahl der untersuchten BUD-Verdachtsfälle bestimmt (3, 14, 26, 31, 36, 55). Bisher beinhalten nur zwei Arbeiten Angaben zur Sensitivität der *IS2404* PCR (15, 39). Meine Arbeitsgruppe führte die bislang größte vergleichende Studie zur Sensitivität diagnostischer Methoden an unterschiedlichen diagnostischen Materialien in Abhängigkeit von Erkrankungsform und Behandlungsart durch (16). Wir ermittelten hierbei die echte Sensitivität der entsprechenden Teste, definiert als die Anzahl der mit diesem Test erzielten positiven Ergebnisse bezogen auf die Anzahl der Patienten, die in allen durchgeführten diagnostischen Testen in mindestens einem dieser Teste positiv getestet wurden. Wir evaluierten als erste Arbeitsgruppe 3 mm Punch-Biopsien als diagnostisches Untersuchungsmaterial. Unsere Daten belegen, dass 3 mm Punch-Biopsien zur Diagnostik ebenso geeignet sind, wie die von Phillips et al untersuchten 4 und 6 mm Punch Biopsien (30). Wir konnten darüber hinaus erstmals zeigen, dass PCR und Kultur von 3 mm Punch-Biopsien aufgrund ihrer hohen Sensitivität uneingeschränkt zur Diagnostik von nicht-vorbehandelten Patienten mit nicht-ulzerativen Läsionen empfohlen werden können. Für ulzerative Läsionen aus allen Behandlungsgruppen erwies sich die PCR Untersuchung von Abstrichen als Methode der Wahl. Früher erhobene Daten meiner Arbeitsgruppe konnten somit bestätigt werden (5). Wir konnten erstmals an einem größeren Patientenkollektiv eine Korrelation zwischen der Dauer antimykobakterieller Therapie vor Probenabnahme und einer signifikanten Abnahme der Sensitivität von PCR und Kultur aufzeigen. Da jedoch auch nach mehrwöchiger Therapie in mehr als 50% der Patienten der Nachweis von *M. ulcerans* DNA möglich war, stützen unsere Ergebnisse die aktuellen TAG-Empfehlungen (53) zur Sicherung der klinischen Diagnose von BUD-Rezidiven durch kulturellen Nachweis. Die von meiner Arbeitsgruppe erstmals beschriebe Isolierung von atypischen Mykobakterien aus BUD-Läsionen unter antimykobakterieller Therapie zeigt jedoch, dass der kulturelle Nachweis allein ohne weitere Bestätigungsteste nicht zur Abgrenzung von Rezidiven und Therapieversagern von Co- oder Superinfektionen geeignet ist.

Therapie

Bis zur offiziellen Empfehlung der antimykobakteriellen Therapie des Buruli Ulkus im Jahr 2004 durch die WHO (52) galt die weiträumige chirurgische Exzision als Therapie der Wahl (48). Therapieerfolg und Rezidivraten variierten je nach angewandter Operationstechnik (1, 9, 22, 44). Chirurgisches Vorgehen und Exzisionsgröße wurden allein von Augenmaß und Erfahrung des Operateurs bestimmt. Um eine mögliche Ausbreitung der Infektion in makroskopisch gesundes Gewebe nachzuweisen, untersuchten wir die Exzisionsränder der Operationspräparate von 20 Patienten mit ulzerativen Läsionen mittels verschiedener Labormethoden auf das Vorliegen von *M. ulcerans*. Obwohl unsere Studienpatienten unter Allgemeinanästhesie operiert, und weite Exzisionen im makroskopisch gesunden Gewebe durchgeführt wurden, ergab die Laboranalyse aller Läsionen eine Ausbreitung der Infektion bis zur Peripherie des exzidierten Gewebes einschließlich der Exzisionsränder. Die Bestimmung der Exzisionsgröße allein aufgrund des makroskopisch gesunden Aspektes des die Läsion umgebenden Gewebes kann somit eine Progression der Infektion in peripheres subkutanes Fettgewebe nicht sicher ausschließen. Innerhalb eines Beobachtungszeitraumes von etwa zwei Monaten während des postoperativen stationären Aufenthaltes trat bei keinem unserer Studienpatienten ein Rezidiv auf. Die klinische Relevanz des Nachweises von *M. ulcerans* in Exzisionsrändern bezüglich des Auftretens von Rezidiven ist jedoch nur durch Langzeitbeobachtung definitiv zu klären. Die Kombination mit antimykobakterieller Therapie erscheint im Falle ulzerativer Läsionen ratsam (6).

Da sich - im Gegensatz zu Ulzera - die Erreger in nicht-ulzerativen Läsionen im Zentrum der Läsion befinden (33), sollte eine weitere Studie klären, ob eine Exzision nodulärer Läsionen und Plaques bakterienhaltiges Gewebe vollständig entfernen, somit als kurative Therapie gelten kann. Wir untersuchten hierfür die Exzisionsränder der Operationspräparate von elf Patienten mit nicht-ulzerativen Läsionen mittels PCR. Der bakteriologische Befund wurde für jedes Einzelsegment mit dem Abstand zwischen Läsions- und Exzisionsrand („chirurgische Distanz“) korreliert. Positive PCR-Ergebnisse waren signifikant mit der „chirurgischen Distanz“ assoziiert. Bei einer „chirurgischen Distanz“ von mehr als 9 mm betrug das Risiko für im Gewebe verbliebene Mykobakterien weniger als 10%, bei einer „chirurgische Distanz“ von mehr als 13 mm weniger als 5%, bei mehr als 25 mm ging das Risiko gegen Null. Aufgrund unserer Daten ist somit davon auszugehen, dass nicht-ulzerative Läsionen unter Wahrung eines ausreichenden Sicherheitsabstandes durch Entfernung des im Läsionszentrum befindlichen infizierten Gewebes kurativ exzidiert werden können. Während für ulzerative Läsionen in jedem Fall die Indikation zur antimykobakteriellen Therapie gegeben ist, sollten

bei der Wahl der Behandlung nicht-ulzerativer Läsionen die möglichen Vorteile der Chirurgie erwogen werden. Bei kleinen Läsionen besteht die Möglichkeit ambulanter Eingriffe. Auch die Chirurgie größerer Läsionen mit einer begrenzten stationären Verweildauer bietet dem Patienten eine weniger zeitaufwändige Behandlungsoption als eine bis zu achtwöchige antimykobakterielle Therapie (17).

Um die Häufigkeit von Rezidiven nach Therapie, sowie das Auftreten sowohl objektiv messbarer als auch subjektiv empfundener funktioneller Einschränkungen der Beweglichkeit („range of motion“, ROM) als Folge therapeutischer Maßnahmen zu untersuchen, führte meine Arbeitsgruppe an einer Kohorte von 126 laborbestätigten BUD-Patienten aus Ghana eine Follow-up Studie durch. Neunundsiebzig Patienten (61%) konnten in ihren Heimat-dörfern aufgefunden, befragt und körperlich untersucht werden. Die Patienten waren vor Einführung der standardisierten antimykobakteriellen Therapie gemäß WHO-Leitlinien (52) in Ghana durch chirurgische Intervention, in 82% mit begleitender - allerdings hinsichtlich Behandlungszeitpunkt und -dauer nicht standardisierter - antibiotischer Therapie behandelt worden. Bei der Mehrzahl der Patienten wurde nach weiträumiger Exzision der Läsion eine Spalthauttranplantation durchgeführt, ein Teil der Exzisionswunden wurde durch primäre Wundnaht versorgt, oder zur Sekundärheilung offen gelassen. Die Rezidivrate in unserer Kohorte betrug neun Prozent. Wir konnten für keinen der betroffenen Patienten spezifische, zur Rezidiventstehung prädisponierende Risikofaktoren ermitteln. Goniometrische Messung der passiven Gelenkbeweglichkeit ergab für 21 (27%) der Studienpatienten reduzierte ROM-Werte. Anamnestisch war für neun dieser Patienten bereits vor der Exzision eine durch die Läsion selbst verursachte Beweglichkeitseinschränkung bekannt, in 12 Fällen handelte es sich um Spätkomplikationen nach therapeutischen Maßnahmen. Reduzierte ROM-Werte waren signifikant mit drei unabhängigen Variablen, nämlich Ausdehnung der Läsion über ein oder mehrer Gelenke, Läsionen des nicht-nodulären Typs, und Anwendung von Spalthauttransplantaten zum Wundverschluss assoziiert. Die objektiv messbaren funktionellen Einschränkungen bei Patienten mit reduzierten ROM-Werten entsprachen nicht dem subjektiv empfundenen Grad der Behinderung. Unter den 21 Patienten mit goniometrisch ermittelten reduzierten ROM-Werten fühlten sich nur 52% in den Aktivitäten ihres täglichen Lebens eingeschränkt, während unter insgesamt 17 Patienten, die subjektive Behinderungen angaben, nur bei 11 (65%) reduzierte ROM-Werte gemessen wurden. Die relativ niedrige Rezidivrate in unserer Studienkohorte im Vergleich zu älteren Studien (1, 22, 44) lässt sich auf die Verbesserung chirurgischer Technik durch kontinuierliche Trainingsmaßnahmen in Ghana, sowie durch den hohen Anteil zusätzlich antimykobakteriell therapierter Patienten

zurückführen. Der relativ hohe Anteil objektiv messbarer sowie subjektiv empfundener funktioneller Einschränkungen und Behinderungen weist auf die Notwendigkeit präventiver, physiotherapeutischer Maßnahmen hin.

Die von uns nachgewiesene Progression einer *M. ulcerans* Infektion in makroskopisch gesundes Gewebe wurde in einer zum gleichen Zeitpunkt veröffentlichten Studie bestätigt. Rondini et al. konnten mittels quantifizierender Real-Time PCR ebenfalls *M. ulcerans* in den Randbereichen chirurgisch exzidierter Ulzera nachweisen (33). Eine weiteren Arbeit der gleichen Autoren dokumentiert darüber hinaus die Entstehung eines Rezidives nach postoperativ im Gewebe verbliebenen Bakterien (34). Aufgrund dieser Befunde ist eine vollständige Entfernung infizierten Gewebes durch chirurgische Exzision nicht anzunehmen, Rezidive können durch alleinige chirurgische Behandlung nicht sicher vermieden werden. Unsere Ergebnisse stützen somit die gegenwärtig gültigen Empfehlungen zur amtimykobakteriellen Therapie ulzerativer Läsionen (3, 7, 13, 19, 28, 52)**.**

Zur chirurgischen Exzision nicht-ulzerativer Läsionen existieren in der Literatur generell nur wenige Arbeiten (1, 21, 48), der Stellenwert der Chirurgie als mögliche Alternative zur antimykobakteriellen Therapie wurde bislang nur von meiner Arbeitsgruppe untersucht. Die operative Therapie nicht-ulzerativer Läsionen ohne vorherige Antibiose wird derzeit nach unseren Empfehlungen in Togo evaluiert.

Kanga et al. und Amofah et al. dokumentierten für rein chirurgisch therapierte Patienten aus der Elfenbeinküste und Ghana Rezidivraten von 17% bzw. 20% (1, 22). Teelken et al. gaben für eine Patientenkohorte aus Ghana, die vor 2001 in den gleichen Behandlungszentren wie unsere Studienpatienten operiert worden war (wobei 29% der Patienten zusätzlich Rifampicin als Monotherapie erhalten hatten), eine Rezidivrate von 35% an (44). Die in unserer Studie für eine Kohorte chirurgisch behandelter, größtenteils zusätzlich antimykobakteriell therapierter BUD-Patienten ermittelte Rezidivrate, liegt im Vergleich zu diesen älteren Daten relativ niedrig. Nur eine Studie aus Benin ermittelte für chirurgisch therapierte Patienten (davon 15% mit begleitender antimykobakterieller Kombinationstherapie) eine unseren Daten vergleichbare niedrige Rezidivrate von sechs Prozent (9). Es ist davon auszugehen, dass die Verbesserung chirurgischer Technik durch kontinuierliche Trainingsmaßnahmen in beiden Ländern zur Senkung der Rezidivraten beitrug. Bei nach derzeitigen WHO-Standards antimykobakteriell therapierten Patienten treten nach aktueller Datenlage in weniger als zwei Prozent Rezidive auf (50, 52). Die Patientenkohorten der genannten älteren Studien wurden nicht (1, 22) bzw. nur in einem geringen Prozentsatz mit antimykobakteriellen Medikamenten

in Form einer Rifampicin-Monotherapie (44) behandelt. In unserer Studie dagegen erhielten bereits 82% der Patienten antimykobakterielle Therapie. Unsere Daten zeigten somit bereits vor Einführung der standardisierten Kombinationstherapie nach WHO-Standards einen positiven Effekt der in unserer Studie angewandten begleitenden, wenngleich nicht standardisierten, antimykobakteriellen Therapie auf die Senkung der Rezidivraten.

Gemäß zum Studienzeitpunkt geltender Definitionen (48) wurde in unserer Patientenkohorte jeder Patient mit einer neuen Läsion, die innerhalb eines Jahres nach Therapie auftrat, als Rezidivpatient klassifiziert. Durch die im Zuge der Einführung standardisierter antimykobakterieller Therapie geänderten, seit 2007 und 2008 geltenden Definitionen werden jedoch gegenwärtig nur Läsionen die mehr als drei Monate nach einem kompletten antimykobakteriellen Therapiezyklus auftreten und durch kulturellen Erregernachweis bestätigt werden, als echte Rezidive eingestuft (53, 54). Ein Teil unserer Rezidivpatienten müsste demnach eher der Kategorie der „non-healers“ zugeordnet werden. Eine Neubewertung unserer Ergebnisse vor dem Hintergrund der aktuellen Definitionen ist allerdings problematisch. Unsere Studienpatienten wurden weder mit der achtwöchigen antimykobakteriellen Therapie nach derzeitigem Standard behandelt, noch bestand unter Feldbedingungen die Möglichkeit der standardisierten Probenabnahme zum kulturellen Erregernachweis – somit sind die Grundvoraussetzungen zur Anwendung der aktuellen Falldefinitionen auf unsere Rezidivpatienten nicht gegeben.

Zur Häufigkeit funktioneller Einschränkungen und Behinderungen als Spätfolgen der Erkrankung selbst sowie therapeutischer Maßnahmen existieren bislang nur wenige Studien. Für zwei Patientenkohorten aus Ghana und der Demokratischen Republik Kongo wurden für 49% bzw. 37% der Patienten Bewegungseinschränkungen nach Therapie dokumentiert (12, 29). Die in unserer Studie aufgrund reduzierter ROM-Werte ermittelten 27% funktionellen Einschränkungen bzw. von 22% der Patienten subjektiv empfundenen Behinderungen entsprechen den von der WHO geschätzten 20-25% (49). Der auch in unserer Studie relativ hohe Anteil an Spätkomplikationen unterstreicht analog zu aktuellen WHO-Empfehlungen die Bedeutung präventiver physiotherapeutischer Maßnahmen für die Behandlung von BUD-Patienten (49, 50).

Weiterführende Studien

Evaluierung von Feinnadelaspiraten im Vergleich zu anderen Untersuchungmaterialien

Mit der Einführung der antimykobakteriellen Therapie im Jahr 2006 gewann die Laborbestätigung der klinischen Diagnose vor Beginn der medikamentösen Therapie zunehmend an Bedeutung (19, 50). Neben dem diagnostischen Abstrich aus ulzerativen Läsionen, sind hierfür vor allem Punch-Biopsien sowie Feinnadelaspirate aus nicht-ulzerativen Läsionen geeignet.

Als Alternative zu Punch-Biopsien empfiehlt die WHO („guidelines for national programmes", http://www.who.int/buruli/control, Stand 2007) gegenwärtig Feinnadelaspirate (FNA) als Untersuchungmaterial für nicht-ulzerative Läsionen, sowie für Ulzera bei denen die Abnahme diagnostischer Abstriche aufgrund vernarbter Ränder nicht möglich ist. Der Vorteil von Feinnadelaspiraten besteht in der Vermeidung der bei der Abnahme von Punch-Biopsien notwendigen Lokalanästhesie, die Eignung derartiger Proben für die Routinediagnostik ist jedoch noch nicht validiert. Ersten Studien zufolge liegen diagnostische Sensitivitäten bei der Untersuchung von FNAs bei 38% für Mikroskopie, 20-27% für Kultur, und 52% für PCR (10). In einer laufenden Studie in Ghana und Togo untersuchen wir gegenwärtig die diagnostischen Sensitivitäten von Mikroskopie und PCR von Feinnadelaspiraten im Vergleich zu diagnostischen Abstrichen, Punch-Biopsien und Proben aus chirurgisch exzidiertem Gewebe. Nach unseren eigenen Beobachtungen ist die Abnahme von Feinnadelaspiraten jedoch insbesondere für Kinder sehr schmerzhaft, so dass in diesen Fällen unseres Erachtens nach Punch-Biopsien vorzuziehen sind. Weiterhin enthalten die Präparate relativ häufig nicht unerhebliche Blutbeimengungen, welche insbesondere die mikroskopische Untersuchung erschweren (Manuskript in Vorbereitung).

Co- und Superinfektionen von BUD-Läsionen durch nicht-tuberkulöse Mykobakterien

Gemäß aktuellen WHO Empfehlungen bestätigt der kulturelle Nachweis von *M. ulcerans* die Verdachtsdiagnose eines BUD-Rezidives (53). Bewachsene Mykobaterienkulturen werden unserer Erfahrung nach in den Laboratorien endemischer Regionen jedoch meist nicht weiter differenziert. Die Diagnose eines *M. ulcerans* wird in der Regel anhand von Kulturmorpholgie und/oder dem Vorliegen säurefester Stäbchen im mikroskopischen Kultur-Präparat gestellt. Zur zuverlässigen Abgrenzung von BUD-Rezidiven und Therapieversagern von Infektionen durch andere nicht-tuberkulöse Mykobakterien ist jedoch eine weitergehende Erregerdifferenzierung erforderlich. Die Mykobakterienisolate unseres ghanaischen

Patientenkollektives wurden zur Bestätigung des Vorliegens von *M. ulcerans* einer *IS2404* PCR unterzogen. Zur Identifikation *IS2404* negativer Isolate wurde eine Kombination molekularbiologischer Methoden (Sequenzanalyse von *rpoB*-, 16S *rRNA*-, ITS-, und 65 kDA *hsp*-Gen) herangezogen. Somit konnten bei einigen, als Rezidiv bzw. „non-healer" klassifizierten BUD-Patienten, Infektionen mit anderen Mykobakterien nachgewiesen werden. Der angewandte diagnostische Algorithmus wird anhand einer kleinen Fallserie von vier Patienten beschrieben (Manuskript in Vorbereitung).

Sequenzbasierte Detektion mit Rifampicin- und Streptomycinresistenz assoziierter Genmutationen in klinischen M. ulcerans Isolaten

Nach Einführung der antimykobakteriellen Therapie des Buruli Ulkus in endemischen Regionen in 2006 ist - analog zu andern Mykobakterieninfektionen - mit der Entwicklung von Medikamentenresistenzen zu rechnen. Da keine Basisdaten zur Resistenzsituation in endemischen Regionen vorlagen, führte meine Arbeitsgruppe im Rahmen des EU-geförderten Forschungsprojektes BURULICO von 2006-2007 eine Pilotstudie zur Detektion resistenzassoziierter Mutationen des *rpoB*- (Rifampicinresistenz) und *rpsL*-Gens (Streptomycinresistenz) an klinischen Isolaten von 162 antimykobakteriell behandelten Patienten aus Ghana durch. Aufgrund unserer Daten ist für den Zeitraum unmittelbar nach Einführung der antimykobakteriellen Kombinationstherapie eine niedrige Rate (<1%) resistenzassoziierter Mutationen anzunehmen. Im Hinblick auf die teils dramatische Resistenzentwicklung bei anderen mykobakteriellen Erkrankungen (TB, Lepra) nach Einführung antibiotischer Therapie halten wir jedoch eine kontinuierliche Überprüfung der Resistenzsituation durch molekulare und konventionelle Testmethoden für erforderlich. Die gegenwärtig angewandten molekularen Testmethoden eigenen sich hauptsächlich für die Untersuchung von Kulturen, die Sensitivität der Analyse von genomischer DNA aus diagnostischen Proben ist um ein Vielfaches geringer zu bewerten. Zum zeitnahen Nachweis resistenzassoziierter Mutationen insbesondere bei Therapieversagern und Rezidivpatienten ist die Entwicklung optimierter, sensitiverer Untersuchungsmethoden erforderlich (Manuskript in Vorbereitung).

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Anwendung unserer Forschungsergebnisse auf andere endemische Regionen

Die in unserem diagnostischen Netzwerk in Ghana gewonnenen Erkenntnisse zu Organisation und Standardisierung von Probenabnahme und Datenmanagement konnten in jüngster Zeit auch für ein Kooperationsprojekt mit der Deutschen Lepra- und Tuberkulosehilfe (DAHW)

genutzt werden. In Zusammenarbeit mit dem DAHW-Regionalbüro Togo werden im Rahmen einer von mir betreuten medizinischen Doktorarbeit seit September 2007 diagnostische Proben von Buruli-Verdachtsfällen in der Abteilung für Infektions- und Tropenmedizin mittels PCR untersucht. In Togo wird derzeit eine Modifikation des für Ghana konzipierten standardisierten Probenabnahmesets inklusive zugehöriger Formulare verwendet, die togoischen Daten werden in der für Ghana entwickelten Datenbank mit erfasst. Gemäß vorläufiger Projektdaten können derzeit etwa 40% der togoischen Buruli-Verdachtsfälle mittels PCR bestätigt werden. Analog zur der in Ghana beobachteten Entwicklung ist durch intensivierte Schulung des chirurgischen Personals, sowie die Erhöhung des Anteils früher Läsionen im Patientengut eine erhebliche Steigerung des Anteils laborbestätigter Fälle für 2009 zu erwarten. Die Etablierung eines diagnostischen Mykobakterienlabors (inklusive DRB-PCR-Diagnostik) im Referenzzentrum Tsévie ist ebenfalls für 2009 geplant. Die Einarbeitung des lokalen Laborpersonals wird anhand unseres ghanaischen Laborhandbuches erfolgen.

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Dry-Reagent-Based PCR as a Novel Tool for Laboratory Confirmation of Clinically Diagnosed *Mycobacterium ulcerans*-Associated Disease in Areas in the Tropics Where *M. ulcerans* Is Endemic

V. Siegmund,[1]* O. Adjei,[2] P. Racz,[1] C. Berberich,[2] E. Klutse,[3] F. van Vloten,[1] T. Kruppa,[2] B. Fleischer,[1] and G. Bretzel[1]

Department of Medical Microbiology and Immunology, Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany,[1] *and Kumasi Centre for Collaborative Research in Tropical Medicine, Kwame Nkrumah University of Science and Technology, Kumasi,*[2] *and Dunkwa Government Hospital, Dunkwa-on-Offin,*[3] *Ghana*

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After tuberculosis and leprosy, Buruli ulcer (BU), caused by *Mycobacterium ulcerans*, is the third most common mycobacterial disease in immunocompetent humans. The disease occurs in tropical countries, with foci in West Africa, Central Africa, and the western Pacific. BU is defined as an infectious disease involving the skin and the subcutaneous adipose tissue characterized by a painless nodule, papule, plaque, or edema, evolving into a painless ulcer with undermined edges and often leading to invalidating sequelae. Due to the fundamental lack of understanding of modes of transmission, disease control in endemic countries is limited to early case detection through improved active surveillance and surgical treatment. The laboratory confirmation of BU is complicated by the absence of a diagnostic "gold standard." Therefore, misclassification and delayed diagnosis of BU may occur frequently, causing a considerable socioeconomic impact in terms of treatment costs due to prolonged hospitalization. In order to respond to the urgent need to develop reliable tools for early case detection and to overcome technical difficulties accompanying the implementation of diagnostic PCR procedures in tropical countries, a dry-reagent-based PCR formulation for the detection of *M. ulcerans* in diagnostic specimens has been developed at the Bernhard Nocht Institute for Tropical Medicine. Following technical and clinical validation, the assay has been successfully installed and field tested at the Kumasi Centre for Collaborative Research in Tropical Medicine, Kumasi, Ghana. Preliminary results show an excellent diagnostic sensitivity of >95%.

After tuberculosis and leprosy, Buruli ulcer (BU) is the third most common mycobacterial disease in immunocompetent humans. The incidence of BU has been on the rise worldwide. The disease occurs in tropical countries, with foci in West Africa, Central Africa, and the western Pacific. The disease mainly affects impoverished inhabitants of remote rural areas, with a preference for children under the age of 15, and is believed to be associated with tropical and subtropical wetlands. Epidemiological studies suggest that swamps and slowly flowing water are the sources of the organism. Recent evidence supports the view that waterborne insects may be involved in the transmission of the infection. However, the precise source of infection is still unknown. The incidence and prevalence of BU worldwide are not precisely defined, as adequate surveillance data based on accurate case confirmation data are lacking. However, prevalences for the disease up to >20% have been reported from various foci in countries where the disease is highly endemic. In Ghana in 1999, for example, the overall crude national prevalence rate of active lesions was 20.7 per 100,000, but the rate was 150.8 per 100,000 in the district where the disease was most endemic (1, 3, 6, 7, 11).

According to World Health Organization standard case definitions, BU is defined as an infectious disease involving the skin and the subcutaneous adipose tissue characterized by a painless nodule, papule, plaque, or edema and evolving into a painless ulcer with undermined edges. The lesion may lead to extensive scarring, contractures, and deformations with possible total loss of articulation function. If left untreated, it may even result in loss of limbs or blindness.

Cases that meet these clinical definitions are considered probable cases. Confirmed cases require a positive laboratory diagnosis. Because BU is associated with nonspecific clinical manifestations and an indolent course, every ulcer or nodule in an area of endemicity should be suspected as a *Mycobacterium ulcerans* infection until proven otherwise. Observations made by various researchers suggest that misclassification of clinically diagnosed BU cases may occur frequently. Early and healed lesions, especially, may be confused with other skin diseases endemic in tropical areas. The differential diagnosis comprises infectious (e.g., abscess, onchoceriasis, leprosy, elephantiasis, yaws, scrofuloderma, mycosis, actinomycosis, herpes, cutaneous leishmaniasis, tropical phagedenic ulcer, venous ulcer, and noma) and other (e.g., insect bites; psoriasis; enlarged lymph nodes; lipoma and other neoplasms; vascular, diabetic, and varicose ulcers; and burns) conditions and poses difficulties, especially in tropical settings with limited access to laboratory facilities (8, 12).

Treatment of BU with antibiotics has been widely unsuccessful. Experimental drug treatment trials are under way. The present standard of treatment is surgical removal of the affected tissue, eventually followed by skin grafting. Due to the

* Corresponding author. Mailing address: Department of Medical Microbiology and Immunology, Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany. Phone: 49 40 42 81 84 67. Fax: 49 40 42 81 84 00. E-mail: siegmund@bni-hamburg.de.

fundamental lack of understanding of modes of transmission, effective prevention strategies have not yet been developed. At present, disease control in countries of endemicity is limited to early detection through improved active surveillance and surgical treatment to prevent the development of severe ulceration and resulting disabilities (3, 6, 15).

The laboratory confirmation of BU is complicated by the absence of a diagnostic "gold standard" and a resulting lack of clarity regarding the sensitivities and specificities of different diagnostic laboratory assays. A positive laboratory diagnosis of *M. ulcerans*-associated disease requires any two of the following findings: detection of acid-fast bacilli in a Ziehl-Neelsen-stained smear, positive culture of *M. ulcerans* (swab or tissue specimen, confirmed by biochemical tests or IS*2404* PCR), histopathological confirmation, and positive IS*2404* PCR (swab and/or tissue specimen) (6, 14, 15). Whereas the sensitivities of smear microscopy and culture are relatively low, histopathology and different PCR assays targeting different regions in *M. ulcerans* IS*2404* and 16S rRNA genes provide diagnostic sensitivities of >90%. PCR can serve as a highly sensitive and reliable tool for presurgical early diagnosis and postsurgical laboratory confirmation of probable cases. Thus, PCR can prevent misclassification and subsequent inadequate treatment of patients in countries of endemicity. In addition, PCR provides reliable laboratory-confirmed incidence and prevalence data (5, 9, 10, 13, 16).

However, conventional PCR techniques in most cases are not available in countries of endemicity. In order to respond to the urgent need to develop reliable tools for early case detection and to overcome technical difficulties accompanying the implementation of conventional diagnostic PCR procedures in tropical countries, a dry-reagent-based PCR formulation for the detection of *M. ulcerans* in diagnostic specimens has been developed at the Bernhard Nocht Institute for Tropical Medicine (BNITM). The method is based on the standard diagnostic IS*2404* PCR developed by Stinear et al. (14), and due to the use of lyophilized reagents, is well adapted to tropical conditions and convenient for application in the field. After technical and clinical validation, the method has been successfully installed at the Kumasi Centre for Collaborative Research in Tropical Medicine (KCCR), Kumasi, Ghana.

This pilot study presents the first validation data obtained under tropical conditions from March 2002 until September 2002.

MATERIALS AND METHODS

Ethical considerations. Ethical clearance for the study was obtained through the Ethics Committee of the School of Medical Sciences, Kumasi, Ghana. Verbal consent was obtained from the study participants and heads of households or guardians.

Mycobacterial strains and DNA standard. The mycobacterial strains used in this study (Table 1) were kindly provided by Françoise Portaels, Institute for Tropical Medicine, Antwerp, Belgium; David Dawson, Queensland Diagnostic and Reference Center for Mycobacterial Diseases, Brisbane, Australia; Ohene Adjei, Kumasi Center for Collaborative Research in Tropical Medicine, Kumasi, Ghana; and Sabine-Rüsch Gerdes, German National Reference Center for Mycobacteria, Borstel, Germany.

M. ulcerans and *Myvobacterium marinum* strains were cultured on Loewenstein-Jensen medium at 32°C; all other mycobacterial strains were cultured at 37°C.

Mycobacterium leprae DNA was kindly provided by Paul Klatser, Institute for Tropical Medicine, Amsterdam, The Netherlands.

TABLE 1. Mycobacterial strains and genomic DNA used in the study

Strain reference no.	Species	Geographical origin	Source[a]
ITM 97-610	*M. ulcerans*	Ghana	ITM
ITM 3129	*M. ulcerans*	Zaire	ITM
ITM 5114	*M. ulcerans*	Mexico	ITM
ITM 5147	*M. ulcerans*	Australia	ITM
ITM 8756	*M. ulcerans*	Japan	ITM
ITM 9146	*M. ulcerans*	Benin	ITM
ITM 94-511	*M. ulcerans*	Ivory Coast	ITM
ITM 98-912	*M. ulcerans*	China	ITM
ITM 97-680	*M. ulcerans*	Togo	ITM
ITM 96-657	*M. ulcerans*	Angola	ITM
ITM 94-1328	*M. ulcerans*	Malaysia	ITM
ITM 5156	*M. ulcerans*	Papua	ITM
QDRLMD 9807	*M. ulcerans*	Australia	QDRLMD
QDRLMD 9808	*M. ulcerans*	Australia	QDRLMD
QDRLMD 9819	*M. ulcerans*	Australia	QDRLMD
QDRLMD 9820	*M. ulcerans*	Australia	QDRLMD
QDRLMD 9885	*M. ulcerans*	Australia	QDRLMD
QDRLMD 9920	*M. ulcerans*	Australia	QDRLMD
QDRLMD 10128	*M. ulcerans*	Australia	QDRLMD
QDRLMD 10137	*M. ulcerans*	Australia	QDRLMD
QDRLMD 10166	*M. ulcerans*	Australia	QDRLMD
QDRLMD 10463	*M. ulcerans*	Australia	QDRLMD
KCCR 207	*M. ulcerans*	Ghana	KCCR
KCCR 216	*M. ulcerans*	Ghana	KCCR
KCCR 221	*M. ulcerans*	Ghana	KCCR
KCCR 4	*M. ulcerans*	Ghana	KCCR
KCCR 05	*M. ulcerans*	Ghana	KCCR
KCCR 05 R/S	*M. ulcerans*	Ghana	KCCR
KCCR 07	*M. ulcerans*	Ghana	KCCR
KCCR 7	*M. ulcerans*	Ghana	KCCR
KCCR 10	*M. ulcerans*	Ghana	KCCR
KCCR 11	*M. ulcerans*	Ghana	KCCR
KCCR 12	*M. ulcerans*	Ghana	KCCR
KCCR 13	*M. ulcerans*	Ghana	KCCR
KCCR 14	*M. ulcerans*	Ghana	KCCR
KCCR 19	*M. ulcerans*	Ghana	KCCR
KCCR 21	*M. ulcerans*	Ghana	KCCR
KCCR D1	*M. ulcerans*	Ghana	KCCR
KCCR D3	*M. ulcerans*	Ghana	KCCR
3240/02; BNITM/M1	*M. marinum*	Germany	Borstel
10368/01; BNITM/M2	*M. lentiflavium*	Germany	Borstel
9679/00; BNITM/M3	*M. tuberculosis* $H_{37}Rv$	Germany	Borstel
3746/02; BNITM/M4	*M. avium*	Germany	Borstel
827/02; BNITM/M5	*M. intracellulare*	Germany	Borstel
BNITM/M6	*M. fortuitum*	Germany	Borstel
BNITM/M7	*M. szulgai*	Germany	Borstel
2968/02; BNITM/M8	*M. xenopi*	Germany	Borstel
6554/01; BNITM/M9	*M. scrofulaceum*	Germany	Borstel
3899/02; BNITM/M10	*M. gordonae*	Germany	Borstel
3709/02; BNITM/M11	*M. kansasii*	Germany	Borstel
2280/02; BNITM/M12	*M. malmoense*	Germany	Borstel
BNITM/M13	*M. chelonae*	Germany	Borstel
BNITM/M14	*M. smegmatis*	Germany	Borstel
Genomic DNA	*M. leprae*	The Netherlands	KIT

[a] ITM, Institute for Tropical Medicine, Antwerp, Belgium; QDRLMD, Queensland Diagnostic and Reference Laboratory for Mycobacterial Diseases; KIT, Institute for Tropical Medicine, Amsterdam, The Netherlands.

Diagnostic specimens. The diagnostic specimens used in this study (swabs, $n = 41$; tissue specimens, $n = 46$) were obtained from 48 clinically diagnosed patients with *M. ulcerans*-associated disease treated at Dunkwa Governmental Hospital, Dunkwa-on-Offin, Upper Denkyira District, Ghana, from March 2002 until September 2002.

Clinical and epidemiological information for each patient was taken from BU1

forms: ulcerative lesions, $n = 37$; nonulcerative lesions, $n = 7$ (nodules, $n = 4$; plaque, $n = 1$; papule, $n = 1$; edema, $n = 1$); clinical data not available, $n = 4$.

The duration of the disease ranged from 1 week to 16 years, with a median duration of 2 months.

To standardize the process of specimen collection, the following criteria were established. Diagnostic swabs (Greiner Bio-One, Essen, Germany) were to be taken from the undermined edges of the lesions before surgery; 10- by 10-mm tissue specimens were to be obtained from surgically excised tissue. In the case of nodules, the tissue specimens should contain a section of the center of the nodule; in the case of ulcers, the specimens should be taken from the edge of the ulcerative lesions containing necrotic tissue sections.

The PCR specimens were stored in 2-ml tubes (Sarstedt, Nümbrecht, Germany) containing 700 μl of cell lysis solution (Puregene DNA isolation kit; Gentra Systems, Indianapolis, Ind.) at room temperature until they were processed.

PCR-negative tissue specimens were subjected to histopathological analysis to exclude other infectious and noninfectious conditions considered for differential diagnosis. Ten- by 10-mm tissue sections were stored in 5-ml tubes (Sarstedt) in 10% formaldehyde, and histopathological analysis was carried out according to standardized criteria (4).

DNA preparation. The Puregene DNA isolation kit was used for DNA isolation with minor modifications evaluated for extracting *M. ulcerans* DNA from tissue samples as described below. All materials mentioned were included in the kit.

Tissue specimens were inactivated at 95°C for 15 min. Subsequently, the specimens were cut, and 5-mm^3 (maximum) pieces were incubated overnight at 55°C in 700 μl of cell lysis solution enriched with proteinase K (Sigma-Aldrich, Munich, Germany) to a final concentration of 300 μg/ml. The proteinase K was inactivated at 95°C for 15 min. After the specimens were cooled to room temperature, egg white lysozyme was added to a final concentration of 250 μg/ml, and the specimens were incubated at 37°C for 1 h.

Processing of swab samples, as well as subsequent procedures of DNA extraction for tissue specimens, were carried out according to the manufacturers' instructions. The DNA pellets were resuspended in 200 μl of DNA hydration solution, and extracts were stored at 4°C until they were further processed.

Selection of oligonucleotides and PCR conditions. (i) Standard reference method. Based on the findings in a recent publication (14), amplification of a specific 492-bp-long DNA sequence in *M. ulcerans* IS*2404* (GenBank accession no. AF003002) by PCR was carried out from 2 μl of target DNA extract using primers MU5 (5′-AGC GAC CCC AGT GGA TTG GT) and MU6 (5′-CGG TGA TCA AGC GTT CAC GA). The reaction volume was 20 μl, containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM (each) deoxynucleoside triphosphate, and 1 U of Ampli-Taq Gold DNA polymerase. All reagents except the oligonucleotides (TibMolbiol, Berlin, Germany) were purchased from Perkin-Elmer, Weiterstadt, Germany. DNA extracts of diagnostic specimens were tested in 10^0 and 10^{-1} dilutions. Hot-start thermal cycling was conducted in a Primus Thermocycler (MWG Biotech, Ebersberg, Germany) according to the following cycling profile: one initial activation step with polymerase at 94°C for 10 min; 40 cycles, each consisting of denaturation at 94°C for 10 s, primer annealing at 58°C for 10 s, and extension at 72°C for 30 s; and one final extension step at 72°C for 15 min. The amplification products were held at 4°C until they were further processed and detected by agarose gel electrophoresis (1.5%) and ethidium bromide staining (1 μg/ml).

(ii) Dry-reagent-based PCR. PuReTaq Ready-To-Go PCR Beads (Amersham Biosciences, Freiburg, Germany) were used to carry out PCR amplification. When brought to a final volume of 25 μl, each reaction mixture contained ~2.5 U of PuReTaq DNA polymerase, 10 mM Tris-HCl [pH 9.0], 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM (each) deoxynucleoside triphosphate, and stabilizers, including bovine serum albumin. The oligonucleotides (MU5 and MU6) were lyophilized (Hetovac; Nunc GmbH, Wiesbaden, Germany) for 20 min (1.150 rpm) in 200-μl reaction tubes (MWG Biotech) and applied to the reaction in a concentration equal to that in the standard reference method. DNA extracts of diagnostic specimens were tested in 10^0 and 10^{-1} dilutions.

Because the reaction conditions of the PuReTaq Ready-To-Go PCR bead Technology were fixed as described above and therefore deviated from the standard reference method, the amount of target DNA was extrapolated and adjusted to 2.5 μl due to the increased reaction volume.

Technical validation and optimization of PCR. (i) Quantified plasmid DNA standard. The 492-bp target region of the standard reference method was amplified, analyzed by gel electrophoresis, and prepared using the NucleoSpin Extract kit (Macherey Nagel, Düren, Germany) according to the manufacturer's instructions. Subsequently, the amplicon was directly cloned via the T/A sticky end using the AdvanTAge PCR cloning kit (Clontech, Heidelberg, Germany).

Plasmid DNA was prepared using the NucleoSpin Plasmid kit (Macherey Nagel). Afterwards, plasmid concentrations were determined photometrically (260 nm). Because the target region is located within a multiple-copy element and is therefore detectable ~50 times per genome (13), the plasmid standard with 1.5 genome equivalents represents ~75 copies of the element.

(ii) Validation by testing diagnostic specimens in comparison to the IS*2404* reference method. Diagnostic specimens used for validation of the dry-reagent-based PCR method were obtained from 48 clinically diagnosed BU patients. (i) Swabs ($n = 19$) and tissue specimens ($n = 16$) from 19 clinically diagnosed BU patients were simultaneously tested at the BNITM by both the standard reference and the dry-reagent-based PCR methods. (ii) After implementation of the method at the KCCR, swabs ($n = 22$) and tissue specimens ($n = 30$) from 30 clinically diagnosed BU patients were tested simultaneously at the KCCR (dry-reagent-based PCR) and the BNITM (standard reference method).

Internal quality control. (i) Extraction control. To exclude false-positive PCR results caused by contamination during DNA extraction, an extraction was performed without a specimen and used as an extraction control. The extraction control was required to produce a negative result after PCR.

(ii) PCR run controls. To exclude false-positive and -negative PCR results, the following control reactions were performed.

(a) Negative control reaction. The reaction mixture contained water instead of template DNA, which should not produce a specific band after PCR.

(b) Positive control reaction. PCR was performed using plasmid DNA containing the specific 492-bp DNA sequence of *M. ulcerans* IS*2404* as a template.

(c) Inhibition control reactions. For quality testing of the DNA extract, inhibition control PCRs were run in parallel with the tested samples. These controls consisted of half of the volume of DNA extract, with the IS*2404* plasmid DNA as a competitor (1.5 genome equivalents). Possible inhibitory effects of substances in the DNA extract were excluded if a clearly visible PCR amplicon of 492 bp was present in the inhibition control reactions.

RESULTS

Analytic sensitivity. To establish a dry-reagent-based PCR for the detection of *M. ulcerans*, IS*2404* was selected as the target region. This target had been proven to be appropriate for diagnostic application, and its use is regarded as the standard reference method routinely used for diagnostic purposes (14).

To determine the analytical sensitivity in a quantified DNA standard, we cloned the amplicon of the standard reference method into *Escherichia coli*.

Replicates of $\log_{10}$ plasmid serial dilutions were amplified by both the standard reference PCR and the dry-reagent-based PCR. It was shown that the sensitivity was excellent: 1.5 genome equivalents per reaction could be detected with the standard reference PCR in 10 of 10 reactions. When testing arithmetically higher dilutions (~0.15 plasmids per reaction), positive standard reference PCR results were occasionally obtained due to the plasmids randomly distributed among the samples. Therefore, the reaction conditions could be considered already optimized.

The same sensitivity, i.e., 1.5 genome equivalents per reaction, was achieved using the dry-reagent-based PCR formulation, indicating comparable analytical sensitivity.

Sensitivity and specificity. (i) Mycobacterial reference strains. To confirm the specificity of the dry-reagent-based PCR, 39 *M. ulcerans* reference strains and 15 different mycobacterial strains (Table 1) were tested with our assay. Genomic DNA preparations were obtained by extraction of culture material. All 39 *M. ulcerans* reference strains tested positive and all 15 other mycobacterial strains tested negative in the dry-reagent-based PCR.

(ii) Dry-reagent-based PCR in comparison to the IS*2404* reference method. Nineteen swabs and 16 tissue specimens

TABLE 2. Accordance and discordance rates (BNITM testing)[a]

Specimen	Result		%	% accordance	Result		%	% discordance
	Dry-reagent-based PCR	Standard reference method			Dry-reagent-based PCR	Standard reference method		
Swab	Positive	Positive	31.6 (6 of 19)		Positive	Negative	5.3 (1 of 19)	
	Negative	Negative	63.2 (12 of 19)		Negative	Positive	0 (0 of 19)	
Total				94.7				5.3
Tissue	Positive	Positive	18.8 (3 of 16)		Positive	Negative	6.3 (1 of 16)	
	Negative	Negative	56.3 (9 of 16)		Negative	Positive	18.8 (3 of 16)	
Total				75.0				25.0

[a] Dry-reagent-based PCR (BNITM)/standard reference method (BNITM).

from 19 patients were tested simultaneously with both methods at the BNITM. The accordance (swabs, 94.7%; tissue specimens, 75%) and discordance (swabs, 5.3%; tissue specimens, 25.0%) rates are presented in Table 2.

After implementation of the dry-reagent-based PCR at the KCCR, 22 swab specimens and 30 tissue specimens from 30 patients were tested simultaneously with both methods (KCCR, dry-reagent-based PCR; BNITM, standard reference method). The accordance (swabs, 95.5%; tissue specimens, 96.7%) and discordance (swabs, 4.5%; tissue specimens, 3.3%) rates are presented in Table 3.

(iii) Diagnostic sensitivity. Totals of 31.6 (6 of 19) and 27.3% (6 of 22) of the swabs tested positive, and 18.8 (3 of 16) and 36.7% (11 of 30) of the tissue specimens produced a positive PCR result; 63.2 (12 of 19) and 68.2% (15 of 22) of the swabs tested negative, and 56.3 (9 of 16) and 60% (18 of 30) of the tissue specimens, i.e., 58.7% of the total number of tissue specimens (27 of 46), produced a negative PCR result (Tables 2 and 3).

Histopathological analysis of 27 PCR-negative tissue specimens. Eleven specimens were inappropriate for diagnostic purposes, as the subcutaneous adipose tissue was either missing or not complete. For two specimens, histopathological results were not available. Unspecific dermatitis was found in two specimens, and one specimen was diagnosed as Kaposi's sarcoma. Two specimens showed signs of parasitic infection, one of those being onchocerciasis. One specimen was confirmed as cutaneous tuberculosis.

The histopathological features of eight specimens were compatible with *M. ulcerans*-associated disease. Six of those could be classified as active disease, one could be classified as inactive (healing) stage, and one case could not be definitely confirmed.

DISCUSSION

At present, control strategies for BU in countries of endemicity are limited to early case detection through improved active surveillance and surgical treatment. A diagnostic gold standard for the laboratory confirmation of BU has not yet been established, and sensitive diagnostic techniques like PCR and histopathology are often not available in areas of endemicity (6). Thus, misclassification and delayed diagnosis may occur frequently. According to a retrospective study carried out in Ashanti Region, Ghana, in 1994 to 1996, the average total treatment costs for a BU patient with advanced ulcerative disease requiring prolonged hospitalization were determined to be U.S.$780 as opposed to U.S.$20 to $30 for early cases. Thus, early case detection and subsequent surgical treatment reduce patient-related treatment costs (2).

In order to respond to the urgent need to develop reliable tools for early case detection, a dry-reagent-based PCR formulation for the detection of *M. ulcerans* in diagnostic specimens has been developed at the BNITM.

The implementation of conventional PCR assays in tropical countries is accompanied by various technical difficulties. The transport and storage of reagents and specimens require cold chains and equipment like generators and voltage stabilizers to prevent damage to the reagents and specimens by repeated freezing and thawing due to regularly occurring power cuts. Furthermore, conventional PCR assays require careful han-

TABLE 3. Accordance and discordance rates (KCCR and BNITM testing)[a]

Specimen	Result		%	% Accordance	Result		%	% Discordance
	Dry-reagent-based PCR	Standard reference method			Dry-reagent-based PCR	Standard reference method		
Swab	Positive	Positive	27.3 (6 of 22)		Positive	Negative	4.5 (1 of 22)	
	Negative	Negative	68.2 (15 of 22)		Negative	Positive	0 (0 of 22)	
Total				95.5				4.5
Tissue	Positive	Positive	36.7 (11 of 30)		Positive	Negative	3.3 (1 of 30)	
	Negative	Negative	60.0 (18 of 30)		Negative	Positive	0.0 (0 of 30)	
Total				96.7				3.3

[a] Dry-reagent-based PCR (KCCR)/standard reference method (BNITM).

dling of reaction components and strict measures by skilled laboratory workers to avoid contamination of reagents.

The use of lyophilized, room-temperature-stable PCR reagents and transport buffer for specimens prevents temperature-dependent transport- and storage-related problems. In addition, lyophilized primers and reaction mixtures are easy to handle and less time-consuming for laboratory staff. The chemicals are not sensitive to climatic conditions, like heat and humidity, and thus, the qualities of reagents and reactions are always equal. The risk of contamination is minimized, because instead of at least eight different liquid components, as in conventional PCR assays, only water and template are added to the lyophilized reagents.

The dry-reagent-based PCR is slightly more expensive than the conventional diagnostic PCR (in the range of €2 to €3, depending on the manufacturer); however, the numerous advantages of the assay clearly outweigh the slight financial imbalance. In general, studies of the cost-effectiveness of implementing highly sensitive diagnostic tools like PCR in areas of high endemicity in terms of reducing total treatment costs are required.

With an analytical sensitivity and specificity equal to those of conventional PCR and accordance rates for diagnostic swabs (n = 19) of 95% (Table 2) for both dry-reagent-based and standard reference methods carried out simultaneously at the BNITM, we considered the dry-reagent-based PCR a reliable diagnostic tool. As the discordance rate of 25% (Table 2) for tissue specimens (n = 16) was attributable to technical aspects of specimen collection, i.e., the specimens subjected to the two methods were not obtained from the same location, there was no evidence for a lower sensitivity of the method for tissue samples.

After implementation of the assay at the KCCR, 52 diagnostic specimens from 30 clinically diagnosed patients were simultaneously tested at the KCCR (dry-reagent-based assay) and BNITM (standard reference method). Accordance rates of >95% (Table 3) for both swabs and tissue specimens suggest that the dry-reagent-based PCR assay is highly reliable and well adapted to application under tropical conditions.

The diagnostic specimens tested for technical validation at the BNITM and for validation under tropical conditions simultaneously at the KCCR and the BNITM show relatively low diagnostic sensitivity (31.6 and 27.3%, respectively, for swabs; 18.8 and 36.7%, respectively, for tissue samples [Tables 2 and 3]). As the aim of this study was to establish and validate the dry-reagent-based diagnostic PCR by comparing it with the standard IS*2404* reference method, all patients with clinically diagnosed *M. ulcerans*-associated disease who presented at Dunkwa Government Hospital during the study period were included in the pilot study. Therefore, specific selection criteria, like the type and size of lesion or duration of the disease, were not applied. Preliminary data from an ongoing study at the KCCR aimed at the PCR confirmation of clinically diagnosed early cases suggest high diagnostic sensitivity (>90%) for early lesions (i.e., where the duration of disease was <6 months). Of the patients included in the validation pilot study, however, 47.9% had lesions older than 6 months (13 of 48; 27.1%) or data were not available (10 of 48; 20.8%). In those cases, the age of the lesions was likely to affect the diagnostic sensitivity, as older lesions might be in an inactive (healing) stage, and thus, bacilli could not be detected.

In order to explain the relatively high number of PCR-negative tissue specimens (27 of 46; 58.7%), they were subjected to histopathology. Histopathological analysis revealed that 40.7% (11 of 27) of the specimens were not appropriate for diagnostic purposes, as the subcutaneous tissue was either missing or incomplete. In 22.2% (6 of 27) of the specimens, either noninfectious (nonspecific dermatitis and Kaposi's sarcoma) or other infectious (tuberculosis, onchocerciasis, and other parasitic infections) conditions were diagnosed. Of the PCR-negative tissue specimens, 29.6% (8 of 27) showed histopathological features compatible with Buruli ulcer but without detectable acid-fast bacilli. One specimen was in the inactive (healing) stage. Older and inactive lesions tend not to contain detectable amounts of bacilli and thus may not produce positive PCR results. Therefore, only early lesions might be considered suitable for molecular diagnosis of *M. ulcerans*-associated disease. Furthermore, these findings clearly support the need to establish the differential diagnosis before classifying and treating a patient for Buruli ulcer. Thus, diagnostic tools to exclude other conditions that might be mistaken for *M. ulcerans*-associated disease are required as well. These findings also stress the importance of accurate specimen collection, as only specimens that are taken from necrotic-tissue areas and that include the subcutaneous tissue allow a reliable diagnosis of *M. ulcerans*-associated disease. Despite the prior definition of criteria for specimen collection, in this pilot study, the technical procedures were not closely monitored, as a close link between the hospital and the laboratory was not yet fully established. The diagnostic sensitivity clearly depends on the quality of specimens, and only a close collaboration between the surgeon and the laboratory guarantees optimized laboratory results.

If these conditions are fulfilled, the dry-reagent-based PCR presented in this study can serve as a reliable and rapid tool for the laboratory confirmation of *M. ulcerans*-associated disease under tropical conditions. Implementation of the method in major treatment centers in areas of endemicity is under way and will provide diagnostics at the district level.

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External quality assurance for the laboratory diagnosis of Buruli ulcer disease in Ghana

G. Bretzel[1,2], V. Siegmund[1,2], J. Nitschke[2], K. H. Herbinger[1], R. Thompson[2], E. Fleischmann[1], B. Fleischer[2] and O. Adjei[3]

1 *Department of Infectious Diseases and Tropical Medicine, University of Munich, Munich, Germany*
2 *Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany*
3 *Kumasi Centre for Collaborative Research in Tropical Medicine, Kumasi, Ghana*

Summary

OBJECTIVE To assure the quality of the laboratory diagnosis of Buruli ulcer disease; microscopy and PCR were subjected to external quality assurance (EQA).

METHODS Slides were read by test laboratory staff, followed by blinded re-reading by the controller. Parallel testing of PCR specimens was carried out at the local and external reference laboratory. Slides and PCR specimens with discordant results were subjected to a second reading/testing by the controller to determine the final result. For training purposes, slides and PCR specimens with discrepant results were subsequently re-read/re-tested under supervision at the test laboratory.

RESULTS *Microscopy*. First reading: concordance rate 82.9%, discordance rate 17.1%, percentage false negatives 27.1% (sensitivity 72.9%), percentage false positives 10.1% (specificity 89.9%). Second reading: concordance rate 97.9%, discordance rate 2.1%, percentage false negatives 4.2% (sensitivity 95.8%), percentage false positives 0.6% (specificity 99.4%). *PCR*. First testing: concordance rate 87.9%, discordance rate 12.1%, percentage false negatives 8.2% (sensitivity 91.8%), percentage false positives 19.1% (specificity 80.9%). Second testing: concordance rate 96.2%, discordance rate 3.8%, percentage false negatives 4.7% (sensitivity 95.3%), percentage false-positives 2.1% (specificity 97.9%).

CONCLUSIONS EQA identified deficiencies in the laboratory performance. Corrective action consisted in on-site training and reduced the number of false-negative and false-positive microscopy and PCR results.

keywords Buruli ulcer diagnosis, quality assurance, microscopy, PCR, Ghana

Introduction

The laboratory diagnosis for Buruli ulcer disease (BUD), caused by *Mycobacterium ulcerans*, is achieved by laboratory assessment of swab and tissue specimens. Currently available laboratory tests include the microscopic detection of acid-fast bacilli (AFB) in swab and tissue smears, PCR analysis and culture of swab and tissue specimens, as well as histopathology. However, laboratory capacity in areas of endemicity is limited. In order to strengthen the diagnostic capacities for BUD in Ghana, a diagnostic network was implemented. Diagnostic specimens were collected in two major BUD treatment centres according to standardized criteria. One local reference laboratory provided microscopy, culture and PCR according to standardized procedures. The implementation of diagnostic laboratory services for BUD at the local reference laboratory was accompanied by external quality assurance (EQA) testing. EQA procedures followed largely the recommendations for external quality assessment for AFB smear microscopy for tuberculosis (Stinear *et al.*, 1999; WHO, 2001; APHL, CDC, IUATLD, KNCV, RIT & WHO, 2002; Siegmund *et al.*, 2005). In this study, the results from the first round of external quality assurance testing of AFB smear microscopy and PCR are reported.

Material and methods

Ethical clearance for the study was sought through the Committee of Human Research Publication and Ethics, School of Medical Sciences, Kwame Nkrumah, University of Science and Technology, Kumasi, Ghana.

Diagnostic specimens and quality assurance testing were assessed in the laboratory between January 2004 and January 2006. Swabs were taken by circling the entire undermined edge of ulcers before surgery. Tissue specimens with a maximum size of 10 × 10 mm were obtained from surgically excised tissue. In the case of pre-ulcerative lesions, the tissue specimens were taken from the centre of the lesion by cutting the centre longitudinally and

horizontally in equal segments. The tissue specimens from ulcerative lesions were taken from the edge of the lesions below the end of the undermined edge. All tissue specimens were located adjacent to each other to guarantee comparable results in all diagnostic tests conducted. For diagnostic purposes, it was mandatory that tissue specimens contained subcutaneous adipose tissue. Swabs and tissue smears for AFB microscopy were prepared from decontaminated material (modified Petroff method) that was subsequently subjected to culture. The smears were stained with the Ziehl–Neelsen technique and read according to the recommended grading scale at the local reference laboratory (WHO, 2001).

Swab and tissue specimens were subjected to a dry-reagent-based PCR (DRB-PCR) adapted to tropical conditions at the local reference laboratory. The external reference laboratory used the standard reference method. Both PCR assays were shown to have a comparable analytical and diagnostic sensitivity (Stinear *et al.*, 1999; Siegmund *et al.*, 2005). In total, we externally tested 287 slides (swab smears, $n = 106$; tissue smears, $n = 181$) and 265 PCR specimens (swab specimens, $n = 98$; tissue specimens, $n = 167$) for quality assurance.

In the context of this study, the local reference laboratory is referred to as test laboratory and the external reference laboratory as controller. The Kumasi Centre for Collaborative Research in Tropical Medicine, Kumasi, Ghana was the test laboratory and the Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany was controller from January 2004 to March 2005; the Department of Infectious Diseases and Tropical Medicine, University of Munich, Germany was controller from April 2005 to January 2006.

Laboratory diagnosis of BUD was implemented at the local reference laboratory only in 2003; thus, no baseline data or information on the performance of the laboratory were available. Therefore, a 100% sample size of microscopy and PCR samples was chosen.

Slides were read by test laboratory staff, and then read again by the controller who was blinded to previous results. Reading took place either at the local reference laboratory 3–6 months after the local reading, or slides were sent to the external reference laboratory for re-reading. All slides with discordant results were subjected to a second reading by the controller to determine the final result. First and second readings by the controller were always conducted by different individuals (APHL, CDC, IUATLD, KNCV, RIT & WHO, 2002). For training purposes, all discrepant slides were subsequently re-read under supervision at the test laboratory.

The test laboratory tested all specimens by DRB-PCR, the controller tested a parallel specimen by the standard reference method in a blinded fashion. Testing by the controller took place with a 3- to 6-month delay because of time of shipment. According to the procedure described for AFB microscopy, specimens with discordant results were subjected to a second testing by the controller to determine the final result. For training purposes, all specimens with discrepant results were re-tested under supervision at the test laboratory.

Parameters determined

In accordance with the recommendations for external quality assessment for AFB smear microscopy for tuberculosis, the following parameters were determined at the first reading/testing: positivity rate at the test laboratory, number of negative slides/specimens per study period, sensitivity of the test laboratory relative to the controller, false negatives and false positives at the test laboratory relating to the final result of the controller, concordance and discordance rate between test laboratory and controller. False-negative slides were classified as low false negatives/minor quantification error (i.e. a negative result of the test laboratory and a result of the controller of <10 AFB/100 fields) and high false negatives/major error (i.e. a negative result of the test laboratory and a result of the controller of ≥'+'). False-positive slides were classified as low false positives/minor quantification error (i.e. a result of the test laboratory of <10 AFB/100 fields and a negative result of the controller) and high false positives/major error (i.e. a result of the test laboratory of ≥'+' and a negative result of the controller) (APHL, CDC, IUATLD, KNCV, RIT & WHO, 2002). At the second reading/testing, we determined concordance and discordance rate between test laboratory and controller, and percentage of false negatives and false positives after the second reading/testing at the test laboratory relating to the final result of the controller.

Statistical analysis (sensitivity, specificity, positive and negative predictive values, positive and negative likelihood ratios) was carried out by EpiInfo 3.3.2.

Results

Acid-fast bacilli microscopy

After the first reading at both laboratories, 55 slides had discordant results. All of these slides with discordant results were re-read by the controller. Six of 17 that were previously reported positive by the controller were corrected and reported negative. Thus, related to the total number of slides subjected to second reading, 6 (10.9%) of the 55 results were revised by the controller.

Table 1 Results of the first reading at the test laboratory relating to the final result of the controller

Results of first reading of slides at test laboratory	Results of reading of slides conducted by controller (final results)		
	Positive	Negative	Total
Positive	86	17	103
Negative	32	152	184
Total	118	169	287

Table 2 Percentage of total false negatives, high false negatives, low false negatives, total false positives and high false positives at the test laboratory

Category of error	Total false negatives	High false negatives	Low false negatives	Total false positives	High false positives
Percentage/number	27.1% (32/118)	17.8% (21/118)	9.3% (11/118)	10.1% (17/169)	10.1% (17/169)

Table 1 shows the results of the first reading at the test laboratory relating to the final result of the controller. The slide positivity rate (SPR) of the test laboratory was 35.9% (103/287), 64.1% of the slides were negative (184/287). The concordance rate after the first reading was 82.9% (238/287), the discordance rate 17.1% (49/287). The percentage of false negatives at the test laboratory was 27.1% (32/118), the percentage of false positives 10.1% (17/169), which corresponds to a sensitivity of 72.9% (86/118) and a specificity of 89.9% (152/169). The positive predictive value was 83.5% (86/103), the negative predictive value was 82.6% (152/184), the positive likelihood ratio was 7.25 ($86 \times 169/118 \times 17$), the negative likelihood ratio was 0.30 ($32 \times 169/118 \times 152$).

Table 2 shows the classification of false negatives and false positives. Twenty-one of the 32 false negative results of the test laboratory were classified as high false negatives/major errors (17.8% of the total volume of positive slides), 11 were classified as low false negatives/minor quantification errors (9.3% of the total volume of positive slides). All 17 false-positive results were classified as high false positives/major errors (10.1% of the total volume of negative slides).

Table 3 shows the results of the test laboratory relating to the final result of the controller after the second reading of the slides with discrepant results at the test laboratory relating to the number of discrepant slides. After the second reading of the 49 discrepant slides at the test laboratory, 27 of 32 previously false-negative results were reported positive, 16 of 17 previously false-positive results were reported negative. Thus, 43 of 49 discrepant results were revised by the test laboratory after the second reading, which corresponds to a concordance rate of 87.8% (43/49). The discordance rate was 12.2% (6/49), sensitivity 84.4% (27/32), specificity 94.1% (16/17), positive predictive value 96.4% (27/28), negative predictive value 76.2% (16/21), positive likelihood ratio 14.34 ($27 \times 17/32 \times 1$), negative likelihood ratio 0.17 ($5 \times 17/32 \times 16$).

Table 4 shows the results of the test laboratory relating to the final results of the controller after the second reading of slides with discrepant results at the test laboratory relating to the total number of slides. The percentage of false negatives dropped from 27.1% to 4.2% (5/118), with 3.4% high false negatives (4/118) and 0.8% low false negatives (1/118). The percentage of high false positives fell from 10.1% to 0.6% (1/169). The second reading resulted in a concordance rate of 97.9% (281/287) and a discordance rate of 2.1% (6/287). Sensitivity was 95.8% (113/118), specificity 99.4% (168/169), positive predictive value

Table 3 Results of the test laboratory relating to the final result of the controller after the second reading of the slides with discrepant results at the test laboratory relating to the number of discrepant slides

Results of second reading of slides at test laboratory	Results of reading of slides conducted by controller		
	Positive	Negative	Total
Positive	27	1	28
Negative	5	16	21
Total	32	17	49

Table 4 Results of the test laboratory relating to the final results of the controller after the second reading of slides with discrepant results at the test laboratory relating to the total number of slides

Results after second reading at test laboratory	Results of reading of slides conducted by controller		Total
	Positive	Negative	
Positive	113	1	114
Negative	5	168	173
Total	118	169	287

Table 5 Results of the first testing at the test laboratory relating to the final result of the controller

Results of first PCR at test laboratory	Results of PCR conducted by controller		Total
	Positive	Negative	
Positive	157	18	175
Negative	14	76	90
Total	171	94	265

99.1% (113/114), negative predictive value 97.1% (168/173), positive likelihood ratio 161.84 (113 × 169/118 × 1) and negative likelihood ratio 0.04 (5 × 169/118 × 168).

PCR

After the first round of testing, 42 specimens with discrepant results were re-tested by the controller. Five positive and five negative results each (10/42, 23.8%) were revised by the controller, 32 (12.1%) discordant results remained for second testing at the test laboratory.

Table 5 shows the results of the first testing at the test laboratory relating to the final result of the controller. The positivity rate of the test laboratory was 66.0% (175/265), 34.0% of the specimens were tested negative (90/265). The concordance rate after the first testing was 87.9% (233/265), the discordance rate 12.1% (32/265). The percentage of false negatives at the test laboratory was 8.2% (14/171), the percentage of false positives 19.1% (18/94), which corresponds to a sensitivity of 91.8% (157/171) and a specificity of 80.9% (76/94). The positive predictive value was 89.7% (157/175), the negative predictive value was 84.4% (76/90), the positive likelihood ratio was 4.79 (157 × 94/171 × 18), the negative likelihood ratio was 0.1 (14 × 94/171 × 76).

Table 6 shows the results of the test laboratory relating to the final result of the controller after the second testing of the specimens with discrepant results at the test laboratory relating to the number of discrepant specimens. After the second testing of the 32 discrepant samples at the test laboratory, six out of 14 previously false-negative results were reported positive, 16 of 18 previously false-positive results were reported negative. Twenty-two of 32 (68.8%) of the discrepant results were revised by the test laboratory after the second testing. The concordance rate was 68.8% (22/32), the discordance rate 31.3% (10/32), sensitivity 42.9% (6/14), specificity 88.9% (16/18), positive predictive value 75.0% (6/8), negative predictive value 66.7% (16/24), positive likelihood ratio 3.86 (6 × 18/14 × 2), negative likelihood ratio 0.64 (8 × 18/14 × 16).

Table 6 Results of the test laboratory relating to the final result of the controller after the second testing of the specimens with discrepant results at the test laboratory relating to the number of discrepant specimens

Results of second PCR at test laboratory	Results of PCR conducted by controller		Total
	Positive	Negative	
Positive	6	2	8
Negative	8	16	24
Total	14	18	32

Table 7 shows the results of the test laboratory relating to the final result of the controller after the second testing of specimens with discrepant results at the test laboratory relating to the total number of specimens. The percentage of false negatives was decreased from 8.2% to 4.7% (8/171), the percentage of false positives from 19.1% to 2.1% (2/94). Second reading resulted in a concordance rate of 96.2% (255/265) and a discordance rate of 3.8% (10/265). Sensitivity was 95.3% (163/171), specificity 97.9% (92/94), positive predictive value 98.8% (163/165), negative predictive value 92.0% (92/100), positive likelihood ratio 44.8 (163 × 94/171 × 2) and negative likelihood ratio 0.05 (8 × 94/171 × 92).

Table 7 Results of the test laboratory relating to the final result of the controller after the second testing of specimens with discrepant results at the test laboratory relating to the total number of specimens

Results of second PCR at test laboratory	Results of PCR conducted by controller		Total
	Positive	Negative	
Positive	163	2	165
Negative	8	92	100
Total	171	94	265

Discussion

The need for ensuring reliable laboratory services as one of the main pillars of successful disease control has been recognized for the laboratory diagnosis of tuberculosis in high prevalence countries. Guidelines for the external quality assessment for AFB smear microscopy have been made available to National TB control programmes and National TB Reference Laboratories (APHL, CDC, IUATLD, KNCV, RIT & WHO, 2002). Given the similarities between the laboratory diagnosis of TB and BUD, the requirement for external quality assessment of the BUD laboratory is obvious and lessons can be learnt from the experiences in the TB sector.

In order to obtain information on laboratory performance and to ensure the reliability of laboratory diagnosis, the implementation of diagnostic laboratory services for BUD in Ghana has been accompanied by external quality assurance for AFB smear microscopy and PCR from the outset. As no baseline data were available, for a study period of 2 years, a 100% sampling of slides and PCR specimens was chosen.

After the first reading, the high rate of high false negatives and high false positives requiring correction was causing concern. After individual training of laboratory staff, the second reading by test laboratory staff could resolve the majority of major errors. Supervised re-reading of known false-negative and false-positive smears identified typical major sources of false-negative and false-positive results, like reading of less than 100 fields or the use of unfiltered staining solutions. Furthermore, treatment of BUD patients with antimycobacterial drugs before surgery as practised in several treatment centres in Ghana resulted in an altered bacterial morphology that hampered reading of slides. Therefore, like in the diagnostic TB laboratory, training clearly can contribute to improving the performance of laboratory staff.

Inclusion of additional treatment centres will result in an increased slide volume that does not allow to maintain a 100% sample size. Further sampling for EQA could be performed according to the Lot Quality Assurance System (LQAS) as recommended in the international EQA guidelines. Experiences from several countries proved already the suitability of LQAS for implementation in the field (Selvakumar *et al.*, 2005). For an estimated volume of 300 negative slides per year at the test laboratory, an SPR of 35%, a sensitivity relative to the controller of 72% as determined in this study, a specificity of 100% and an acceptance number of false negatives of 0, the recommended annual sample size would be 23 slides per year (APHL, CDC, IUATLD, KNCV, RIT & WHO, 2002). If additional microscopy centres were to be included in the diagnostic network, we suggest to begin with 100% sampling at these centres to obtain reliable baseline data on the performance of the new laboratories.

External quality assurance of PCR rendered 8.2% false negatives and 19.1% false positives at the test laboratory. Interpretation of these results, however, must take into account that PCR testing at both laboratories was carried out on different specimens. Although adjacent pieces of tissue were taken, the possibility of one of the two specimens not containing *M. ulcerans* cannot be excluded. Therefore, future EQA of PCR in the described setting will utilize extracted DNA instead of specimens. However, even if identical source material is used, repeated testing of weakly positive PCR specimens may still render variable results (Siegmund *et al.*, 2005). Supervised re-testing of PCR specimens at the test laboratory reduced the false negatives to 4.7%, the false positives to 2.1%. In view of methodological considerations, however, it is difficult to judge if these revised results are mainly due to the intra-assay variability of PCR or can be considered a result of training. Nevertheless, the authors consider regular training for PCR laboratory staff essential in order to eliminate factors that may influence the outcome of diagnostic results. In the above-described setting, for instance, low-resolution gel imaging contributed to over-interpretation of questionable gel pictures. That source of false-positive PCR results could be resolved by training.

Due to laboratory-related costs (€13–15 per test/specimen, own data), the calculation of sample sizes for PCR quality assurance cannot follow statistical models, but will rather be determined by financial considerations. For the Ghanaian setting, the next round of PCR quality assurance at the external reference laboratory will cover 10% of the specimens assessed at the local reference laboratory.

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Corresponding Author G. Bretzel, Department of Infectious Diseases and Tropical Medicine, University of Munich, Leopold Str. 5, D-80802 Munich, Germany. Tel.: +498921803618; Fax: +49336112; E-mail: bretzel@lrz.uni-muenchen.de

Assurance de qualité externe pour le laboratoire de diagnostic de l'ulcère de Buruli au Ghana

OBJECTIF Afin d'assurer la qualité du diagnostic de laboratoire pour l'ulcère de Buruli (UB), la microscopie et la PCR ont été soumises à une assurance de qualité externe.

MÉTHODES Les lames ont été lues par le personnel de laboratoire et relues de façon aveugle par le vérificateur. Parallèlement, un test des échantillons par PCR a été réalisé dans le laboratoire de référence local et externe. Les lames et PCR avec des résultats discordants ont été soumises à une seconde lecture/test par le vérificateur afin de déterminer le résultat final. Dans un but de formation du personnel, lames et échantillons PCR ont été de nouveau relues/re-testés sous supervision dans le laboratoire contrôlé.

RÉSULTATS *Microscopie*; 1ere lecture: concordance 82,9%, discordance 17,1%, faux négatifs 27,1% (sensitivité 72,9%), faux positifs 10,1% (spécificité 89,9%). 2nde lecture: concordance 97,9%, discordance 2,1%, faux négatifs 4,2% (sensitivité 95,8%), faux positifs 0,6% (spécificité 99,4%). *PCR*; 1er test: concordance 87,9%, discordance 12,1%, faux négatifs 8,2% (sensitivité 91,8%), faux positifs 19,1% (spécificité 80,9%). 2nd test: concordance 96,2%, discordance 3,8%, faux négatifs 4,7% (sensitivité 95,3%), faux positifs 2,1% (spécificité 97,9%).

CONCLUSIONS L'assurance de qualité externe a identifié des déficiences dans la performance du laboratoire. Des actions correctives ont consisté à la formation sur place ce qui a réduit le nombre de résultats faux négatifs et faux positifs pour la microscopie et la PCR.

mots clés diagnostic de l'ulcère de Buruli, assurance de qualité, microscopie, PCR, Ghana

Control de calidad externo para el diagnóstico en laboratorio de la Úlcera de Buruli en Ghana

OBJETIVO Con el fin de asegurar la calidad del diagnóstico de laboratorio de la úlcera de Buruli (UB), se sometió a un control de calidad externo (CCE) la microscopía y la PCR

MÉTODOS Las laminas fueron leídas por el personal de laboratorio y releídas por un controlador ciego al resultado previo. Paralelamente se analizaron las muestras mediante PCR, tanto a nivel local como en un laboratorio de referencia externo. Las láminas y muestras de PCR con resultados discordantes se sometieron a una segunda lectura/prueba que llevó a cabo el controlador, determinando el resultado final. Las láminas/muestras de PCR con resultados discrepantes fueron, con fines formativos, releídas/analizadas por el personal local bajo supervisión.

RESULTADOS Microscopía. Primera lectura: tasa de concordancia 82.9%, tasa de discordancia 17.1%, porcentaje de falsos negativos 27.1% (sensibilidad 72.9%), porcentaje de falsos positivos 10.1% (especificidad 89.9%). Segunda lectura: tasa de concordancia 97.9%, tasa de discordancia 2.1%, porcentaje de falsos negativos 4.2% (sensibilidad 95.8%), porcentaje de falsos positivos 0.6% (especificidad 99.4%). PCR. Primer análisis: tasa de concordancia 87.9%, tasa de discordancia 12.1%, porcentaje de falsos negativos s 8.2% (sensibilidad 91.8%), porcentaje de falsos positivos 19.1% (especificidad 80.9%). Segundo análisis: tasa de concordancia 96.2%, tasa de discordancia 3.8%, porcentaje de falsos negativos 4.7% (sensibilidad 95.3%), porcentaje de falsos positivos 2.1% (especificidad 97.9%).

CONCLUSIONES El CCE identificó deficiencias en el desempeño del laboratorio. La acción correctiva consistió en entrenar al personal local, reduciendo en número de falsos negativos y falsos positivos por microscopía y PCR.

palabras clave úlcera de Buruli, diagnóstico, control de calidad, microscopía, PCR, Ghana

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A stepwise approach to the laboratory diagnosis of Buruli ulcer disease

G. Bretzel[1,2], V. Siegmund[1,2], J. Nitschke[1,2], K. H. Herbinger[1], W. Thompson[3], E. Klutse[4], K. Crofts[5], W. Massavon[6], S. Etuaful[7], R. Thompson[8], K. Asamoah-Opare[8], P. Racz[2], F. Vloten[2] van, C. Berberich[8], T. Kruppa[8], E. Ampadu[9], B. Fleischer[2] and O. Adjei[8]

1 *Department of Infectious Diseases and Tropical Medicine (DITM), University of Munich, Munich, Germany*
2 *Bernhard Nocht Institute for Tropical Medicine (BNITM), Hamburg, Germany*
3 *Agogo Presbyterian Hospital, Agogo, Ghana*
4 *Dunkwa Governmental Hospital, Dunkwa-on-Offin, Ghana*
5 *Humanitarian Aid Relief Team (HART), Provo, UT, USA*
6 *Goaso Hospital, Goaso, Ghana*
7 *St Martin's Catholic Hospital, Agroyesum, Ghana*
8 *Kumasi Centre for Collaborative Research in Tropical Medicine (KCCR), Kumasi, Ghana*
9 *National Buruli Ulcer Control Programme (NBUCP), Accra, Ghana*

Summary

OBJECTIVE In view of technical and financial limitations in areas of endemicity, the current practice and recommendations for the laboratory diagnosis of Buruli ulcer disease (BUD) may have to be reconsidered. We reviewed diagnostic results in order to explore options for a modified, more practicable, cost-effective and timely approach to the laboratory diagnosis of BUD.

METHODS Diagnostic specimens from 161 clinically diagnosed BUD patients from four different treatment centres in Ghana were subjected to laboratory analysis. The positivity rates of the laboratory assays were compared.

RESULTS The number of laboratory-confirmed clinically diagnosed BUD cases with one positive confirmative test was 20% higher than that with two positive confirmative tests. The specificity of microscopy (MIC) and PCR was 96.6% and 100%, respectively. Subsequent analysis of specimens from surgically excised pre-ulcerative tissue-by-tissue MIC and tissue PCR rendered 65% laboratory-confirmed BUD cases. Subsequent analysis of diagnostic swabs from ulcerative lesions by swab smear MIC and swab PCR rendered 70% of laboratory-confirmed BUD cases.

CONCLUSIONS The specificity of the diagnostic tests used in this study suggests that one positive diagnostic test may be considered sufficient for the laboratory confirmation of BUD. Subsequent application of different diagnostic tests rendered a laboratory confirmation of 65% pre-ulcerative and of 70% ulcerative lesions. Implementation of a stepwise, subsequent analysis of diagnostic specimens will result in considerable cost saving compared with simultaneous testing of specimens by several diagnostic assays.

keywords Buruli ulcer, PCR, laboratory diagnosis, reduction of costs, Ghana

Introduction

The laboratory confirmation of Buruli ulcer disease (BUD), caused by *Mycobacterium ulcerans*, can be achieved by analysis of swab and tissue specimens by means of microscopy (MIC), culture, PCR and histopathology. According to current WHO recommendations, a positive diagnosis requires two positive laboratory tests (WHO 2001). In practice, however, these recommendations are difficult to realize for several reasons.

Previously available data comparing different diagnostic tests applied on different diagnostic specimens suggest a wide range of diagnostic sensitivities, varying from as low as < 40% (swab smear MIC) to > 95% (PCR and histopathological analysis of tissue specimens) (Guimaraes-Peres *et al.* 1999; N'Guessan *et al.* 2001; WHO 2001; Whitney *et al.* 2002). A combination of highly sensitive tests (PCR and histopathology) performed on invasively collected tissue specimens would render the two required positive results with the highest probability. However, the availability of sophisticated laboratory assays in areas of endemicity is limited. In general, diagnostic services for BUD are largely restricted to reference centres. Furthermore, technical difficulties, such as the instability of

reagents under tropical conditions, hamper molecular laboratory tests. Histopathology is rarely available in resource poor countries.

In addition, in most cases, close links between reference laboratories and peripheral treatment centres do not exist. Thus, testing of specimens in reference centres may not be performed on a regular basis. If carried out, the turnaround time for results may be too long to allow a pre-surgical diagnosis. Thus, diagnostic information is available only after the patients have already been treated surgically.

Only a few district level laboratories located in major treatment centres have started out doing swab smear MIC for the laboratory diagnosis of BUD. Laboratory capacity at district level is limited. Diagnostic tests available – if any – are restricted to MIC. Therefore, on-site testing of specimens with several assays may not be possible.

In general, restricted health budgets often prevent a comprehensive laboratory analysis. In view of these limitations, the current diagnostic practice and recommendations, i.e. simultaneous testing of diagnostic specimens with different laboratory methods in order to obtain two positive results, may have to be reconsidered. In order to strengthen the diagnostic capacity in 2003, a diagnostic network was initiated in Ghana with financial support of the Volkswagen foundation. The network initially comprised two major treatment centres, one local and two external reference laboratories providing all diagnostic laboratory assays and quality assurance. A close cooperation with the National Buruli Ulcer Control Programme in Ghana has been established. The collaboration has been extended to other Ghanaian BUD treatment centres. At the outset of the project, a dry-reagent-based PCR (DRB-PCR) adapted to tropical conditions was implemented at the local reference laboratory (Siegmund *et al.* 2005). Diagnostic specimens were subjected to MIC, PCR and culture (local reference laboratory), as well as histopathology and external quality assurance (external reference laboratories).

The diagnostic results were reviewed in order to explore options for a modified, more practicable, cost-effective and timely approach to the laboratory diagnosis of BUD. Special attention was given to the applicability of diagnostic methods that allow pre-surgical diagnosis replacing post-surgical laboratory confirmation.

Materials and methods

Ethical clearance and informed patient consent

Ethical clearance for the study was sought through the Committee of Human Research Publication and Ethics, School of Medical Sciences, University of Science and Technology, Kumasi, Ghana. Informed patient consent was obtained before surgery.

Inclusion criteria

Clinically diagnosed BUD patients with pre-ulcerative and ulcerative lesions were eligible for participation, if their disease had lasted <6 months ('early lesions'), if we could obtain complete sets of diagnostic specimens with accompanying clinical information and if laboratory consensus results were confirmed by quality assurance.

Treatment centres

Participating treatment centres were Agogo Presbyterian Hospital, Agogo, Asante Akim North District, Ashanti Region; Dunkwa Government Hospital, Dunkwa-on-Offin, Upper Denkyira District, Central Region; Goaso Hospital, Goaso, Ahafo Ano District, Brongh Ahafo Region and St Martin's Catholic Hospital, Agroyesum, Amansie West District, Ashanti Region.

Standardised collection of patient data and diagnostic specimens

Specimens were collected by the local surgeons before (swabs) and after (tissue specimens) surgery according to standardized criteria (Siegmund *et al.* 2005). Briefly, swabs were taken by circling the entire undermined edge of ulcers before surgery. Tissue specimens with a maximum size of 10×10 mm were obtained from surgically excised tissue after surgery. In case of pre-ulcerative lesions, the tissue specimens were taken from the centre of the lesion by cutting the centre longitudinally and horizontally in four equal segments. The tissue specimens for ulcerative lesions were taken from the edge of the lesions below the end of the undermined edge containing necrotic tissue sections. Tissue specimens for different laboratory tests were located adjacent to each other to guarantee comparable results in all diagnostic tests. For diagnostic purposes, tissue specimens had to contain subcutaneous adipose tissue, otherwise specimens were rejected.

In order to provide optimal conditions for storage and transport of the specimens, standardized specimen bags containing all required items and containers were distributed to the hospitals. Relevant patient information was recorded on a laboratory data collection form.

Diagnostic laboratories

The Kumasi Centre for Collaborative Research in Tropical Medicine (KCCR), Kwame Nkrumah University of Science

and Technology (KNUST), Kumasi, Ghana, served as the local reference laboratory. The external reference laboratories were at Bernhard Nocht Institute for Tropical Medicine (BNITM), Hamburg, Germany and the Department of Infectious Diseases and Tropical Medicine (DITM), University of Munich, Germany.

Diagnostic methods and quality assurance

In this study, the diagnostic results obtained by swab smear MIC, swab DRB-PCR, tissue MIC and tissue DRB-PCR at the local reference laboratory were selected for further evaluation. The laboratory analysis followed standardized procedures. Smears were prepared from decontaminated material and stained with the Ziehl-Neelsen technique. DRB-PCR was carried out as described elsewhere (WHO 2001; Bretzel *et al.* 2005; Siegmund *et al.* 2005).

A test result was only accepted if verified by quality assurance testing, i.e. rereading of slides for MIC and parallel testing of specimens by the standard method for PCR (Stinear *et al.* 1999). All quality assurance procedures were conducted at the BNITM and DITM. In case of contradicting results between local and external laboratories, the tests were repeated at both laboratories. Only consensus results were communicated to the hospitals, otherwise the test result was considered 'not determined'.

Tissue specimens from patients with negative MIC and PCR results were subjected to histopathology at the BNITM in order to establish the differential diagnosis (Guarner *et al.* 2003). Specimens from patients with only one single positive test result were also analysed by histopathology in order to verify the diagnosis of BUD.

Patients and specimens

Between January 2003 and August 2005, diagnostic specimens from 161 patients (ulcers: $n = 94$ and nodules: $n = 67$) were subjected to laboratory analysis.

Comparison of positivity rates of different laboratory assays

In this study, the positivity rate of an assay was calculated by determining the proportion of specimens that tested positive in the respective assay referring to the population of clinically diagnosed BUD cases. The following positivity rates were determined:

- *Pre-ulcerative lesions:* tissue MIC and tissue PCR (post-surgical laboratory confirmation).
- *Ulcerative lesions:* swab smear MIC (pre-surgical diagnosis) and tissue MIC (post-surgical laboratory confirmation), swab smear MIC and swab PCR (pre-surgical diagnosis), swab PCR (pre-surgical diagnosis) and tissue PCR (post-surgical laboratory confirmation), tissue MIC and tissue PCR (post-surgical laboratory confirmation).

Based on the positivity rate of each test, the diagnostic yield of a combination of different methods was determined by calculating the additional diagnostic yield gained by each additional method starting from the most simple laboratory assay as reference value.

Diagnostic sensitivity of microscopy and PCR

The diagnostic sensitivity of MIC and PCR was determined as the proportion of positive test results related to the total number of clinically diagnosed BUD patients.

Statistical analysis

Statistical analysis (odds ratio, *P*-value and chi-square test) was carried out by EPIINFO 3.3.2. (CDC, Atlanta, GA, USA).

Cost analysis

The costs of each laboratory assay were calculated based on the costs for laboratory reagents and material as used in this study.

Results

Of 161 patients, 85 (52.8%) could be confirmed by at least two or more positive laboratory tests at the local reference centre. Of 161 patients 114 (70.8%), were confirmed by at least one positive laboratory test (29 of those had only one single positive test result). The laboratory tests carried out at the local reference laboratory did not provide any positive result for 47 (29.2%) patients.

Histopathological analysis of tissue specimens from four patients with single positive MIC results (swab $n = 2$ and tissue $n = 2$) confirmed BUD in two cases and excluded BUD in two cases (false positives). Histopathological analysis of tissue specimens from 25 patients with single positive PCR results confirmed BUD in all cases.

Fifty-nine patients had positive swab and/or tissue MIC results. The positive MIC results of 57 patients were confirmed by at least one additional test method. The MIC result of two patients was determined false positive by histopathology. The specificity of MIC was 96.6% (57/59).

One hundred eight patients had positive swab and/or tissue PCR results. All PCR results were confirmed by at least one additional test method. Histopathology did not identify false-positive results. The specificity of PCR was 100% (118/118).

Histopathological analysis of the 47 MIC- and PCR-negative specimens identified an additional 13 BUD cases (nodules: $n = 5$ and ulcers: $n = 8$). BUD was excluded in 34 cases, an alternative diagnosis was available for 23 of these patients [leprosy $n = 1$, bacterial abscess $n = 3$, parasitic infections $n = 7$ (5 of those onchocerciasis), bacterial ulceration $n = 1$, chronic ulceration $n = 1$, granulomatous inflammation $n = 1$, unspecific chronic and acute dermatitis $n = 9$].

The positivity rates for MIC and PCR in pre-ulcerative and ulcerative lesions are shown in Tables 1–5.

Diagnostic sensitivity of MIC and PCR in pre-ulcerative lesions was tissue MIC 40.3% (27/67), tissue PCR 62.7% (42/67); for ulcerative lesions: swab smear MIC 29.8% (28/94), swab PCR 66.0% (62/94), tissue MIC 42.6% (40/94), tissue PCR 57.4% (54/94).

Table 1 Comparison of tissue microscopy (MIC) and tissue polymerase chain reaction (PCR) in pre-ulcerative lesions ($n = 67$)

	Tissue PCR		
In pre-ulcerative lesions	Positive	Negative	Total
Tissue MIC			
Positive	25 (37.3)	2 (3.0)	27 (40.3)
Negative	17 (25.4)	23 (34.3)	40 (59.7)
Total	42 (62.7)	25 (37.3)	67 (100.0)

Values are given in %.
The positivity rates as well as the percentage of negative results for tissue MIC and tissue PCR are indicated. OR = 18.75 (95% CI: 3.51–133.05), $P = 0.000015$ and $\chi^2 = 18.78$.

Table 2 Comparison of swab smear microscopy (MIC) and tissue MIC in ulcerative lesions ($n = 94$)

	Tissue MIC		
In ulcerative lesions	Positive	Negative	Total
Swab smear MIC			
Positive	20 (21.3)	8 (8.5)	28 (29.8)
Negative	20 (21.3)	46 (48.9)	66 (70.2)
Total	40 (42.6)	54 (57.4)	94 (100.0)

Values are given in %.
The positivity rates as well as the percentage of negative results for swab smear MIC and tissue MIC are indicated. OR = 5.75 (95% CI: 1.98–17.21), $P = 0.000236$ and $\chi^2 = 13.60$.

Diagnostic yield of the combination of different methods and false positives

For pre-ulcerative lesions, the subsequent application of tissue MIC and tissue PCR rendered positive results in 44 (65.7%) of all 67 patients; for 23 (34.3%) of all 67

Table 3 Comparison of swab smear microscopy (MIC) and swab polymerase chain reaction (PCR) in ulcerative lesions ($n = 94$)

	Swab PCR		
In ulcerative lesions	Positive	Negative	Total
Swab smear MIC			
Positive	26 (27.7)	2 (2.1)	28 (29.8)
Negative	36 (38.3)	30 (31.9)	66 (70.2)
Total	62 (66.0)	32 (34.0)	94 (100.0)

Values are given in %.
The positivity rates as well as the percentage of negative results for swab MIC and swab PCR are indicated. OR = 10.83 (95% CI: 2.21–71.97), $P = 0.000337$ and $\chi^2 = 12.85$.

Table 4 Comparison of swab polymerase chain reaction (PCR) and tissue PCR in ulcerative lesions ($n = 94$)

	Tissue PCR		
In ulcerative lesions	Positive	Negative	Total
Swab PCR			
Positive	49 (52.1)	13 (13.8)	62 (66.0)
Negative	5 (5.3)	27 (28.7)	32 (34.0)
Total	54 (57.4)	40 (42.6)	94 (100.0)

Values are given in %.
The positivity rates as well as the percentage of negative results for swab PCR and tissue PCR are indicated. OR = 20.35 (95% CI: 5.88–75.51), $P < 0.000001$ and $\chi^2 = 34.71$.

Table 5 Comparison of tissue microscopy (MIC) and tissue polymerase chain reaction (PCR) in ulcerative lesions ($n = 94$)

	Tissue PCR		
In ulcerative lesions	Positive	Negative	Total
Tissue MIC			
Positive	33 (35.1)	7 (7.4)	40 (42.6)
Negative	21 (22.3)	33 (35.1)	54 (57.4)
Total	54 (57.4)	40 (42.6)	94 (100.0)

Values are given in %.
The positivity rates as well as the percentage of negative results for tissue MIC and tissue PCR are indicated. OR = 7.41 (95% CI: 2.53–22.47), $P = 0.000024$ and $\chi^2 = 17.88$.

patients, no positive result could be obtained. Tissue MIC provided two false-positive results (Table 6).

In case of ulcerative lesions, subsequent application of swab smear MIC and swab PCR, both methods for pre-surgical laboratory diagnosis, rendered positive results in 64 (68.1%) of all 94 patients; for 30 (31.9%) of all 94 patients, no positive result could be obtained. False-positive results did not occur (Table 7).

As shown in Table 8, subsequent application of tissue MIC and tissue PCR, both methods for post-surgical laboratory confirmation, rendered positive results in 61 (64.9%) of all 94 patients; for 33 (35.1%) of all 94 patients, no positive result could be obtained. False-positive results did not occur.

Table 9 reveals that the subsequent application of pre-surgical and post-surgical laboratory methods rendered

Table 6 Pre-ulcerative lesions ($n = 67$): Diagnostic yield of tissue microscopy (MIC), additional diagnostic yield of tissue polymerase chain reaction (PCR), combined diagnostic yield MIC/PCR, percentage of patients without laboratory confirmation by tissue MIC/PCR and the percentage of false positives related to the number of positive results in the respective test

Type of lesion	Pre-ulcerative (nodule)			
Application	Post-surgical laboratory confirmation			
Diagnostic yield	Diagnostic yield (tissue MIC)	Additional diagnostic yield (tissue PCR)	Combined diagnostic yield (tissue MIC and tissue PCR)	No laboratory diagnosis by tissue MIC/PCR
	40.3 (27/67)	25.4 (17/67)	65.7 (44/67)	34.3 (23/67)
False positives	7.4 (2/27)	0 (0/17)	4.5 (2/44)	–

Values are given in %.

Table 7 Ulcerative lesions ($n = 94$), pre-surgical diagnosis: Diagnostic yield of swab smear microscopy (MIC), additional diagnostic yield of swab polymerase chain reaction (PCR), combined diagnostic yield swab MIC/PCR, percentage of patients without laboratory confirmation by swab MIC and PCR and the percentage of false positives related to the number of positive results in the respective test

Type of lesion	Ulcerative			
Application	Pre-surgical laboratory diagnosis			
Diagnostic yield	Diagnostic yield (swab smear MIC)	Additional diagnostic yield (swab PCR)	Combined diagnostic yield (swab smear MIC and swab PCR)	No laboratory diagnosis by swab smear MIC/swab PCR
	29.8 (28/94)	38.3 (36/94)	68.1 (64/94)	31.9 (30/94)
False positives	0 (0/28)	0 (0/36)	0 (0/64)	–

Values are given in %.

Table 8 Ulcerative lesions ($n = 94$): Diagnostic yield of tissue microscopy (MIC), additional diagnostic yield of tissue polymerase chain reaction (PCR), combined diagnostic yield tissue MIC/PCR, percentage of patients without laboratory confirmation by tissue MIC and PCR and the percentage of false positives related to the number of positive results in the respective test

Type of lesion	Ulcerative			
Application	Post-surgical laboratory confirmation			
Diagnostic yield	Diagnostic yield (tissue MIC)	Additional diagnostic yield (tissue PCR)	Combined diagnostic yield (tissue MIC and tissue PCR)	No laboratory diagnosis by tissue MIC/tissue PCR
	42.6 (40/94)	22.3 (21/94)	64.9 (61/94)	35.1 (33/94)
False positives	0 (0/40)	0 (0/21)	0 (0/61)	–

Values are given in %.

Table 9 Ulcerative lesions ($n = 94$): Diagnostic yield of a combination of pre-surgical and post-surgical methods [swab smear microscopy (MIC), swab polymerase chain reaction (PCR), tissue MIC and tissue PCR], percentage of patients without laboratory confirmation by all methods and the percentage of false positives related to the number of positive results in the respective test

Type of lesion	Ulcerative					
Application	Pre-surgical laboratory diagnosis		Post-surgical laboratory confirmation		All	
Diagnostic yield	Diagnostic yield (swab smear MIC)	Additional diagnostic yield (swab PCR)	Additional diagnostic yield (tissue MIC)	Additional diagnostic yield (tissue PCR)	Combined diagnostic yield (swab smear MIC, swab PCR, tissue MIC, tissue PCR)	No laboratory diagnosis by combination of all methods
	29.8 (28/94)	38.3 (36/94)	4.3 (4/94)	2.1 (2/94)	74.5 (70/94)	25.5 (24/94)
False positives	0 (0/28)	0 (0/36)	0 (0/5)	0 (0/2)	0 (0/70)	–

Values are given in %.

Table 10 Simultaneous testing of 100 tissue specimens [microscopy/polymerase chain reaction (MIC/PCR)] *vs.* stepwise subsequent testing of diagnostic specimens

Pre-ulcerative lesions				
Number of patients ($n = 100$)	Tissue MIC	Tissue PCR	Histopathology	Total
Simultaneous testing				
Number of specimens	100	100	35	
Cost (€)	50	1500	175	1725
Stepwise testing				
Number of specimens	100	60	35	
Cost (€)	50	900	175	1125

Cost calculations for the stepwise approach are based on the assumption that 40% of specimens can be diagnosed by tissue MIC, an additional 25% by tissue PCR, and 35% by histopathological analysis. Costs per specimen/test: MIC: €0.5, PCR: €15, histopathology: €5

positive results in 70 (74.5%) of all 94 patients; for 24 (25.5%) of all 94 patients, no positive result could be obtained. False-positive results did not occur.

Table 10 (pre-ulcerative lesions) and Table 11 (ulcerative lesions) show the costs and cost reduction, respectively, of simultaneous *vs.* stepwise subsequent testing of diagnostic specimens of 100 patients with all available laboratory tests.

Discussion

Structural, technical and financial limitations often hamper the comprehensive laboratory diagnosis of BUD in areas of endemicity. Therefore, the current diagnostic recommendations, i.e. obtaining two positive laboratory results for a positive diagnosis, may have to be reconsidered (WHO 2001). A more practicable and cost-effective approach to the laboratory diagnosis of BUD considering availability

Table 11 Simultaneous testing of 100 swab and tissue specimens [swab microscopy (MIC)/swab polymerase chain reaction (PCR)/tissue MIC/tissue PCR] *vs.* stepwise subsequent testing of diagnostic specimens

Ulcerative lesions						
Ulcers ($n = 100$)	Swab MIC	Swab PCR	Tissue MIC	Tissue PCR	Histopathology	Total (€)
Simultaneous testing						
Number of specimens	100	100	100	100	30	
Cost (€)	50	1500	50	1500	150	3250
Stepwise testing (a)						
Number of specimens	100	70	30	25	22	
Cost (€)	50	1050	30	25	330	1485
Stepwise testing (b)						
Number of specimens	100	70	–	–	30	
Cost(€)	50	1050	–	–	150	1250

Cost calculations for the stepwise approach are based on the assumption that 30% of specimens can be diagnosed by swab smear MIC, an additional 40% by swab PCR. For the remaining 30%, two options are shown: (a) tissue MIC (additional 5% positive results) followed by tissue PCR (additional 3% positive results) and histopathology (22% remaining), (b) only histopathology. Costs per specimen/test: MIC: €0.5, PCR: €15, histopathology: €5

and feasibility of diagnostic methods is required. In this study a stepwise diagnostic approach, i.e. the subsequent application of different diagnostic assays, focusing on pre-surgical diagnosis was evaluated.

The number of laboratory-confirmed clinically diagnosed BUD cases with one positive confirmative test was 20% higher than that with two or more positive confirmative tests. In this study, the specificity of PCR was determined to be 100%. Previous histopathological analysis of PCR-positive specimens also resulted in a specificity of 100% (Bretzel *et al.* 2005). Therefore, single positive PCR results guarantee a reliable diagnosis of BUD. The specificity of swab smear and tissue MIC may not be considered sufficient for the detection of *M. ulcerans*. In this study, however, the specificity of MIC as determined by additional confirmatory tests was 96.6% (57/59). With 1.8% (2/114) false positives related to the total number of positive patients, the authors consider one diagnostic test sufficient for the laboratory confirmation of BUD (van der Werf *et al.* 2005).

Based on the diagnostic yield of the laboratory assays analysed in this study, a stepwise application of diagnostic tests is recommended. Tissue specimens from pre-ulcerative lesions should first be subjected to tissue MIC (40% positive results). If MIC is negative, subsequently tissue PCR (additional 25% positive results) should be carried out. According to the data presented in this study, the combination of these methods allows the laboratory confirmation of approximately 65% of clinically diagnosed BUD cases with pre-ulcerative lesions. In view of the safety of laboratory personnel, grinding of tissue for tissue MIC should be carried out under a laminar flow. Alternatively, all specimens may be subjected to PCR. Only histopathological analysis can achieve a diagnosis for the remaining 35% of cases.

For patients with ulcerative lesions, diagnostic swabs should first be examined by swab smear MIC (30% positive results). If MIC is negative, subsequently swab PCR (additional 40% positive results) should be carried out. According to the data presented in this study, the combination of these methods allows non-invasive pre-surgical diagnosis of approximately 70% of clinically diagnosed BUD cases with ulcerative lesions.

Laboratory confirmation of the remaining 30% of cases may be attempted by post-surgical analysis of excised tissue by MIC and PCR. However, considering the low additional diagnostic yield of tissue analysis, PCR analysis of tissue must be carefully considered in view of the laboratory costs. Therefore, analysis of the remaining 30% of cases by histopathology may be advisable, if links with a reference centre providing histopathology are established.

Implementation of a stepwise, subsequent analysis of diagnostic specimens will result in considerable cost saving. Compared with simultaneous testing of diagnostic swabs and tissue specimens by MIC, PCR and histopathology, stepwise, successive testing of specimens saves up to 35% of diagnostic costs in pre-ulcerative lesions and up to 60% of diagnostic costs in ulcerative lesions.

For pre-surgical diagnosis in pre-ulcerative lesions and in ulcerative lesions with negative swab samples, punch biopsies may be used (Phillips *et al.* 2005). However, the unit costs of €2 to €3 per biopsy needle must be considered. For very small pre-ulcerative lesions, punch biopsies may not be appropriate, as the surface area of the lesion might not be sufficient to take specimens for several diagnostic tests.

Timely on-site laboratory diagnosis of BUD requires the availability of laboratory capacity at district level. In collaboration with the National Buruli Ulcer Control Programme in Ghana, decentralization of diagnostic facilities is underway. BUD MIC and DRB-PCR laboratories have been established in selected treatment centres at district level, laboratory staff is being trained at the local reference laboratory, and an external quality assurance system for these laboratories has been installed. Large-scale on-site assessment of diagnostic specimens from selected BUD treatment centres will further explore the practicability and cost-effectiveness of the proposed stepwise approach to the laboratory diagnosis of BUD.

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Corresponding Author Gisela Bretzel, Department of Infectious Diseases and Tropical Medicine, Ludwig Maximilians University of Munich, Leopoldstrasse 5, D-80802 Munich, Germany. Tel.: +49 89 2180 3618; Fax: +49 89 336112; E-mail: bretzel@lrz.uni-muenchen.de

Une approche par étape pour le diagnostic de laboratoire de l'ulcère de Buruli

OBJECTIFS Vu les limitations techniques et financières dans les zones endémiques, la pratique actuelle et les recommandations pour le diagnostic de laboratoire de la maladie de l'ulcère de Buruli (UB) pourraient être à revoir. Nous avons analysé des résultats de diagnostic afin de d'investiguer des options pour un diagnostic de laboratoire modifié pour l'UB qui soit plus pratique, avec un bon rapport coût/efficacité et une approche plus opportune.
MÉTHODES Des spécimens de 161 patients UB cliniquement diagnostiqués dans quatre centres de traitement différents du Ghana ont été analysés en utilisant des tests de laboratoire et les taux de positivité ont été comparés.
RÉSULTATS Le nombre de cas d'UB cliniquement diagnostiqués et confirmés par un seul test positif de laboratoire était 20% plus élevé que celui par deux tests positifs de laboratoire. La spécificité de la microscopie et de la PCR était de 96,6% et 100% respectivement. Une analyse subséquente par microscopie et par PCR, de spécimens provenant d'excision de tissu en state pré ulcéré a révélé 65% de cas d'UB confirmés au laboratoire. L'analyse subséquente par microscopie et par PCR sur des prélèvements sur tiges de coton de lésions ulcérées a révélé 70% de cas d'UB confirmés au laboratoire.
CONCLUSIONS La spécificité des tests de diagnostic utilisés dans cette étude suggère qu'un seul test positif peut être considéré suffisant pour la confirmation de laboratoire de l'UB. L'application subséquente de différents tests de laboratoire a permis une confirmation de laboratoire pour 65% de cas de lésions en stade pré ulcéré et 70% de cas de lésions ulcérées. L'implémentation d'une analyse par étape des spécimens permettra de réduire considérément les coûts par rapport au test simultané des spécimens par plusieurs tests de diagnostic.

mots clés ulcère de Buruli, PCR, microscopie, réduction des coûts, Ghana

Enfoque escalonado del diagnóstico en laboratorio de la úlcera de Buruli

OBJETIVO En vista de las limitaciones técnicas y económicas existentes en las áreas endémicas, debería reconsiderarse la práctica y recomendaciones actuales para el diagnóstico en laboratorio de la úlcera de Buruli (UB). Hemos revisado los resultados del diagnóstico en laboratorio de la UB, con el fin de explorar opciones para un nuevo enfoque más práctico, costo-efectivo y oportuno.
MÉTODOS Se analizaron 161 especimenes de pacientes con diagnóstico clínico de UB de cuatro centros diferentes en Ghana. Se comparó la tasa de positividad de las muestras.
RESULTADOS El número de casos clínicos de UB confirmados por laboratorio con una prueba positiva confirmatoria fue un 20% más alta que con dos pruebas confirmatorias. La especificidad de la microscopía y la PCR fue del 96.6% y 100% respectivamente. Análisis subsiguientes de especimenes diagnósticos de frotis de lesiones ulcerosas mediante microscopía y PCR dieron un 70% de los casos de UB previamente confirmados en el laboratorio.
CONCLUSIONES La especificidad de las pruebas diagnósticas utilizadas en este estudio sugiere que una prueba diagnóstica positiva puede considerarse como suficiente para la confirmación en laboratorio de la UB. La aplicación subsiguiente de diferentes técnicas diagnósticas tuvo una confirmación del 65% para lesiones pre-ulcerativas y un 70% para las ulcerativas. La implementación de un análisis escalonado de los especimenes diagnósticos resultaría en un ahorro considerable, comparado con una evaluación simultánea de los especimenes utilizando diferentes pruebas diagnósticas.

palabras clave Úlcera de Buruli, PCR, microscopía, ahorro, Ghana

MAJOR ARTICLE

Dry Reagent–Based Polymerase Chain Reaction Compared with Other Laboratory Methods Available for the Diagnosis of Buruli Ulcer Disease

Vera Siegmund,[1,2] Ohene Adjei,[3] Jörg Nitschke,[2] William Thompson,[4] Erasmus Klutse,[5] Karl Heinz Herbinger,[1] Ruth Thompson,[3] Felicitas van Vloten,[2] Paul Racz,[2] Bernhard Fleischer,[2] Thomas Loescher,[1] and Gisela Bretzel[1,2]

[1]Department of Infectious Diseases and Tropical Medicine, Ludwig-Maximilians University of Munich, Munich, and [2]Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany; and [3]Kumasi Centre for Collaborative Research in Tropical Medicine, Kwame Nkrumah University of Science and Technology, Kumasi, [4]Agogo Presbyterian Hospital, Agogo, and [5]Dunkwa Governmental Hospital, Dunkwa-on-Offin, Ghana

Background. Because of the multifaceted clinical presentation of Buruli ulcer disease, misclassification of clinically diagnosed cases may occur frequently. Laboratory tests for the confirmation of suspected cases include microscopic examination, culture, polymerase chain reaction (PCR), and histopathologic examination. However, microscopic examination, the only test usually available in areas of endemicity, has a low sensitivity.

Methods. To make a highly sensitive diagnostic method locally available, dry reagent–based PCR (DRB-PCR), which is well adapted to tropical conditions, was pilot-tested in Ghana. Subsequently, the assay was used for the routine diagnosis of Buruli ulcer disease over a period of 2 years. The method was compared with other diagnostic tests to evaluate its performance under field conditions.

Results. The interassay agreement rate between DRB-PCR and standard PCR was 91.7% for swab specimens and 95% for tissue specimens. Among all of the locally available tests, DRB-PCR revealed the highest overall positivity ratio. Sixty percent of patients with clinical diagnoses of Buruli ulcer disease had the diagnoses confirmed by DRB-PCR of swab or tissue specimens, compared with 30%–40% of patients who had diagnoses confirmed by microscopic examination of swab or tissue specimens. The positivity ratio of DRB-PCR varied considerably when analyzed per treatment center. Standardization of specimen collection resulted in a 30% increase in the positivity ratio of the assay, compared with that in the pilot-testing phase.

Conclusions. DRB-PCR is a reliable tool for the diagnosis of Buruli ulcer disease. However, PCR assays are suitable for detection only during early stages of the disease, when samples still contain bacilli. The quality of clinical diagnosis and the quality of diagnostic specimens strongly influence the positivity ratio.

Buruli ulcer disease (BUD) is an infectious disease caused by *Mycobacterium ulcerans* that involves the skin and the subcutaneous adipose tissue. In the majority of cases, the disease starts as a painless papule, plaque, or nodule that evolves into a large ulceration with characteristic undermined edges. Destructive lesions with extensive scarring and contractures of the limbs are common. Because of the multifaceted clinical presentation, misclassification of clinically diagnosed cases of BUD may occur frequently. After tuberculosis and leprosy, BUD has become the third most common mycobacterial disease in immunocompetent humans worldwide, and the incidence of BUD is increasing in tropical countries. The incidence and prevalence of BUD worldwide are not precisely defined, because adequate surveillance data based on accurate case confirmation data are lacking. Thus far, disease control in countries where BUD is endemic is limited to early case detection through improved active surveillance and surgical excision of lesions followed by skin grafting [1–6].

Laboratory confirmation of suspected cases prevents misclassification, ensures adequate treatment, and provides reliable incidence and prevalence data that, in turn, may support the development of new disease-control strategies. Currently available laboratory tests include microscopic detection of acid-fast bacilli in

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Reprints or correspondence: Dr. Vera Siegmund, Dept. of Infectious Diseases and Tropical Medicine, Ludwig-Maximilians University of Munich, Leopoldstrasse 5, D-80802 Munich, Germany (vera.siegmund@lrz.uni-muenchen.de).

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swab and tissue smears, PCR analysis and culture of swab and tissue specimens, and histopathologic examination. The sensitivity of smear microscopic examination and culture is relatively low, whereas histopathologic examination and PCR provide high diagnostic sensitivities, except during the healing stage of the disease. However, the latter diagnostic tests are rarely available in countries where BUD is endemic. In particular, the application of PCR techniques in tropical regions presents technical difficulties. Transportation and storage of reagents require cold chains that are often interrupted by frequent power outages. In addition, conventional PCR assays require careful handling of reaction components by skilled laboratory workers, as well as strict measures to avoid contamination. To facilitate the PCR diagnosis of BUD in countries where the disease is endemic, a dry reagent–based PCR (DRB-PCR) was installed and pilot-tested at the Kumasi Centre for Collaborative Research in Tropical Medicine (Kumasi, Ghana) in 2003. Because the method uses lyophilized reagents, it is well adapted to tropical conditions [1, 7–11]. Subsequently, over a period of 2 years, a study of the performance of the method under field conditions and a comparison with other diagnostic methods, including standard PCR (used in according to Stinear et al. [12]), was performed.

MATERIALS AND METHODS

Ethical clearance and informed patient consent. Ethical clearance for the study was provided by the Committee of Human Research Publication and Ethics at the School of Medical Sciences at the University of Science and Technology (Kumasi, Ghana). Informed consent was obtained from patients before surgery.

Inclusion criteria and treatment centers. During the study period (January 2004–January 2006), all patients who had received a clinical diagnosis of BUD and who had preulcerative or ulcerative lesions and a duration of the disease of <6 months ("early lesions") were included at 4 of the following treatment centers: Agogo Presbyterian Hospital (Agogo, Asante Akim North District, Ashanti Region; hospital A), Dunkwa Government Hospital (Dunkwa-on-Offin, Upper Denkyira District, Central Region; hospital D), Goaso Hospital (Goaso, Ahafo Ano District, Brongh Ahafo Region; hospital G), and St. Martin's Catholic Hospital (Agroyesum, Amansie West District, Ashanti Region; hospital AG).

Diagnostic laboratories and tests. Microscopic examination, culture, and DRB-PCR were performed serially over time at the Kumasi Centre for Collaborative Research in Tropical Medicine (Kumasi, Ghana) and at the Kwame Nkrumah University of Science and Technology (Kumasi, Ghana). Histopathologic examination was performed at the Bernhard Nocht Institute for Tropical Medicine (Hamburg, Germany), and standard PCR was performed at the Department of Infectious Diseases and Tropical Medicine at Ludwig-Maximilians University of Munich (Munich, Germany). Diagnostic specimens were sent and tested in batches.

Standardized collection of diagnostic specimens. Local surgeons collected swab specimens before surgery and tissue specimens after surgery according to standardized criteria. In case of ulcerative lesions, 3 diagnostic swab specimens (tested by microscopic examination, culture, DRB-PCR, and standard PCR) and 4 tissue specimens (tested by microscopic examination, culture, DRB-PCR, standard PCR, and histopathologic examination) were collected. In case of preulcerative lesions, 4 tissue specimens (tested by microscopic examination, culture, DRB-PCR, standard PCR, and histopathologic examination) were subjected to laboratory analysis. Swab specimens were obtained by circling the entire undermined edge of the ulcers before surgery. Tissue specimens with a maximum size of $10mm \times 10mm$ were obtained from surgically excised tissue. For preulcerative lesions, the tissue specimens were obtained from the center of the lesion by cutting it longitudinally and transversally into 4 equal segments. The tissue specimens for ulcerative lesions were obtained from the edge of the lesions

Table 1. Diagnostic specimens, transportation media, and diagnostic tests.

Specimen, transportation medium	Diagnostic test
Tissue	
PANTA, 5 mL	Microscopic examination and culture
CLS, 700 μL	DRB-PCR
CLS, 700 μL	Standard PCR
Ten percent buffered neutral formalin, 5 mL	Histopathologic examination
Swab	
PANTA, 5 mL	Microscopic examination and culture
CLS, 700 μL	DRB-PCR
CLS, 700 μL	Standard PCR

NOTE. CLS, cell lysis solution (Gentra Systems); DRB-PCR, dry reagent–based PCR; PANTA, polymyxin B, amphotericin B, nalidixic acid, trimethoprim, azlocillin, Dubos broth base, and Dubos medium albumin (Becton Dickinson Biosciences).

below the end of the undermined edge containing all necrotic tissue sections. Tissue specimens for different laboratory tests were located adjacent to each other to guarantee maximum comparability of results of all diagnostic tests. Tissue specimens had to contain subcutaneous adipose tissue to be included in the analysis [13, 14].

To provide optimal conditions for storage and transportation of specimens, standardized specimen collection bags containing all required items and containers, as well as standardized, laboratory data entry forms, were distributed to the hospitals. The diagnostic specimens and the respective transportation and storage media for each laboratory test conducted are shown in table 1. Using the media shown in table 1, diagnostic specimens could be stored for a minimum of 6 months at room temperature until further processing.

Diagnostic methods applied. All reagents were stored and used according to the manufacturer's instructions.

Culture, microscopic examination, and histopathologic examination. Swab and ground tissue specimens for culture were decontaminated by the Petroff method, inoculated on Löwenstein-Jensen media, and incubated at 32°C for 6 months [9]. Swab and tissue smears for microscopic examination were prepared from decontaminated material and stained with the Ziehl-Neelsen technique [9]. Histopathologic examination of tissue specimens was conducted according to standardized procedures [15].

PCR. DNA from swab and tissue specimens was prepared using the Puregene DNA isolation kit (Genomic DNA Purification Kit, Gentra Systems), with minor modifications [7]. For DRB-PCR, oligonucleotides MU5 and MU6 [12] were lyophilized in reaction tubes. PuReTaq Ready-To-Go PCR Beads (Amersham Biosciences) were added and dissolved in water. The stability of primers (up to 1 year) was monitored by positive control reactions. Standard PCR (IS2404 PCR) was performed in accordance with Stinear et al. [12]. Both PCR methods included a negative extraction control and positive, negative, and inhibition controls [7]. The thermal cycling protocol was as follows: 94°C for 10 min, followed by 40 cycles at 94°C for 10 s, 58°C for 10 s, and 72°C for 30 s, with a final cycle at 72°C for 15 min. The amplification products were held at 4°C until they were processed further by agarose gel electrophoresis.

Table 2. Distribution of preulcerative and ulcerative lesions per treatment center.

Treatment center	No. (%) of preulcerative lesions (n = 98)	No. (%) of ulcerative lesions (n = 132)	No. (%) of total lesions (n = 230)
Agogo	49 (45)	61 (55)	110 (47.8)
Agroyesum	6 (50)	6 (50)	12 (5.2)
Dunkwa	27 (43)	36 (57)	63 (27.4)
Field	0 (0)	12 (100)	12 (5.2)
Goaso	16 (48)	17 (52)	33 (14.3)

Specificity of PCR methods. Primer validation by BLAST algorithm and testing of 48 mycobacterial species and 21 other organisms by PCR resulted in 100% specificity of the standard PCR [12]. Identical primers, thermal cycling profiles, and comparable reaction conditions predicted 100% specificity of the DRB-PCR. Our own technical validation data (on 15 mycobacterial species that are common in human infections) [7] confirmed the specificity of the assay.

Patients and specimens. All patients who received a clinical diagnosis of BUD underwent surgery. Forty patients from hospital A had received previous short-term antibiotic treatment (mean duration, 7 days). Three hundred fifty diagnostic specimens from 218 patients who had received clinical diagnoses of BUD at 4 different hospitals and 12 patients with suspected cases from the field (132 patients with ulcerative lesions and 98 patients with preulcerative lesions) were subjected to laboratory analysis. The preulcerative lesions consisted of 72 nodules, 1 papule, and 25 plaques. One hundred thirty-two swab specimens and 218 tissue specimens were analyzed by microscopic examination, culture, DRB-PCR, and standard PCR; 167 tissue specimens were subjected to histopathologic examination (table 2).

Parameters determined. To determine the interassay agreement rates (defined as the agreement rates between DRB-PCR and standard PCR), 2 swab specimens (from ulcerative lesions) and 2 adjacent tissue specimens (from ulcerative and preulcerative lesions) were subjected to DRB-PCR (at Kumasi Centre for Collaborative Research in Tropical Medicine) and standard PCR (at the Department of Infectious Diseases and Tropical Medicine at the Ludwig-Maximilians University of Munich). The agreement rates between both methods were determined as the percentage of concordant results per type of specimen and per type of lesion and were expressed as overall agreement rates and agreement rates per treatment center.

The positivity ratio was defined as the proportion of specimens among all tested specimens that yielded positive results by a certain diagnostic method. The overall positivity ratio of DRB-PCR was compared with that of standard PCR, microscopic examination, culture, and histopathologic examination. To determine hospital-related factors that might influence the quality of laboratory diagnosis, the positivity ratio of each test method was determined per treatment facility.

Statistical analysis. For statistical analysis, approximative tests (χ^2 tests) and exact tests (Fisher's exact tests) were performed with Stata software, version 9.0 (Stata). Statistical analysis (95% CIs) considered the lower number of histopathologic specimens.

RESULTS

Agreement rates between DRB-PCR and standard PCR (interassay agreement rate) overall and per hospital. As shown in table 3, the overall agreement rate between DRB-PCR and standard PCR was 91.7% ($P < .01$) for swab specimens and 95.0% ($P < .01$) for all tissue specimens. Table 3 also shows the interassay agreement rates between DRB-PCR and standard PCR per hospital. Interassay agreement rates for centers A and D were 93.4% and 97.2%, respectively, for swab specimens and 97.3% and 93.7%, respectively, for tissue specimens.

Overall positivity ratios of all diagnostic tests conducted. Table 4 shows the overall positivity ratios of DRB-PCR versus all other diagnostic tests conducted. The overall positivity ratio of DRB-PCR of swab and tissue specimens was 60.6%, and the overall positivity ratio of standard PCR of swab specimens and tissue specimens was 67.4% and 61.5%, respectively. Overall positivity ratios for other diagnostic tests ranged from 0% (culture of swab specimens) to 77.8% (histopathologic analysis of tissue specimens).

Positivity ratio of all diagnostic test methods per treatment center. Table 4 also shows the positivity ratios of DRB-PCR versus all other diagnostic test methods, per treatment center. DRB-PCR of swab specimens provided the highest positivity ratios at treatment centers A, AG, and D (67.2%, 66.7%, and 63.9%, respectively), and DRB-PCR of tissue specimens provided the highest positivity ratios at centers A and AG (71.8% and 83.3%, respectively). The lowest positivity ratio of DRB-PCR of tissue specimens was detected at center D (41.3%).

Differential diagnosis by histopathologic examination. Among all 167 specimens, BUD was confirmed in 130 specimens and excluded in 37 specimens, by histopathologic examination. An alternative diagnosis was available for 27 of the patients who were confirmed not to have BUD (1 patient had leprosy; 3 patients had a bacterial, granulomatous, or unspecific

Table 3. Interassay agreement rates between dry reagent–based PCR (DRB-PCR) and standard PCR (overall agreement rates and agreement rates per treatment center).

Specimen, treatment center	Concordant results of DRB-PCR and standard PCR			No. of tested specimens	Interassay agreement rate, %	*P*
	Negative	Positive	Total			
Swab						
A	16	41	57	61	93.4	<.01[a]
AG	2	3	5	6	83.3	.40[a]
D	12	23	35	36	97.2	<.01[a]
F	8	3	11	12	91.7	.02[b]
G	5	8	13	17	76.5	.03[a]
All centers	43	78	121	132	91.7	<.01[a]
Ulcerative tissue						
A	18	42	60	61	98.4	<.01[a]
AG	1	4	5	6	83.3	.33[a]
D	19	13	32	36	88.9	<.01[a]
G	6	9	15	17	88.2	<.01[a]
All centers	44	68	112	120	93.3	<.01[b]
Preulcerative tissue						
A	12	35	47	49	95.9	<.01[a]
AG	1	4	5	6	83.3	.33[a]
D	14	13	27	27	100	<.01[a]
G	9	7	16	16	100	<.01[a]
All centers	36	59	95	98	96.9	<.01[a]
All tissue						
A	30	77	107	110	97.3	<.01[a]
AG	2	8	10	12	83.3	.35[a]
D	33	26	59	63	93.7	<.01[a]
G	15	16	31	33	93.9	<.01[a]
All centers	80	127	207	218	95.0	<.01[a]

NOTE. A, Agogo; AG, Agroyesum; D, Dunkwa; DRB-PCR, dry reagent–based PCR; F, Field; G, Goaso.

[a] Determined using Fisher's exact test.

[b] Determined using χ^2 test.

Table 4. Positivity ratios per treatment center and overall positivity ratios per type of specimen.

Test, treatment center	Swab specimens		Ulcerative tissue specimens		Preulcerative tissue specimens		Total tissue specimens	
	No. of positive specimens/total no. of specimens	Positivity ratio, % (95% CI)	No. of positive specimens/total no. of specimens	Positivity ratio, % (95% CI)	No. of positive specimens/total no. of specimens	Positivity ratio, % (95% CI)	No. of positive specimens/total no. of specimens	Positivity ratio, % (95% CI)
Microscopic examination								
A	25/61	41.0	36/61	59.0	26/49	53.1	62/110	56.4
AG	2/6	33.3	2/6	33.3	2/6	33.3	4/12	33.3
D	10/36	27.8	9/36	25.0	10/27	37.0	19/63	30.2
F	1/12	8.3	NA	NA	NA	NA	NA	NA
G	3/17	17.6	7/17	41.2	1/16	6.3	8/33	24.2
All centers	41/132	31.1 (23.2–39.0)	54/120	45.0 (36.1–53.9)	39/98	39.8 (30.1–49.5)	93/218	42.7 (36.1–49.2)
Culture								
A	0/61	0	2/61	3.3	4/49	8.2	6/110	5.5
AG	0/6	0	1/6	16.7	0/6	0	1/12	8.3
D	0/36	0	1/36	2.8	1/27	3.7	2/63	3.2
F	0/12	0	NA	NA	NA	NA	NA	NA
G	0/17	0	0/17	0	0/16	0	0/33	0
All centers	0/132	0	4/120	3.3 (0.1–6.5)	5/98	5.1 (0.7–9.5)	9/218	4.1 (1.5–6.8)
DRB-PCR								
A	41/61	67.2	42/61	68.9	37/49	75.5	79/110	71.8
AG	4/6	66.7	5/6	83.3	5/6	83.3	10/12	83.3
D	23/36	63.9	13/36	36.1	13/27	48.1	26/63	41.3
F	4/12	33.3	NA	NA	NA	NA	NA	NA
G	8/17	47.1	10/17	58.8	7/16	43.8	17/33	51.5
All centers	80/132	60.6 (52.3–68.9)	70/120	58.3 (49.5–67.2)	62/98	63.3 (53.7–72.8)	132/218	60.6 (54.1–67.0)
Standard PCR								
A	45/61	73.8	43/61	70.5	35/49	71.4	78/110	70.9
AG	4/6	66.7	5/6	83.3	4/6	66.7	9/12	75.0
D	24/36	66.7	17/36	47.2	13/27	48.1	30/63	47.6
F	4/12	33.3	NA	NA	NA	NA	NA	NA
G	12/17	70.6	10/17	58.8	7/16	43.8	17/33	51.5
All centers	89/132	67.4 (59.4–75.4)	75/120	62.5 (53.8–71.2)	59/98	60.2 (50.5–69.9)	134/218	61.5 (55.0–67.9)
Histopathologic examination								
A	NA	NA	38/46	82.6	34/39	87.2	72/85	84.7
AG	NA	NA	5/6	83.3	2/2	100.0	7/8	87.5
D	NA	NA	24/30	80.0	15/24	62.5	39/54	72.2
F	NA	NA	NA	NA	NA	NA	NA	NA
G	NA	NA	7/9	77.8	5/11	45.5	12/20	60.0
All centers	NA	NA	74/91	81.3 (73.3–89.3)	56/76	73.7 (63.8–83.6)	130/167	77.8 (71.5–84.1)

NOTE. A, Agogo; AG, Agroyesum; D, Dunkwa; DRB-PCR, dry reagent–based PCR; F, Field; G, Goaso; NA, not available.

abscess; 2 patients had chronic or bacterial ulceration of the skin; 8 patients had parasitic infections [5 of these infections were onchocerciasis]; 2 patients had nonspecific inflammation; 9 patients had nonspecific, chronic, or acute dermatitis; 1 patient had fibrosis; and 1 patient had an enlarged lymph node).

DRB-PCR versus histopathologic examination. Table 5 compares the results of DRB-PCR with those of histopathologic examination. Of the 130 specimens confirmed by histopathologic examination, 105 were smear positive for acid-fast bacilli. DRB-PCR yielded positive results for 89 of these specimens and negative results for 16 of these specimens. Of the 37 specimens that were negative by histopathologic examination, 1 specimen was positive by DRB-PCR.

DISCUSSION

To facilitate the application of PCR for the diagnosis of BUD in countries where the disease is endemic, a DRB-PCR was installed at the Kumasi Centre for Collaborative Research in Tropical Medicine. The method was used under routine conditions for 2 years and compared with other diagnostic tests, including standard PCR.

Although the reagents are slightly more expensive, DRB-PCR offers considerable advantages over conventional PCR assays for application in resource-poor countries. The use of lyophilized reagents, which are stable at room temperature for at least 1 year, facilitates shipment and storage. The assay is less sensitive to tropical climatic conditions, such as heat and humidity, as well as the freezing and thawing effects that are consequences of frequent power outages. The reduced number of work steps shortens the performance time and minimizes the risk of contamination. The simplified handling of the test allows rapid familiarization with the method; laboratory staff without previous PCR training were capable of performing the assay correctly after <1 week.

The overall agreement rates between DRB-PCR and standard PCR of 91.7% for swab specimens and 95.0% for tissue specimens were similar to those obtained in the pilot study [7]. The observed deviations of up to 8% between the 2 assays are probably because of the use of different swab and tissue specimens for PCR analysis at the Kumasi Centre for Collaborative Research and at the Department of Infectious Diseases and Tropical Medicine at the Ludwig-Maximilians University of Munich. Although adjacent tissue specimens were subjected to both methods, the possibility of one specimen containing fewer bacilli than the other specimen or containing no bacilli cannot be excluded. Likewise, if several swab specimens are collected for different tests, the second or third swab specimen is likely to contain fewer bacilli than the first specimen. In addition, it is well known that, even if identical source material is used, repeated testing of material containing few bacilli may render variable results. In view of the agreement rates, DRB-PCR is a reliable method for the laboratory diagnosis of BUD [7, 13].

Table 5. Results of dry reagent–based PCR (DRB-PCR) versus histopathologic examination.

	Histopathologic examination result			
	BUD (n = 130)		Not BUD (n = 37)	
DRB-PCR result	AFB positive	AFB negative	AFB negative	Total
Positive	89	14	1	104
Negative	16	11	36	63
Total	105	25	37	167

NOTE. Data are no. of specimens. AFB, smear for acid-fast bacilli; BUD, Buruli ulcer disease.

When analyzed according to the origin of specimens, the agreement rates differed among hospitals, with the highest interassay agreement rates being detected at centers A and D (table 3). The specimen collection guidelines were developed in close collaboration with these treatment centers, training of hospital staff was performed at least twice yearly by staff from the Department of Infectious Diseases and Tropical Medicine at the Ludwig-Maximilians University of Munich, and regular technical assistance for specimen collection was provided by staff from the Kumasi Centre for Collaborative Research. Because of the remoteness of the other centers and the irregular patient intake, specimens were submitted without regular assistance from laboratory staff. Therefore, we conclude that the quality of laboratory diagnosis strongly depends on the quality of specimens and the training status of the hospital staff collecting the specimens.

The overall positivity ratio of DRB-PCR of swab specimens (60.6%) was nonsignificantly lower than the overall positivity ratio of standard PCR of swab specimens (67.4%). Overall positivity ratios for tissue specimens (60.6% by DRB-PCR and 61.5% by standard PCR) were comparable. Among all tests locally available at the Kumasi Centre for Collaborative Research, DRB-PCR showed significantly higher overall positivity ratios (60.6% for swab and tissue specimens) than did microscopic examination (31.1% for swab specimens and 42.7% for tissue specimens) and culture (0% for swab specimens and 4.1% for tissue specimens). In general, compared with the pilot study, the overall positivity ratio of DRB-PCR of swab and tissue specimens increased by 30% [7]. This increase can be mainly attributed to the patient inclusion criteria in this study (duration of lesions of <6 months, compared with all stages of the disease in the pilot-study patients) and the standardized specimen collection.

The low overall positivity ratio for cultures was likely to be caused by presurgical, short-term antimycobacterial treatment, which was common practice in Ghana during the study period. Because of the long generation time of *M. ulcerans*, culture, in

general, cannot be considered to be a tool for the timely diagnosis of BUD.

The positivity ratio of each test method showed considerable variation when analyzed per hospital. Also, in that respect, the positivity ratio of a test depends on the quality of the diagnostic specimen submitted for analysis. In general, the collection of diagnostic swab specimens (circling the entire undermined edge of the lesion) is less error-prone than the collection of diagnostic tissue specimens. The tissue specimen has to be obtained from the edge of the lesions below the end of the undermined edge containing necrotic tissue sections and the subcutaneous adipose tissue, where the bacilli are present. In specimens collected from excised tissue, the correct location is difficult to define, because the end of the undermined edge may not be clearly recognizable after excision. Therefore, errors may occur. positivity ratios of DRB-PCR of swab specimens from hospitals A (67.2%) and D (63.9%) were comparable. However the positivity ratio of the corresponding ulcerative tissue specimens from hospital D (36.1%) was considerably lower than that for hospital A (68.9%). These findings suggest that the collection of swab specimens did not pose any difficulties in either of these hospitals; however, problems with the collection of tissue specimens may have occurred in hospital D.

In addition, the quality of the clinical diagnosis, and, thus, the selection of patients for laboratory diagnosis, strongly influence the positivity ratio of a test. Onchocerciasis nodules, for example, may be frequently misclassified as BUD. Table 4 shows that only 48% of the preulcerative specimens from hospital D, which is located in an area where onchocerciasis is endemic, were confirmed by PCR. Histopathologic analysis of the preulcerative specimens that had negative PCR results revealed that 18.5% of the preulcerative lesions from hospital D were, in fact, onchocerciasis nodules. Onchocerciasis did not occur in the preulcerative specimens from hospital A, which is not located in an area of endemicity, and 75% of these specimens were confirmed by PCR.

Histopathologic examination confirmed 77.8% of all suspected cases of BUD. The majority of lesions from patients with positive histopathologic results and negative PCR results progressed toward the healing stage, thus containing few or no bacilli. This finding reflects the major limitation of diagnostic PCRs leading to false-negative results, compared with histopathologic examination. PCR is a suitable diagnostic tool only for early stages of the disease, when sufficient amounts of bacilli are present. However, false-negative histopathologic examination results also occur. One patient who presented with the typical clinical picture of BUD, positive results of microscopic examination of swab specimens, and positive results of PCR of swab and tissue specimens was not considered to have BUD by histopathologic examination. Thus, a true gold standard for the laboratory confirmation of BUD does not exist.

The specificity of the IS2404 PCR for clinically relevant mycobacteria was investigated elsewhere [7, 12]. The only mycobacterial species detectable with IS2404 PCR, *Mycobacterium liflandii,* is present only in environmental samples [16]. Therefore, false-positive PCR reactions in clinical samples do not occur.

The results of this study show that DRB-PCR is a reliable tool for the laboratory confirmation of cases clinically diagnosed as BUD by analysis of swab and tissue specimens. However, the performance of the assay is clearly linked to the quality of clinical diagnosis and specimen collection. To grant reliable results, the laboratory should not limit its activities to the laboratory work but should collaborate closely with the treatment centers with regard to training and supervision of specimen collection.

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

Karl-Heinz Herbinger,[1] Ohene Adjei,[4] Nana-Yaa Awua-Boateng,[4] Willemien A. Nienhuis,[8] Letitia Kunaa,[4] Vera Siegmund,[1] Jörg Nitschke,[1,3] William Thompson,[6] Erasmus Klutse,[7] Pius Agbenorku,[5] Alexander Schipf,[2,9] Simone Reu,[2] Paul Racz,[3] Bernhard Fleischer,[3] Marcus Beissner,[1] Erna Fleischmann,[1] Kerstin Helfrich,[1] Tjip S. van der Werf,[8] Thomas Löscher,[1] and Gisela Bretzel[1]

Departments of [1]Infectious Diseases and Tropical Medicine and [2]Pathology, Ludwig-Maximilians University of Munich, Munich, and [3]Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany; [4]Kumasi Centre for Collaborative Research in Tropical Medicine and [5]Reconstructive Plastic Surgery and Burns Unit, Department of Surgery, School of Medical Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, [6]Agogo Presbyterian Hospital, Agogo, and [7]Dunkwa Governmental Hospital, Dunkwa-on-Offin, Ghana; [8]University Medical Centre Groningen, University of Groningen, Groningen, The Netherlands; and [9]Institute of Pathology, Kantonsspital Luzern, Luzern, Switzerland

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

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Reprints or correspondence: Dr. Karl-Heinz Herbinger, Dept. of Infectious Diseases and Tropical Medicine, Ludwig-Maximilians University of Munich, Leopoldstrasse 5, D-80802 Munich, Germany (herbinger@lrz.uni-muenchen.de).

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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 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 PANTA (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,

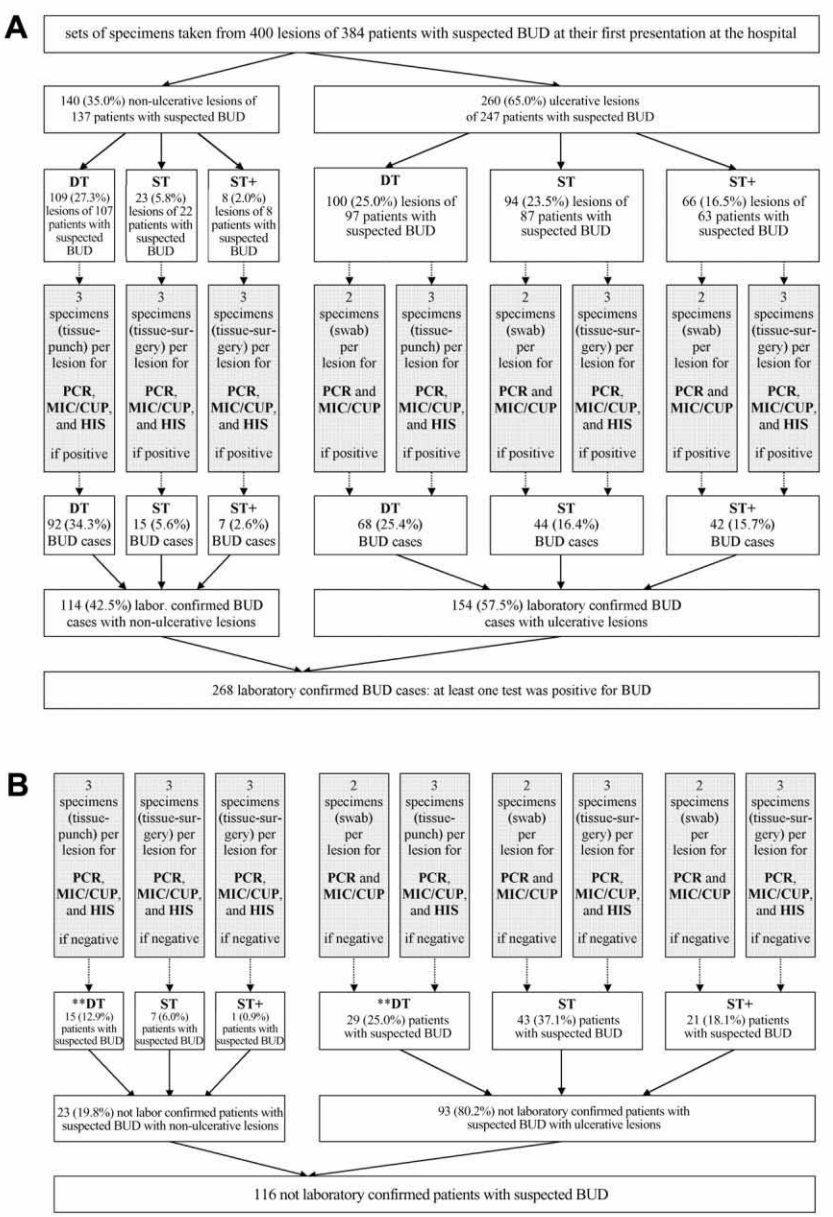

Figure 1. Flow chart of the study procedure. Specimens (swab samples [swab], punch biopsy tissue specimens [tissue-punch], and surgically excised tissue specimens [tissue-surgery]) obtained from 384 patients with suspected Buruli ulcer disease (BUD) from 3 different treatment groups (drug treatment [DT], 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 (220 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 with a swab sample with positive IS2404 PCR) 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 culture plus confirmatory IS2404 PCR. The sensitivity of PCR was significantly higher than that of microscopic examination and culture plus confirmatory IS2404 PCR ($P < .01$), with no statistically significant difference between microscopic exami-

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.

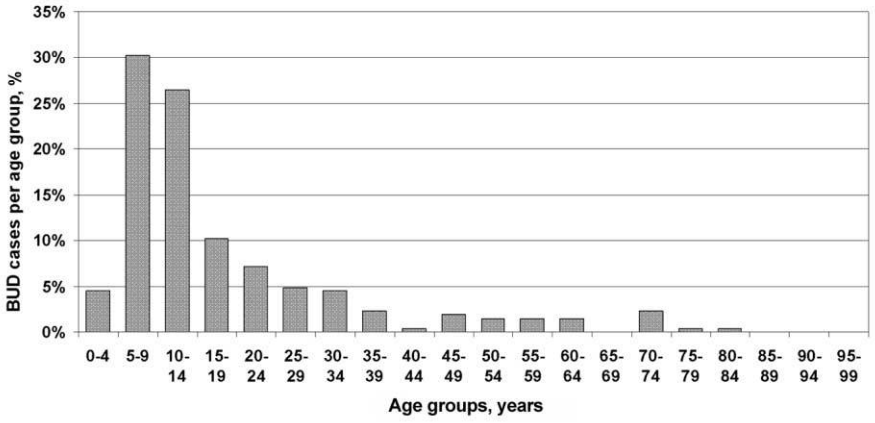

Figure 2. Age distribution of 268 patients with laboratory-confirmed Buruli ulcer disease (BUD). For 3 patients, age was unknown. Age range was 2–80 years, the mean age was 18 years, and the median age was 12 years. For the group of patients who received drug treatment, patients with suspected BUD who 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.

nation and culture plus confirmatory IS2404 PCR ($P = .054$) (table 3).

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 culture plus confirmatory IS2404 PCR analysis of tissue specimens. PCR was significantly more sensitive than microscopic examination and culture plus 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 culture plus 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 culture plus confirmatory IS2404 PCR analysis of swab samples and tissue specimens. PCR was statistically significantly more sensitive than microscopic examination and culture plus 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 culture plus 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 culture plus confirmatory IS2404 PCR were statistically significantly higher in the group of untreated patients than in the group of patients treated for >40 days (for

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.

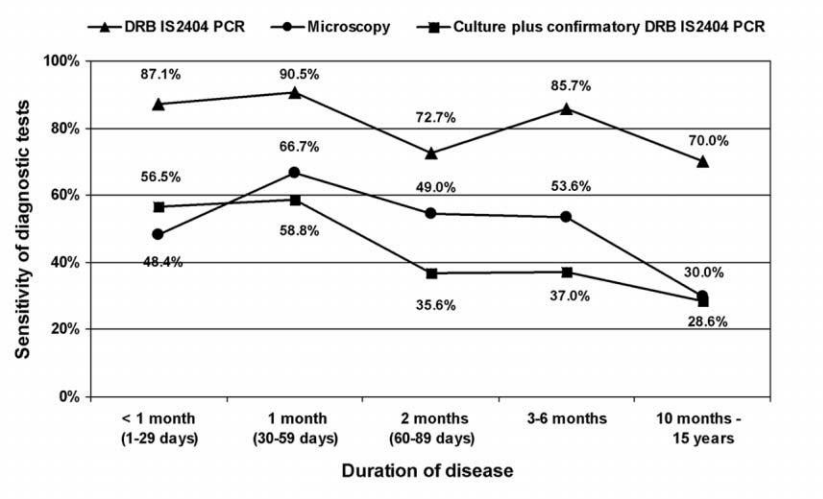

Figure 3. Sensitivity of diagnostic tests (dry reagent–based [DRB] IS2404 PCR, microscopic examination, and culture plus confirmatory DRB IS2404 PCR) by duration of disease among 112 patients with laboratory-confirmed Buruli ulcer disease and ulcerative lesions who had not received previous antibiotic 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.

PCR) or >20 days (for culture plus confirmatory IS2404 PCR). No statistically significant difference was found for microscopic examination (figure 4).

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 culture plus 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 culture plus 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

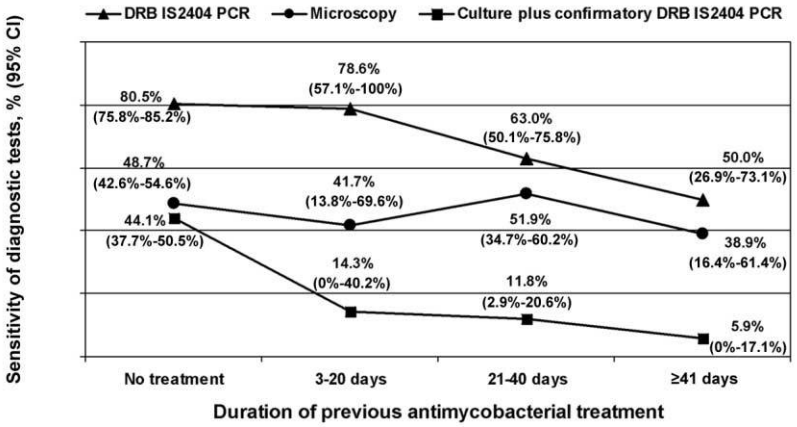

Figure 4. Sensitivity of diagnostic tests (dry reagent–based [DRB] IS2404 PCR, microscopy, and culture plus confirmatory DRB IS2404 PCR) among 49 patients with laboratory-confirmed cases of Buruli ulcer disease who received surgical treatment with previous antimycobacterial treatment (7 with preulcerative 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.

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]. Culture plus confirmatory 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 culture plus 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 culture plus 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.

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Potential conflicts of interest. All authors: no conflicts.

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Post-surgical assessment of excised tissue from patients with Buruli ulcer disease: progression of infection in macroscopically healthy tissue

G. Bretzel[1,2], V. Siegmund[1,2], P. Racz[1], F. van Vloten[1], F. Ngos[3], W. Thompson[4], P. Biason[5], O. Adjei[6], B. Fleischer[1] and J. Nitschke[1,5]

1 *Bernhard Nocht Institute for Tropical Medicine (BNITM), Hamburg, Germany*
2 *Department of Infectious Diseases and Tropical Medicine, University of Munich, Germany*
3 *Hôpital de District, District de Santé d'Akonolinga, Akonolinga, Cameroon*
4 *Agogo Presbyterian Hospital, Agogo, Ghana*
5 *Médecins sans Frontières (MSF), Geneva, Switzerland*
6 *Kumasi Centre for Collaborative Research in Tropical Medicine (KCCR), University of Science and Technology, Kumasi, Ghana*

Summary

OBJECTIVE The current standard of treatment of Buruli ulcer disease (BUD) is surgical excision of lesions. Excision size is determined macroscopically assuming the complete removal of all infected tissue. However, dissemination of infection beyond the excision margins into apparently healthy tissue, possibly associated with recurrences, cannot be excluded in this way. To assess the central to peripheral progression of *Mycobacterium ulcerans* infection and the mycobacterial infiltration of excision margins, excised tissue was examined for signs of infection.

METHODS 20 BUD lesions were excised in general anaesthesia including all necrotic and subcutaneous adipose tissue down to the fascia and at an average of 40 mm into the macroscopically unaffected tissue beyond the border of the lesion. Tissue samples were subjected to PCR and histopathology.

RESULTS Although the bacillary load decreased from central to peripheral, *M. ulcerans* infection was detected throughout all examined tissue specimens including the peripheral segments as well as excision margins of all patients. During the post-operative hospitalization period (averaging 2 months) no local recurrences were observed.

CONCLUSION Available data suggest a correlation of surgical techniques with local recurrences. The results of this study indicate the unnoticed early progression of mycobacterial infection into macroscopically healthy tissue. Thus, the removal of all infected tissue cannot always be verified visually by the surgeon. Provided that long-term follow up of patients with positive excision margins will establish the clinical relevance of these findings, on-site laboratory assessment of excised tissue in combination with follow up may contribute to reduce recurrence rates.

keywords Buruli surgery, exision margins, bacterial dissemination, histopathology, PCR

Introduction

After tuberculosis and leprosy, Buruli ulcer disease (BUD) has become the third, in some West African countries even the second most or most common mycobacterial disease in immunocompetent humans (Amofah *et al.* 2002; Debacker *et al.* 2004). BUD occurs in tropical countries with foci in West Africa, Central Africa, and the Western Pacific. It is defined as an infectious disease involving mainly subcutaneous adipose tissue and the skin, characterized by a painless nodule, papule, plaque or oedema, evolving into a painless ulcer with undermined edges. It often leads to invalidating sequelae such as extensive scarring, contractures of joints with debilitating deformities and severe functional deficiency. Formal clinical trials to evaluate the use of anti-mycobacterial drugs have not yet been conducted, although anecdotal reports (WHO study group for drug treatment of Buruli ulcer, personal communication) suggest that some of these drugs used in combination with surgery or alone might have a beneficial effect. To date disease control in endemic countries is limited to early case detection through improved active surveillance and surgical excision of lesions. The current standard of treatment is surgical removal of all the affected tissue and part of the surrounding tissue, eventually followed by skin grafting

(Meyers *et al.* 1996; Asiedu & Etuaful 1998; WHO 2000,2001; King *et al.* 2001).

Recurrence rates after different surgical techniques between 6.1% and 32% have been reported (Amofah *et al.* 1998; Kanga *et al.* 2003; Debacker *et al.* 2005). Teelken *et al.* (2003) describe large differences in treatment outcome between two hospitals applying different surgical practices.

To date the extent of the surgical excision depends on the surgeon's clinical experience alone. Figure 1 shows an ulcer before treatment. The excision size as shown in Figure 2 was determined based on the assumption of complete removal of all infected tissue.

However, dissemination of infection beyond the excision margins into macroscopically healthy tissue cannot be excluded visually. In general, little is known about the dissemination of bacilli and immunopathological changes in BUD lesions. With the intention to develop standards for determining the surgical excision size a laboratory-based approach to assess the spread of infection in BUD lesions was established. A pilot study on the post-surgical assessment of excised tissue was carried out in collaboration with Médecins sans Frontières (MSF), the Hôpital de District, District de Santé d'Akonolinga, Akonolinga, Cameroon (November–December 2003) and the Agogo Presbyterian Hospital, Agogo, Ghana (January until August 2004).

Materials and methods

Ethical clearance and informed patient consent

Ethical clearance for the study was sought through the Ministry of Health, Cameroon and the Committee of Human Research Publication and Ethics, School of Medical Sciences, University of Science and Technology, Kumasi, Ghana. Informed patient consent was obtained before surgery.

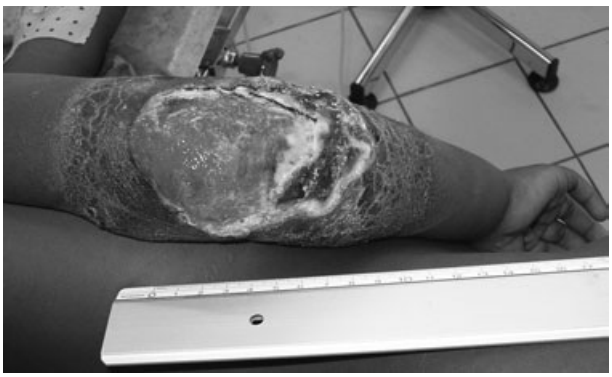

Figure 1 Lesion before surgery (6 months old, diameter 90 mm).

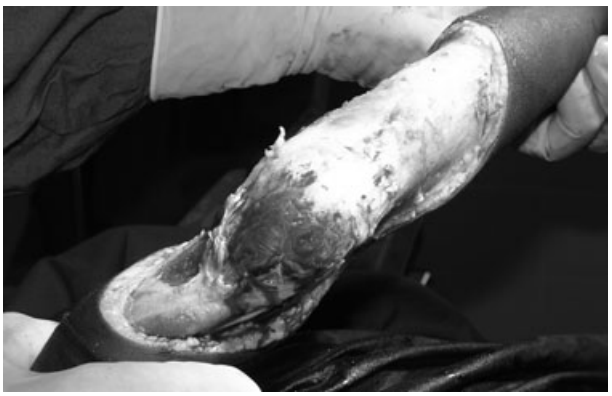

Figure 2 Lesion after surgery.

Inclusion criteria for study patients

Patients were eligible for inclusion if they fulfilled the following criteria:

- Laboratory confirmation of *M. ulcerans* disease by both, PCR (Stinear *et al.* 1999) and histopathology (Guarner *et al.* 2003). Due to the long generation time of *M. ulcerans* culture, results were not considered as inclusion criteria.
- Duration of the disease not more than 6 months.
- Non-ulcerative (nodules and plaques) and ulcerative forms (elevated margins, moderate localized oedema, absence of dermatosclerosis).
- Diameter of the lesion less than 10 cm.

Selection of patients eligible for the study and specimen collection was performed by the surgeon.

Patients, operative techniques and specimen collection

Twenty patients seeking treatment of BUD during the study period meeting the inclusion criteria (female: $n = 10$, male: $n = 10$, median age 19 years (range: 7–60 years) were included in the study (Akonolinga $n = 10$, Agogo $n = 10$). The lesions (ulcers: $n = 19$, nodules: $n = 1$) were localized at the lower limbs ($n = 8$), upper limbs ($n = 7$), back of the foot ($n = 2$), back of the hand ($n = 1$), abdominal wall ($n = 2$). The clinical aspects of these patients did not indicate any concomitant diseases such as tuberculosis or AIDS. Due to ethical reasons screening for HIV and other conditions was not part of the study.

All operations were performed under general anaesthesia. A pneumatic tourniquet was applied at the extremities. After disinfection the intended incision line was marked by a sterile pen. Excisions including all necrotic and subcutaneous adipose tissue down to the fascia were performed

into the macroscopically unaffected tissue beyond the border of the lesion and were directed from peripheral to central. The average diameter of the excised tissue between the border of the lesion and the peripheral excision margin was 40 mm (range: 20–60 mm).

Excised tissue was kept in sterile cotton gauze moistened with sterile sodium hydrochloride. Specimens were taken with a sterile scalpel, gradient sections were cut from peripheral to central to avoid contamination. All specimens comprised skin and subcutaneous adipose tissue down to the fascia, where the bacilli are present.

Hospitalization and follow up of study patients

All study patients with ulcerative lesions received skin transplants and were hospitalized for approximately 2 months until complete healing. During the hospitalization period no local recurrences were observed.

Laboratory confirmation and laboratory methods

To assess the eligibility of patients for the study diagnostic specimens from the edge of the ulcer including necrotic tissue and all levels of the skin and subcutaneous adipose tissue down to the fascia were subjected to PCR and histopathology.

PCR, culture and histopathology were performed according to standardized procedures (Stinear *et al.* 1999; WHO 2001; Guarner *et al.* 2003). PCR results are indicated as 'positive' or 'negative', inhibited reactions were excluded from analysis. The histopathological classification of specimens as definite cases was based on the presence of acid-fast bacilli (AFB) and/or characteristical histopathological features. The bacillary load was graded as follows: absent, mild (1–5 AFB seen with 40 × objective), moderate (≥ 6 AFB seen with 40 × objective), or marked (AFB seen with 20 × objective as clumps or colonies) (Guarner *et al.* 2003). When AFB were absent and histopathological features did not allow a definite diagnosis as BUD, specimens were considered suspect cases unless they had other diagnoses that could account for a clinical nodule or ulcer. According to Meyers (1995) the healing stage of Buruli ulcer was added as a third group to the classification. Specimens were cultured to obtain *M. ulcerans* isolates for further studies, however, due to the long generation time of *M. ulcerans* and the low sensitivity of cultures, the results are not reported in this study (WHO 2001).

Gradient specimens

To assess the progression of infection from the inner edge of the ulcer up to the outer margin of the excised lesion, 20 gradient specimens were taken from excised tissue as follows: three parallel tissue sections (maximum size 50 × 15 mm) were cut from peripheral to central and subjected to PCR, culture and histopathology. Each section was cut in segments (maximum size 10 × 5 mm) (Figure 3). According to the size of the lesion the number of segments per set of gradients ranged from 6 to 3 (six segments: $n = 2$, five segments: $n = 6$, four segments: $n = 5$, three segments: $n = 7$). Altogether 83 tissue segments were taken for analysis. The diameter of the tissue sections was measured (starting point nodule: centre of the lesion, starting point ulcerative lesion: edge of the ulcer) and documented.

All specimens were placed in test tubes (Sarstedt, Nümbrecht, Germany) containing 4 ml 10% formol for histopathology, 700 µl Cell Lysis Solution (Puregene DNA Isolation Kit, Gentra Systems, Indianapolis, USA) for PCR, 5 ml transport medium (Dubos Broth base + PANTA + Dubos Broth medium albumin, Becton Dickinson Diagnostic Systems, Heidelberg, Germany) for culture.

Excision margins

To assess if the margins of the excised tissue were free of bacilli, 20 sets of margin specimens (each margin segment maximum size 10 × 5 mm) were taken from either selected sites of the margin (large lesions) or the entire marginal tissue (small excisions) and subjected to PCR (Figure 4).

Anatomical reasons and the quality of excised tissue respectively allowed the laboratory analysis of 16 sets of margins only. The samples of the remaining four patients were either lacking subcutaneous adipose tissue or the

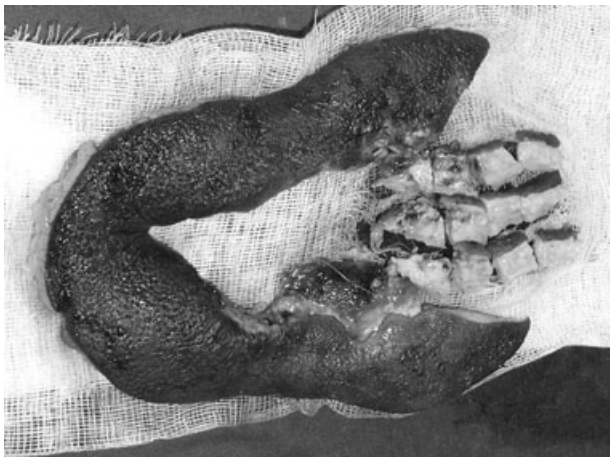

Figure 3 Gradient segments (after cutting turned to the side to display subcutaneous adipose tissue).

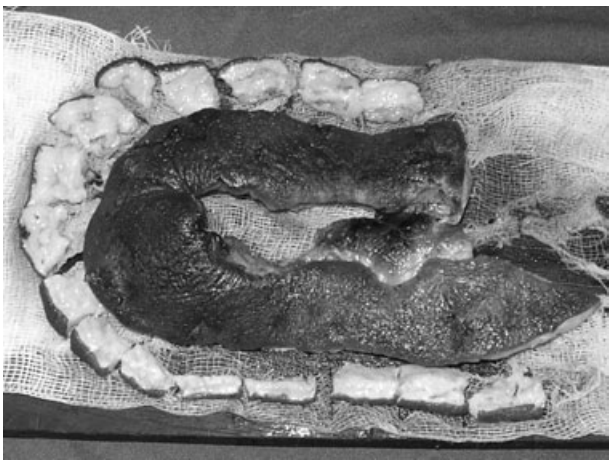

Figure 4 Margin segments (after cutting turned to the side to display subcutaneous adipose tissue.

excision margins were frayed, thus making it impossible to collect adequate specimens. In view of anatomical reasons it was impossible to take three parallel sets of specimens for histopathology, culture and PCR in analogy with the gradient samples. Therefore, only PCR analysis of margin samples was carried out.

According to the size of the lesion and the quality of the excised tissue the number of margin segments per set of margin ranged from 2 to 25 (2 segments: $n = 2$, 3 segments: $n = 1$, 5 segments: $n = 1$, 11 segments: $n = 1$, 14 segments: $n = 1$, 17 segments: $n = 1$, 19 segments: $n = 1$, 20 segments: $n = 7$, 25 segments, $n = 1$). PCR specimens were placed in test tubes (Sarstedt, Nümbrecht, Germany) containing 700 µl Cell Lysis Solution (Puregene DNA Isolation Kit, Gentra Systems, Indianapolis, USA).

Results

Gradients

As indicated in Table 1 after laboratory analysis of 20 sets of gradients 16/20 (80%) sets of gradients showed a positive result in all segments in both PCR and histopathology. 3/20 (15%) sets of gradients were found partially positive in PCR analysis (2/3, 2/4, 2/4 PCR positive segments, respectively), and positive in all segments in histopathology analysis. 1/20 (5%) set of gradients tested partially positive in PCR analysis (3/5 segments) and partially positive in histopathology (4/5 segments). 0/20 (0%) sets of gradients were found PCR positive in all segments and only partially histopathology positive. The overall accordance rate of PCR and histopathology results was 93.9% (77/82 segments were positive in both tests).

The peripheral segment of all histopathology gradient sections was positive for BUD [detection of AFB in 20/20 (100%) peripheral segments]. In general, as shown in Table 1, the quantity of AFB as detected by histopathology decreased from the central (segment a) to the peripheral segment. PCR analysis detected *M. ulcerans* DNA in 17/20 (85%) peripheral segments.

PCR did not detect *M. ulcerans* DNA in 6/82 (7.3%) specimens with a positive histopathology result. In most cases (5/7) PCR negative segments correlate with absence or low numbers of AFB as seen in histopathological analysis. Only histopathology could provide a definite diagnosis of BUD in all cases.

Margins

After laboratory analysis of all 16 sets of margins the specimens could be grouped as follows:

- 100% margin segments completely PCR positive ($n = 10$);
- >50% of segments per set of margin PCR positive [14/20 (70 %) $n = 1$];
- <50% of segments per set of margin PCR positive [total: $n = 5$: 1/6 (16%) $n = 1$; 7/19, 3 inhibited (37%) $n = 1$; 2/25, 2 inhibited (8%) $n = 1$; 1/20, 9 inhibited (5%) $n = 1$; 7/20, 3 inhibited (35%) $n = 1$)];
- 100% margin segments completely PCR negative ($n = 0$).

Discussion

To date surgical excision with subsequent skin grafting is still considered the most promising form of treatment of BUD. Nevertheless, despite broad acceptance surgical treatment has been discussed controversially. Consensus has been reached that wide surgical excisions of BUD lesions (i.e. comprising all necrotic tissue and the subcutaneous adipose tissue down to the fascia as well as macroscopically unaffected tissue beyond the peripheral border of the lesion) are to be performed (Cornet *et al.* 1992; Josse *et al.* 1995; Aguiar & Stenou 1997; van der Werf *et al.* 1999; WHO 2000).

However, standards for the extent of excision into macroscopically healthy tissue do not exist. Therefore, the surgeon's subjective experience alone determines the size of excision margins.

Only a few publications deal with surgical techniques (Ouattara *et al.* 2002, 2003, 2004; Knipper *et al.* 2004). Three case–control studies on recurrence rates and non-healed wounds after excision and skin grafting have been

Table 1 Polymerase chain reaction (PCR) and histopathology results of gradient segments

	Patient	Segment a	Segment b	Segment c	Segment d	Segment e	Segment f
PCR	A/19	Pos	Pos	Pos	Pos	Pos	NA
Histo.	A/19	AFB mild Definite BU	AFB mild Definite BU	AFB moderate Definite BU	AFB moderate Definite BU	AFB mild Definite BU	NA
PCR	A/20	Pos	Pos	Pos	Pos	Pos	NA
Histo.	A/20	AFB moderate Definite BU	AFB mild Definite BU	AFB absent Healing stage	AFB mild Definite BU	AFB mild Definite BU	NA
PCR	A/21	Pos	Pos	Pos	Pos	Pos	Pos
Histo.	A/21	AFB marked Definite BU	AFB marked Definite BU	AFB moderate Definite BU	AFB mild Definite BU	AFB absent Definite BU	AFB moderate Definite BU
PCR	A/22	Pos	Pos	Pos	NA	NA	NA
Histo.	A/22	AFB marked Definite BU	AFB moderate Definite BU	AFB mild Definite BU	NA	NA	NA
PCR	A/23	Pos	Pos	Pos	NA	NA	NA
Histo.	A/23	AFB mild Definite BU	AFB absent Definite BU	AFB mild Definite BU	NA	NA	NA
PCR	A/24	Pos	Pos	Pos	Pos	Pos	NA
Histo.	A/24	AFB marked Definite BU	AFB marked Definite BU	AFB marked Definite BU	AFB mild Definite BU	AFB mild Definite BU	NA
PCR	A/30	Pos	Neg	Pos	Neg	Pos	NA
Histo.	A/30	AFB mild Definite BU	AFB mild Definite BU	AFB mild Definite BU	AFB absent Not BU	AFB mild Definite BU	NA
PCR	A/33	Pos	Pos	Pos	Pos	Pos	Pos
Histo.	A/33	AFB marked Definite BU	AFB marked Definite BU	AFB marked Definite BU	AFB mild Definite BU	AFB mild Definite BU	AFB mild Definite BU
PCR	A/34	Pos	Pos	Neg	Neg	NA	NA
Histo.	A/34	AFB moderate Definite BU	AFB moderate Definite BU	AFB mild Definite BU	AFB mild Definite BU	NA	NA
PCR	A/38	Pos	Neg	Pos	NA	NA	NA
Histo.	A/38	AFB moderate Definite BU	AFB moderate Definite BU	AFB mild Definite BU	NA	NA	NA
PCR	K1	Pos	Pos	Pos	Pos	NA	NA
Histo.	K1	AFB absent Definite BU	AFB mild Definite BU	AFB mild Definite BU	AFB mild Definite BU	NA	NA
PCR	K 2	Pos	Pos	Pos	NA	NA	NA
Histo.	K 2	AFB marked Definite BU	AFB marked Definite BU	AFB moderate Definite BU	NA	NA	NA
PCR	K 3	Pos	Pos	Pos	Pos	Pos	NA
Histo.	K 3	AFB marked Definite BU	AFB marked Definite BU	AFB marked Definite BU	AFB marked Definite BU	AFB marked Definite BU	NA
PCR	K 4	Pos	Pos	Pos	NA	NA	NA
Histo.	K 4	AFB absent Definite BU	AFB absent Definite BU	AFB mild Definite BU	NA	NA	NA
PCR	K 5	Pos	Pos	Pos	Pos	NA	NA
Histo.	K 5	AFB marked Definite BU	AFB marked Definite BU	AFB marked Definite BU	AFB mild Definite BU	NA	NA
PCR	K 7	Pos	Pos	Pos	NA	NA	NA
Histo	K 7	AFB moderate Definite BU	AFB mild Definite BU	AFB mild Definite BU	NA	NA	NA
PCR	K 9	Pos	Pos	Pos	Pos	NA	NA
Histo.	K 9	AFB marked Definite BU	AFB marked Definite BU	AFB moderate Definite BU	AFB mild Definite BU	NA	NA
PCR	K 12	Pos	Pos	Pos	Pos	Pos	NA
Histo.	K 12	AFB marked Definite BU	AFB marked Definite BU	AFB marked Definite BU	AFB mild Definite BU	AFB mild Definite BU	NA

Table 1 (Continued)

	Patient	Segment a	Segment b	Segment c	Segment d	Segment e	Segment f
PCR	K 15	Pos	Pos	Neg	Neg	NA	NA
Histo.	K 15	AFB moderate Definite BU	AFB mild Definite BU	AFB mild Definite BU	AFB moderate Definite BU	NA	NA
PCR	K 18	Pos	Pos	Pos	NA	NA	NA
Histo.	K 18	AFB marked Definite BU	AFB marked Definite BU	AFB marked Definite BU	NA	NA	NA

Segment a, central segment; BU, Buruli ulcer; histo, histopathology (AFB and histopathological classification); AFB, acid fast bacilli; pos, positive; neg, negative; NA: not available.

published: Amofah *et al.* (1998) report on 50 pre-ulcerative lesions from Ghana with a recurrence rate of 16% during a 1 year follow-up. Excision was performed under local anaesthesia with subsequent primary suturing. Recurrences are attributed to secondary bacterial infections and the surgical technique applied. Teelken *et al.* (2003) compare the surgical outcome after excisions of ulcers in two hospitals in Ghana and found non-healed ulcers (retrospectively not distinguishable from recurrences) in 18% (wide excision) and 47% respectively (excision close to border of lesion) of the cases subjected to follow up.

Kanga *et al.* (2003) report on 346 BUD cases from three hospitals in Ivory Coast with recurrence rates of 32%, 25%, and 11% (median 17.1%), within 1 year after excision. The low recurrence rate of 11% is attributed to the professional expertise of the surgeons. Kanga *et al.* discuss if a mycobacterial infiltration into the apparently healthy tissue of the excision margins could explain local recurrences.

The available data suggest two crucial issues for the patient outcome:

- Painless surgery and wide excision, especially in children, are possible only with general, spinal or regional anaesthesia. Local anaesthesia, as often performed in pre-ulcerative cases, can make it difficult to achieve sufficient excision sizes. Moreover, local injection of liquid anaesthetic drugs may further promote peripheral diffusion of AFB into the adjacent subcutaneous tissue. Furthermore, as a general rule, healing of a septic process by secondary intention is achieved by laying the wounds open for later skin grafting or secondary suturing. Thus, local anaesthesia followed by primary suturing may favour recurrences as suggested by Amofah *et al.* (1998).
- If a 'wide' excision into macroscopically intact tissue beyond the border of the lesion is performed, a mycobacterial infiltration into subcutaneous adipose tissue cannot be excluded visually. An excision size that seems clinically sufficient from the surgical point of view may therefore, due to macroscopically invisible progression of infection, be insufficient to be curative.

In order to assess the progress of infection from the centre to the periphery of the lesion and to examine the excision margins for signs of infection excised tissue from 20 patients from two hospitals in Cameroon and Ghana was subjected to laboratory analysis. The study patients were operated under generalized anaesthesia followed by skin grafting, thus local anaesthesia and primary suturing as possible risk factors for local recurrence could be excluded.

Although according to the healthy aspect of the excision line the presence of bacilli was not to be expected, signs of infection were detected in all segments of all sets of gradient specimens. Furthermore, in the majority of cases PCR rendered positive results for the complete set of margin segments, indicating the presence of *M. ulcerans* infection around the entire excision margins.

These findings indicate that the 'wide' excision into macroscopically healthy tissue that has been performed in all cases and was assumed to be curative from the surgical point of view, in fact was not sufficient to remove all infected tissue. The macroscopic aspect of apparently healthy tissue does not correlate with the dissemination of infection into subcutaneous adipose tissue. Thus, a reliable determination of excision margins sufficient to remove all infected tissue based on macroscopic criteria alone is not possible.

In this study the average excision diameter of 40 mm did not result in *M. ulcerans* free margins. In order to develop standards for the excision size, further studies on specimens from areas beyond the excision line are required. The clinical relevance of these findings is yet to be proved. Short-term follow up of the study patients during hospitalization for a period averaging 2 months until completion of skin grafting did not reveal any local recurrences. However, to establish a correlation between

the presence of persisting *M. ulcerans* infection in excision margins and the risk of local recurrences a long-term follow up is required. Quantification of the bacterial load in margin segments according to the method described by Rondini *et al.* (2003) may support these investigations by elucidating the question if a minimal bacterial load is required for the development of recurrences and if the self-healing potential can be related to the bacterial load.

On-site assessment of excised tissue may provide an option to guarantee the complete removal of infected tissue. According to the method described by Siegmund *et al.* (2005) pre-surgical analysis of punch biopsies taken from the intended incision line could help identify the adequate excision size. Post-surgical assessment of excision margins as carried out in this study may contribute to identify patients with incompletely removed infected tissue. In this study the sensitivity of PCR was slightly lower than histopathological analysis, which could be explained by the fact that specimens from parallel tissue sections, and not one and the same specimen, were subjected to analysis with both methods. To gain the maximum diagnostic sensitivity, PCR negative specimens could be re-examined by histopathology, if this technique is available in the respective setting. Patients who tested positive must be subjected to a regular follow-up of at least 12 months or even to early re-operation in case of persisting wounds or recurrences. As a prerequisite to achieve such long-term follow up however, capacities for surveillance of patients in their villages must be made available. In addition, the possibility of supportive antimycobacterial treatment should be considered to reduce recurrence rates. Drug trials evaluating antimycobacterial therapy alone and in combination with surgery conducted by research groups from Belgium and the Netherlands are scheduled to commence in 2005 (Francoise Portaels, Tjip van der Werf, personal communication).

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Authors

G. Bretzel (corresponding author) and **V. Siegmund**, Bernhard Nocht Institute for Tropical Medicine (BNITM), Hamburg; Department of Infectious Diseases and Tropical Medicine (DITM), University of Munich, Germany. Tel.: +49 8921 803618; Fax: +49 89 336112; E-mail: bretzel@lrz.uni-muenchen.de

P. Racz, **F. van Vloten** and **B. Fleischer**, Bernhard Nocht Institute for Tropical Medicine (BNITM), Hamburg, Germany.

F. Ngos, Hôpital de District, District de Santé d'Akonolinga, Akonolinga, Cameroon.

W. Thompson, Agogo Presbyterian Hospital, Agogo, Ghana.

P. Biason, Médecins sans Frontières (MSF), Geneva, Switzerland.

O. Adjei, Kumasi Centre for Collaborative Research in Tropical Medicine (KCCR), University of Science and Technology, Kumasi, Ghana.

J. Nitschke, Bernhard Nocht Institute for Tropical Medicine (BNITM), Hamburg, Germany; Médecins Sans Frontières (MSF), Geneva, Switzerland.

Excision of Pre-Ulcerative Forms of Buruli Ulcer Disease: A Curative Treatment?

K.-H. Herbinger, D. Brieske, J. Nitschke, V. Siegmund, W. Thompson, E. Klutse, N.Y. Awua-Boateng, E. Bruhl, L. Kunaa, M. Schunk, O. Adjei, T. Löscher, G. Bretzel

Abstract

Background: Previous investigations have revealed that *Mycobacterium ulcerans* is extensively distributed spatially throughout ulcerative lesions, including in the margins of excised tissue. In contrast, bacilli in pre-ulcerative lesions are assumed to be concentrated in the center of the lesion. In order to assess the extent to which the surgical excision of pre-ulcerative lesions is capable of removing all infected tissue, we subjected the excision margins of pre-ulcerative lesions to laboratory analysis.

Patients and Methods: Eleven patients with laboratory-confirmed pre-ulcerative lesions were included in the study. The diameter of the lesion and excised tissue and the "surgical distance" between the border of the lesion and excision margin were measured. The entire excision margin was cut into segments and subjected to IS2404 PCR.

Results: The results from the PCR analysis on the samples of excision margins were highly significantly associated with the surgical distance ($p < 0.001$). The margin samples of nodules were significantly more often PCR positive than the plaques ($p = 0.025$). The size of the lesion and the size of the excised tissue did not significantly influence the PCR results. Statistically, a surgical distance of more than 9 mm was found to reduce the risk of remaining infected tissue to less than 10%, that of 13 mm to reduce the risk to less than 5%, and that of 25 mm to reduce the risk to nearly 0%.

Conclusion: The results of this study show that in pre-ulcerative Buruli ulcer disease, bacilli may extend beyond the actual size of the lesion and that there is a strong correlation between the presence of *M. ulcerans* in the margin samples and the surgical distance. Excision with a surgical distance of 25 mm avoided the risk of remaining mycobacteria in this study. However, no recurrences occurred in the patients with *M. ulcerans*-positive excision margins. The need of postoperative antimycobacterial treatment in these patients remains to be determined.

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Introduction

Buruli ulcer disease (BUD) caused by *Mycobacterium ulcerans* is an infectious disease involving the skin and the subcutaneous adipose tissue. It has been reported or suspected in 30 countries worldwide, with a focus and increasing case numbers in West Africa [1–3]. The disease initially presents as painless nodule, papule, plaque, or edema (pre-ulcerative forms), evolving into a painless ulcer with characteristically undermined edges (ulcerative form). While mortality is low, serious long-term sequelae are common. Contractures and deformities originating from self-healing processes or surgical treatment of late, extensive lesions may result in severe functional deficiencies [4, 5].

Various distributions of pre-ulcerative vs ulcerative forms have been reported. In Côte d'Ivoire, *Kanga* et al. observed only 4% of nodular and 6.5% of edematous forms vs 89.5% of ulcerated cases [6]. In Benin, the frequency of pre-ulcerative cases was reported to be 37.4% (7.6% nodules, 29.1% plaques, 0.7% edema) [3]. In Ghana, 42.6% of the clinically diagnosed BUD patients presented with nodules (31.3%), plaques (10.9%), or papules (0.4%) [7].

Due to the characteristically clinical presentation of ulcerative lesions, experienced clinicians are generally

K.-H. Herbinger *(corresponding author)*, **D. Brieske, V. Siegmund, M. Schunk, T. Löscher, G. Bretzel**
Dept. of Infectious Diseases and Tropical Medicine (DITM), Ludwig-Maximilian University Munich, Leopoldstrasse 5, 80802 Munich, Germany; Phone: (+49/89) 2180-3618, Fax: -336112, e-mail: herbinger@lrz.uni-muenchen.de
J. Nitschke
Bernhard Nocht Institute for Tropical Medicine (BNITM), Hamburg, Germany
W. Thompson
Agogo Presbyterian Hospital, Agogo, Ghana
E. Klutse
Dunkwa Governmental Hospital, Dunkwa-on-Offin, Ghana
N.Y. Awua-Boateng, E. Bruhl, L. Kunaa, O. Adjei
Kumasi Centre for Collaborative Research in Tropical Medicine (KCCR), Kumasi, Ghana

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able to establish the diagnosis on clinical grounds, with the analysis of diagnostic swabs providing laboratory confirmation in the majority of cases. In contrast, pre-ulcerative lesions have uncharacteristic clinical features and, consequently, a variety of possible differential diagnoses exists. An accurate clinical diagnosis is therefore more difficult, and priority should be given to the laboratory confirmation or differential diagnosis of these cases based on an analysis of tissue specimens [7–9].

The effect of antibiotic treatment alone or in combination with surgery has recently come under investigation. Data from Ghana, Benin, and Australia suggest that there is a benefit to treating pre-ulcerative and ulcerative forms of BUD with antibiotics [9–13]. A small Australian case series of early ulcerative lesions lead to the formulation of a treatment algorithm for ulcerative lesions and cellulitis that includes perioperative antimycobacterial treatment, surgical debridement with 3- to 5- mm margins (to be repeated until the margins are microscopically clear of acid-fast bacilli (AFB), followed by at least 2 months of antimycobacterial treatment [14]. Depending on the size and type of the lesion, current provisional World Health Organization guidelines recommend combination therapy with rifampicin and streptomycin for 4–8 weeks [9–13].

Previous investigations of the spatial distribution of *M. ulcerans* in ulcerative lesions have revealed the presence of bacilli throughout the lesions as well as in macroscopically healthy excision margins [15, 16]. This finding indicates that even wide surgical excision of ulcerative lesions cannot guarantee the complete removal of all infected tissue. Remaining bacilli, however small the amount, are closely related to the possibility of recurrences, a finding which supports the need for antimycobacterial treatment in ulcerative lesions. For nodular forms, however, bacilli have been shown to be concentrated in the center of the lesion [17]. Therefore, excision of pre-ulcerative forms with sufficiently wide excision margins may provide for the removal of all infected tissue. Data and recommendations on the management of pre-ulcerative lesions are scarce [5, 18, 19]. Observations made during a follow-up study in Ghana (own unpublished data) suggest that functional limitations rarely occur following the excision of nodules and plaques, whereas surgical treatment of ulcerative lesions often results in disabilities caused by extensive scarring associated with grafting. In-patient time was found to be significantly shorter for nodules and plaques than for ulcerative lesions.

As several reasons may favor the surgical treatment of pre-ulcerative lesions, this study was undertaken to investigate if, and under which conditions, primary surgery of nodules and plaques alone, i.e. without previous antimycobacterial treatment, may be curative.

Patients and Methods

Patients, Specimens, and Laboratory Analysis

Eleven patients with laboratory-confirmed pre-ulcerative lesions (nodules n = 7, plaques n = 4) without previous antimycobacterial treatment were eligible for this study. The patients underwent routine surgical excision in Agogo Presbyterian Hospital, Agogo, Ghana (nodules n = 3, plaques n = 3), and Dunkwa Governmental Hospital, Dunkwa-on-Offin, Ghana (nodules n = 4, plaques n = 1) in 2006. As is the case for specimens for routine laboratory confirmation, the samples used for analysis of the excision margins were taken from surgically excised tissue. Due to the introduction of antimycobacterial treatment in Ghana in 2006, only a limited number of pre-ulcerative lesions from patients treated by surgery only could be analyzed. Information on the treatment outcome was obtained by follow-up of the study patients 12–18 months after the surgical intervention.

Ethical clearance for the study was provided by the Committee of Human Research Publication and Ethics at the School of Medical Sciences at the University of Science and Technology, Kumasi, Ghana. Informed patient consent was obtained from patients before surgery.

Before surgery, the excision line comprising an area of macroscopically unaffected tissue was determined and marked by a sterile pen, and the medio-lateral and proximo-distal diameters of the lesion and excision were measured in situ with disposable paper rulers (Figure 1a). Excisions, including all subcutaneous adipose tissue down to the fascia, were performed. The excised tissue was spread out on a table on sterile cotton gauze. Based on the anatomical location, which had been determined prior to the surgery, the position of the ex-

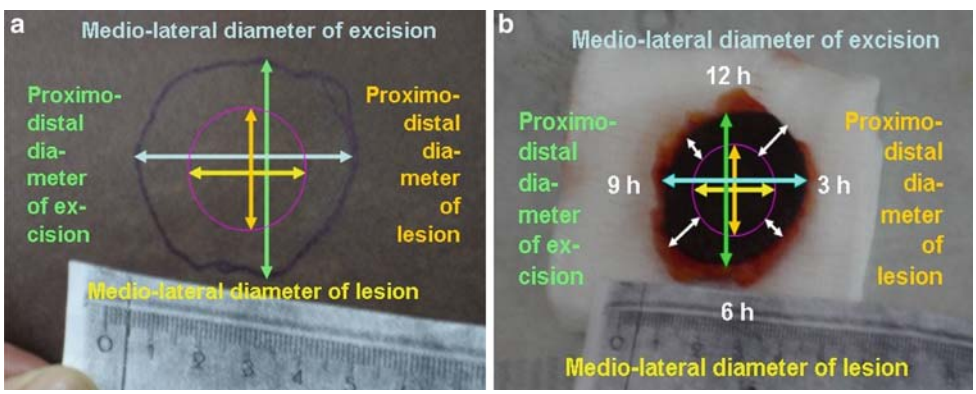

Figure 1. a) Marking of excision line and in situ measurement of proximo-distal and medio-lateral diameters of lesion and excision. b) Measurement of proximo-distal (12 h–6 h) and medio-lateral (3 h–9 h) diameters of lesion and excision in excised tissue according to the previously determined anatomical position of the excised tissue. Surgical distance: distance between the border of the lesion and the margin of excised tissue (white arrows).

cised tissue was marked in a clockwise direction (proximal: 12 h, distal: 6 h). Due to possible post-surgical changes in the proportions of the excised tissue relative to the in situ measurements, the proximo-distal (12 h–6 h) and medio-lateral (9 h–3 h) diameters of the lesion as well as of the total excision were measured again. These measurements served as the basis for further analysis. The distance between the macroscopically visible border of the lesion and the outer excision margin is referred to as the ''surgical distance'' (Figure 1b). In order to obtain as accurate measurements of the lesion as possible, the bottom side of the tissue was also examined for better identification of the necrotic area.

The entire excision margin of each lesion was cut off (Figure 2a) and divided into segments with a sterile scalpel. Depending on the size of the lesion and excision, the size of the margin samples could slightly differ among the patients, with a maximum size of 10 mm × 10 mm. For each margin segment, the surgical distance was measured individually (Figure 2b). Diagnostic specimens were taken from the center of the lesion according to standardized criteria and subjected to microscopy, culture, IS2404 PCR, and histopathology according to standard procedures [7, 8]. A total of 150 margin segments were collected, stored in cell lysis solution (Gentra Systems, Minneapolis, MN) and tested for *M. ulcerans* by IS2404 PCR. Briefly, DNA from tissue specimens was prepared using the Puregene DNA Isolation kit (Genomic DNA Purification kit, Gentra Systems), and the IS2404 PCR was performed according to *Stinear* et al. [20]. In order to exclude false positive PCR results due to contamination, for each PCR extraction, negative controls were performed in a standardized manner [7]. Given the 100% specificity of the IS2404 PCR for clinical samples demonstrated in several studies [7, 8, 12], it was not considered necessary to confirm positive PCR results of margin samples by histopathology.

Statistical Analysis

All analyses were performed using Stata software, ver. 9.0. (Stata Corp., College Station, TX). For simple statistical analyses, approximative tests (χ^2 tests) and exact tests (Fisher's exact tests) were applied. For calculating the association between the dependent variable (PCR results for the samples of the margins) and the independent variables, single and multiple logistic regression were used. In our analysis, we included the following independent variables: Hospital (Agogo, Dunkwa), sex and age of patients, type of pre-ulcerative lesion (nodule, plaque), location of pre-ulcerative lesion, duration between first occurrence of lesion and excision, size of lesion, size of excised tissue, and surgical distance.

Results

Baseline Data

The baseline data of the study patients are summarized in table 1. Of 11 patients (nine females, two males) with pre-ulcerative lesions, seven had nodules and four had plaques. Six of them were operated on in Agogo and five in Dunkwa. The youngest patient was 2 years old, and the oldest was 50 years old (arithmetic mean of age 13.45 years).

The pre-ulcerative lesions were localized as follows: six on thigh or lower leg, two on the arm, and one each on the hip, back, and foot. The duration between the first occurrence of the lesion and excision was between 1 and 20 weeks (arithmetic mean of duration: 4.5 weeks). The smallest lesion measured 47 mm^2 (6 mm × 10 mm) and the largest 707 mm^2 (30 mm × 30 mm). The mean size of the lesions was 279 mm^2 (corresponding to approximately 19 mm × 19 mm). The smallest sample of excised tissue measured 176 mm^2 (14 mm × 16 mm), and the largest measured 4,712 mm^2 (75 mm × 80 mm). The mean size of the excised tissue was 1,783 mm^2 (corresponding to approximately 48 cm × 48 mm). The surgical distance was between 1 mm and 43 mm (arithmetic mean of surgical distance: 17 mm) depending on the anatomical conditions of the patient.

The number of margin samples collected per patient ranged from 4 to 27, depending on the size of the pre-ulcerative lesion (arithmetic mean: 13.6 margin samples per patient). This difference was corrected by multiple logistic regression (all data of margin samples were adjusted for patients).

All patients were considered to be healed when discharged from hospital. Our follow-up of the study patients 12–18 months after the surgical intervention revealed no recurrences.

Association between PCR Results and Independent Variables

The IS2404 PCR analysis obtained positive results for 20 (13.3%) of the 150 margin samples. The association between PCR results and independent variables is summarized in table 2. The detection of *M. ulcerans*

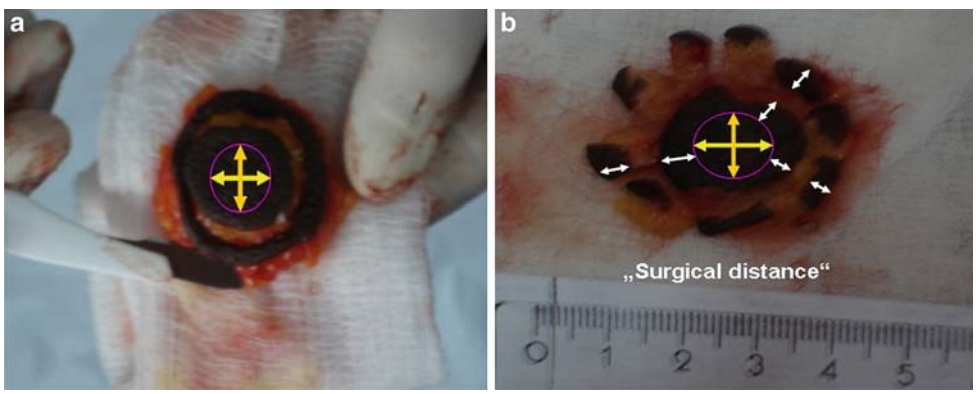

Figure 2. a) Abscission of excision margin. b) Cutting into margin segments with a maximum size of 10 × 10 mm. The ''surgical distance'' was determined for each margin segment individually.

Table 1
Patients with laboratory-confirmed pre-ulcerative lesions.

Number of patients	Hospital	Age (years)	Sex[a]	Number of samples	Type of lesion[b]	Location of lesion	Duration[c] (weeks)	Size of L[d] (mm^2)	Size of E[e] (mm^2)	Surgical distance[f] (mm)	PCR[g]
1	Dunkwa	4	F	9	N	Arm	2	47	327	5–12	3/9 (33.3%)
2	Dunkwa	11	F	12	P	Leg	2	314	1,734	10–20	1/12 (8.3%)
3	Dunkwa	6	F	11	N	Leg	1	314	707	2–8	3/11 (27.3%)
4	Agogo	12	M	17	N	Leg	3	314	2,827	15–23	0/17 (0%)
5	Agogo	23	F	15	P	Arm	1	176	1,616	12–23	0/15 (0%)
6	Agogo	2	M	24	P	Back	N.A.	311	3,792	21–29	0/24 (0%)
7	Agogo	3	F	27	N	Leg	8	314	4,712	17–43	2/27 (7.4%)
8	Agogo	13	F	4	N	Hip	8	79	176	1–5	2/4 (50%)
9	Agogo	18	F	6	P	Foot	20	174	307	2–3	1/6 (16.7%)
10	Dunkwa	50	F	17	N	Leg	4	707	2,919	11–19	0/17 (0%)
11	Dunkwa	6	F	8	N	Leg	2	314	491	2–3	8/8 (100%)
p-value[h]	0.169	0.220	0.304	–	0.025*	0.166	0.184	0.127	0.628	< 0.001*	–

* Significant p-values (< 0.050); N.A.: not available; [a] Sex: F, female; M, male; [b] Type of pre-ulcerative lesion of Buruli Ulcer: N, nodule; P, Plaque; [c] Duration between occurrence of lesion and excision; [d] L = Size of surface of pre-ulcerative lesion (nodule, plaque); [e] E = Size of surface of excised tissue; [f] Surgical distance: distance between the margin of the pre-ulcerative lesion and the margin of excised tissue; [g] Number of positive margin samples/number of all tested (positive and negative) margin samples. Tested by IS2404 PCR for *M. ulcerans*; [h] p-value of the association between the dependent variable (PCR result for *M. ulcerans*) and the independent variables, adjusted for surgical distance

Table 2
Association between PCR results of the margins of surgically excised tissue and different independent variables (without and with adjustment for surgical distance).

Number	Independent variable	p-value not adjusted	p-value adjusted[a]
1	Hospital (Agogo, Dunkwa)	0.014*	0.169
2	Age of patient	0.014*	0.220
3	Sex of patient	0.025*	0.304
4	Type of pre-ulcerative lesion[b]	0.006*	0.025*
5	Location of pre-ulcerative lesion	0.183	0.166
6	Duration[c]	0.122	0.184
7	Size of pre-ulcerative lesion	0.078	0.127
8	Size of excised tissue	0.243	0.628
9	Surgical distance	<0.001*	–

*Significant p-values (< 0.050); [a] Adjusted for surgical distance; [b] Type of pre-ulcerative lesion of Buruli Ulcer: nodule or plaque; [c] Duration between first occurrence of lesion and excision

depended significantly on the surgical distance (the larger the distance, the smaller the probability for the margin sample to be PCR positive; $p < 0.001$). Fractional polynomial regression showed that a surgical distance of more than 9 mm reduced the risk for any mycobacteria remaining after excision to less than 10%, a surgical distance of more than 13 mm reduced the risk to less than 5%, and a surgical distance of more than 25 mm reduced the risk to nearly 0% (Figure 3). This regression includes all margin samples collected from nodules and plaques. Regression after stratification in the different types of pre-ulcerative lesions showed x-axes crossing at 26.8 mm for nodules and 22.9 mm for plaques. Patients with nodules had a higher risk of being tested positively than patients with plaques ($p = 0.025$, adjusted for surgical distance).

The PCR results also depended on the age of the patients. Older patients had a significantly smaller risk of positive margin samples ($p = 0.014$). This association was confounded by the surgical distance. The surgical distance among older patients was larger than that among younger ones ($p = 0.007$). The x-axes crossing was 29.3 mm for children (2–6 years), 19.2 mm for the adolescents (11–18 years), and 18.5 mm for the two adult patients (23 and 50 years). After adjustment, the association between age of the patients and the PCR result was not significant ($p = 0.220$, adjusted for surgical distance).

The independent variables hospital (Agogo Presbyterian Hospital, Dunkwa Government Hospital) and sex showed a significant association with the PCR results ($p = 0.014$ and $p = 0.025$, respectively), but they were also significantly associated with the variable surgical distance.

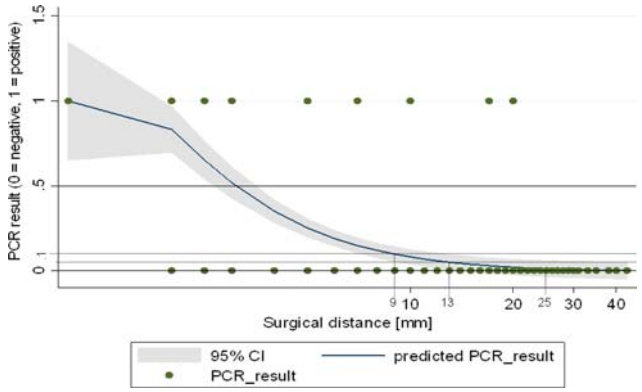

Figure 3. Regression (fractional polynomial): correlation between the PCR results of the margin samples and the surgical distance (distance between the macroscopically visible border of pre-ulcerative lesion and the margin of excised tissue). Y-axes in log scale. Probability of a predicted positive PCR result at a surgical distance of more than 9.0 mm is below 10%, that at a surgical distance of more than 13 mm is below 5%, and that at a surgical distance of more than 25 mm is nearly 0%.

Multiple logistic regression was able detect this confounding (hospital: $p = 0.169$, adjusted for surgical distance; sex: $p = 0.304$, adjusted for surgical distance).

The independent variables location of lesion ($p = 0.166$), duration between first occurrence of lesion and excision ($p = 0.184$), size of lesion ($p = 0.127$), and size of excision ($p = 0.628$) did not significantly influence the PCR results of the margin samples.

Discussion

In accordance with recent promising results, antibiotic treatment of BUD alone or in combination with surgery becomes increasingly important [9–14]. The benefit of treating ulcerative lesions with antibiotics is absolute and not open to controversy. Even if antibiotic treatment alone is not curative and subsequent surgery is required, antimycobacterial treatment can reduce the surface area of the lesions, resulting in a less extensive excision. In addition, in view of the extensive spatial distribution of the bacilli and their spread into macroscopically healthy excision margins, prior antimycobacterial treatment increases the chances of curative secondary surgery [15–17].

For pre-ulcerative lesions, however, other aspects should be taken into consideration. In a follow-up study in Ghana (own unpublished data), the duration of the mean in-patient time after surgery for ulcerative lesions, 84.0 days [95% confidence interval (CI) 65.3–102.7 days], exceeded the 8-week course of antibiotics. In contrast, the mean in-patient time after surgical treatment of pre-ulcerative lesions, 33.4 days, was significantly shorter (95% CI 21.2–45.1 days; $p = 0.002$). No significant difference in the mean in-patient time between nodules and plaques was found ($p = 0.264$). Primary surgery of these lesions therefore offers a less time-consuming treatment option for the patient than antimycobacterial treatment. In the same follow-up study, functional limitations, such as a reduced range of motion of joints after primary surgical excision, were not observed for patients with nodules (0/12) and only in a minority of patients with plaques (3/13: 23%). In this respect, there are no advantages of antibiotic treatment over the surgical treatment of pre-ulcerative lesions.

Uncharacteristic clinical features and a variety of possible differential diagnoses of pre-ulcerative lesions (e.g., onchocercosis, leprosy, leishmaniasis, bacterial abscesses, lipoma) necessitate laboratory analysis to establish the diagnosis. Punch biopsies or fine needle aspirates (FNA) enable a pre-surgical laboratory assessment of tissue specimens. However, the possibilities of establishing the differential diagnosis are limited. Fine needle aspirate specimens are not suitable for histopathological analysis, and punch biopsies have been found to be too small to allow a reliable histopathological diagnosis in many cases (personal communication, *P. Racz*, Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany and *A. Schipf*, Kantonsspital Luzern, Switzerland). Surgically excised tissue still provides the best option for a reliable histopathological differential diagnosis of pre-ulcerative lesions, which also constitutes a valid argument for surgical excision in these cases [7–9].

The results of this study suggest a strong correlation between the surgical distance and the detection of *M. ulcerans* in margin samples by IS2404 PCR. Safe surgical distances of at least 25 mm theoretically reduce the risk of remaining infected tissue to 0%, whereas a surgical distance of at least 13 mm or 9 mm results in a risk of less than 10 or 5%, respectively. We therefore conclude that primary surgery has the potential to be curative in terms of pre-ulcerative lesions if the anatomical conditions allow excision with safe surgical distances. In view of these results, surgical debridement with 3- to 5-mm margins as recommended by *Chin-Lenn* et al. [14] does not seem to be sufficient for a curative excision of pre-ulcerative lesions. In the Australian case series, microscopic analysis was used to determine if the margins were clear of AFB. However, the application of a more sensitive method, such as IS2404 PCR, which allows the detection of small quantities of bacilli in microscopically negative tissue specimens, suggests the need for wider surgical distances.

Surgical excision of pre-ulcerative lesions can either be carried out on an outpatient basis or with in-patient times significantly shorter than for those necessary for antimycobacterial therapy. Primary surgery can remove most of the infected tissue located in the center of the lesion, and it offers the possibility for reliable laboratory diagnosis and differential diagnosis through the assessment of surgically excised tissue specimens.

In patients where anatomical conditions do not allow sufficiently wide excisions, a combination of primary

surgery and subsequent antimycobacterial treatment could be considered. However, as no recurrences were observed in seven of our study patients with *M. ulcerans*-positive excision margins, the need for postoperative antimycobacterial treatment in such patients remains to be determined. Further studies with a larger sample size are required to evaluate the benefit of a combination of primary surgery and subsequent drug treatment in pre-ulcerative lesions.

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Outcome of Patients with Buruli Ulcer after Surgical Treatment with or without Antimycobacterial Treatment in Ghana

Mirjam Schunk,* William Thompson, Erasmus Klutse, Jörg Nitschke, Kwame Opare-Asamoah, Ruth Thompson, Erna Fleischmann, Vera Siegmund, Karl-Heinz Herbinger, Ohene Adjei, Bernhard Fleischer, Thomas Loscher, and Gisela Bretzel

Department of Infectious Diseases and Tropical Medicine (DITM), Ludwig-Maximilians University of Munich, Munich, Germany; Agogo Presbyterian Hospital, Agogo, Ghana; Dunkwa Governmental Hospital, Dunkwa-on-Offin, Ghana; Bernhard Nocht Institute for Tropical Medicine (BNITM), Hamburg, Germany; Kumasi Centre for Collaborative Research and Tropical Medicine (KCCR), Kwame Nkrumah University of Science and Technology Kumasi, Ghana

Abstract. This study assesses the frequency of recurrences and treatment outcome after surgery of buruli ulcer disease (BUD) with or without concomitant antimycobacterial treatment. Of 129 laboratory-confirmed BUD patients who underwent surgery in two treatment centers in Ghana, 79 (61%) were retrieved for follow-up 4–29 months after the initial treatment. Among 7 (9%) recurrent cases no significant association was found between recurrences and clinical or treatment specific factors including antimycobacterial treatment. In 21 (27%) patients, a reduced range of motion (ROM) of one or more joints was detected. Lesions other than nodules, joint involvement, and skin grafting were identified as independent risk factors. Functional limitations hampering daily activities were perceived by 22% of the patients. Compared with other studies the recurrence rate was relatively low, functional limitations were, however, frequent. This emphasizes the need for improvement of pre- and post-treatment wound care as well as rehabilitation programs.

INTRODUCTION

Mycobacterium ulcerans, the causative agent of buruli ulcer disease (BUD), has a wide geographical distribution in the tropical and subtropical belt, yet the clinical manifestation occurs in focal disease clusters. BUD is most common in humid, remote rural areas that are close to stagnant or slow moving bodies of water. Only in the last decade, with dramatically growing numbers of cases reported mainly from West African countries, BUD assumed importance as an emerging infectious disease. This prompted the establishment of the Global Buruli Ulcer Initiative by the World Health Organization (WHO) in 1998.[1] Presently BUD is acknowledged to be the third most frequent mycobacterial disease in humans after tuberculosis and leprosy.[2] The condition has been reported or suspected in > 30 countries worldwide.[3] West Africa is the most affected region. In Ghana, a national case search was conducted in 1999, and cases of BUD were detected in all 10 regions of the country with a peak prevalence rate of 150.8 per 100,000 in the Amansie West district.[4] Children between 2 and 15 years of age are most affected, the lesions occur predominantly on the limbs.[5] The mode of transmission is not certain, although transmission through aquatic insects has been suggested.[6,7] After inoculation of *M. ulcerans* into the subcutaneous tissue the infection initially presents as painless papule, nodule, plaque, or—rarely—as an extensive edema. Later, ulcerative skin lesions develop. The ulcers progress slowly, they are usually painless and there are, besides bone involvement, no systemic signs of infection.[8] Although mortality is very low, the frequency of long-term sequelae is high.[9–11] Contractures and deformities causing restricted movements of affected limbs are often the result of uncontrolled self-healing processes or surgical treatment at a late, far progressed stage of disease.

Until recently wide surgical excision of BUD lesions was the only treatment option. Because there was evidence that a combined antibiotic treatment with rifampicin and streptomycin has the potential to inhibit growth of *M. ulcerans* WHO issued provisional guidelines recommending standard antimycobacterial therapy in 2004.[12,13] Presently, prospective drug trials are being conducted comparing the efficacy of different dosages and durations of antimycobacterial treatment in different stages of BUD. According to the lesion category (category I, single lesion < 5 cm; category II, single lesion 5–15 cm; category III, single lesion > 15 cm and multiple lesions), different treatment schemes are applied (antibiotic treatment alone or in combination with surgery).[3] To improve the quality of clinical management, the need for follow-up studies to monitor the treatment outcome has been emphasized.[12]

The purpose of this study was to assess the frequency of recurrences after surgical treatment of BUD with or without concomitant antimycobacterial treatment in two treatment centers in highly endemic areas in Ghana and to record the treatment outcome with regard to functional limitations as late sequelae of BUD.

MATERIALS AND METHODS

Study area and treatment procedures. The follow-up was conducted in the catchment area of two treatment centers for BUD in Ghana. One center, the Agogo Presbyterian Hospital, is located in the Asante Akim North District in the Ashanti Region; the other, Dunkwa Government Hospital, is situated in Dunkwa-on-Offin, Upper Denkyira District in the Central Region. BUD is highly prevalent in both areas.[4] In each treatment center one surgeon especially trained and experienced in surgical treatment of BUD performed the operations on all study subjects. For wound closure mesh skin grafting was considered the standard technique; in some cases, however, primary suturing was applied or the wound was left open for spontaneous healing. When surgery was carried out physiotherapy could not yet be offered; therefore, prevention of disability strategies were limited to providing adequate wound care and promoting early movement. Splints were not routinely applied.

*Address correspondence to Mirjam Schunk, Department of Infectious Diseases and Tropical Medicine (DITM), Ludwig-Maximilians University of Munich, 80802 Munich, Germany. E-mail: schunk@lrz.uni-muenchen.de

Patients and inclusion criteria. One hundred twenty-nine patients with a laboratory-confirmed BUD infection who received surgical treatment in the period from September 2003 to September 2005 in one of the two sites were included in the study. Laboratory confirmation was carried out by microscopy of Ziehl-Neelsen–stained smears, culture on Loewenstein-Jensen media, *IS2404* polymerase chain reaction (PCR), or histopathological examination according to standardized procedures.[14,15]

Data collection. Subject-specific epidemiological and clinical data was extracted from WHO "BU 01" BUD surveillance forms and hospital records, if available.

In total 16 field trips were conducted in February and March 2006: 11 in the area of Agogo and 5 in the Dunkwa area. We conducted participant interviews using a semi-structured questionnaire to collect information on the pre- and post-treatment history with special focus on possible recurrences and sequelae. For children, the guardian was interviewed.

To record the delay in seeking medical care, the time period from noticing a possible BUD lesion to presentation in a treatment center was documented. In addition, the site of the initial BUD lesion was examined and information on the size and the state of healing was recorded. For documentation, a photograph was taken. In case of joint involvement, the passive range of motion (ROM) was measured using a standard goniometer (E-Z Read Goniometer 30 cm; Rolyan Jamar, Russka, Germany). ROMs were recorded by two observers according to the Neutral-Zero Measuring Method and SFTR (sagittal, frontal, and transverse rotation) documentation.[16] If any skin lesion was present, the site was clinically assessed according to WHO BUD case definition[1] and documented. In case of an ulcer, swab specimens for laboratory confirmation (microscopy/culture/PCR) were collected. At the time of the study, assessment of punch biopsies or fine needle aspirates were not among the established methods for the routine diagnosis of BUD in Ghana; therefore, the collection of tissue specimens from non-ulcerative lesions was not possible in the field. All patients with new or not healed lesions were referred to the respective treatment center.

Definition of recurrence. In accordance to the definition of recurrences established by WHO in 2001,[1] in this study, a recurrent case was defined as a patient with previous surgical treatment with or without concomitant antimycobacterial therapy for *M. ulcerans* who presented with a further BUD lesion at the same or a different site within 1 year after the end of the last treatment.

Lesions that were present at follow-up were judged as BUD recurrences if the clinical features matched the BUD case definitions and the diagnosis was confirmed with at least one positive laboratory test. Clinically diagnosed BUD lesions that developed after the initial surgical excision and were subjected to surgery before follow-up were counted as interim recurrences. All clinically diagnosed interim recurrences were included, even if laboratory confirmation was not performed.

The time period was calculated using the date of initial surgery (as documented in the BU 01 form) and the date of follow-up. For interim recurrences, information from the patients was correlated with the documentation in the corresponding treatment center.

It must be noted that following the WHO recommendation and implementation of antimycobacterial treatment, the definition of recurrence was revised by the WHO technical advisory group. Since 2007, only new and culture confirmed BUD lesions occurring > 3 months after completion of a full course of antibiotic treatment (possibly followed by surgery), which resulted in complete healing of the initial lesion, are considered recurrences. In contrast, lesions occurring within 3 months after completion of a full course of antibiotics in the same or adjacent area are regarded ongoing cases (i.e., non-healers). In both cases, a patient is considered to have completed treatment after 56 doses of antibiotics in 8 weeks.[17,18]

Because treatment of our study subjects was primarily surgical, and none of the patients received a full course of the standardized antimycobacterial treatment according to the current WHO definitions, we consider the revised definition of recurrence not applicable.

Treatment outcome. Outcome was assessed by recording the frequency of a reduced ROM in one or more joints and the perceived functional limitations caused by BUD sequelae.

A reduced ROM was recorded when the ROM deviated from the international standardized normal range of motion.[16] A functional limitation was recorded if the patient reported to have difficulties with daily activities because of sequelae of the BU infection.

Statistical analysis. For categorical data analysis, the χ^2 test and Fisher exact test were used, and for continuous variables, Student's *t* tests were applied. In addition, we constructed multiple logistic regression models for detecting potential confounders among the analyzed variables. Records with missing data for a particular variable were excluded from analysis of that variable. Calculations were done by software package SAS version 9.1.[19] *P* values < 0.05 were considered as statistically significant.

Ethical clearance and informed patient consent. Ethical clearance for the study was obtained through the Ethics Committee of Human Research Publication and Ethics, School of Medical Sciences, University of Science and Technology, Kumasi, Ghana. Informed consent was obtained from the participants or their guardians before beginning any study-related interventions.

RESULTS

Followed up patients. Of a total of 129 laboratory-confirmed BUD patients, 79 (61%) were retrieved for follow-up examination within a study period of 2 months. In the catchment area of Agogo, 56 (63%) of 89 patients could be followed up; in the catchment area of Dunkwa, 23 (58%) of 40 patients were retrieved. Of the 50 lost patients, 29 had a wrong address, 11 moved from the region, 3 died, and 7 could not be followed up for other reasons. The group lost to follow-up was comparable with the study participants in terms of sex, age, and year of initial treatment (data not shown).

Baseline characteristics of the followed-up patients are shown in Table 1. Of the 79 followed-up patients, 65 (82%) received antibiotic treatment in addition to surgery as recorded in BU 01 forms and/or hospital records. In 11 of these cases, no records could be retrieved on the type and duration of treatment; however, in the BU 01 form, it was stated that the respective patients were treated with antimycobacterial drugs. In 54 cases, hospital records with precise documentation were available: 13 patients were treated with rifampicin only, one was treated with streptomycin only, and 40 patients were treated with a combination of both. The mean duration of ther-

TABLE 1
Baseline characteristics of the follow-up samples

Variables	Agogo ($N = 56$)	Dunkwa ($N = 23$)	Total ($N = 79$)
Sex (female), *N* (%)	28 (50)	10 (43)	38 (48)
Age (years)			
Range (minimal-maximal)	2–72	3–43	2–72
Mean (SD)	17.1 (15.1)	18.3 (11.3)	17.5 (14.0)
Median	11	14	13
Delay in seeking medical care (in days)			
Range (minimal-maximal)	7–180	7–120	7–180
Mean (SD)	41.8 (35.5)	46.2 (38.1)	43.0 (34.7)
Median	30	30	30
Kind of medical care, *N* (%)			
Not known	2 (4)	10 (43)	12 (15)
Ambulant operation and post treatment	3 (5)	6 (26)	9 (11)
Treatment as in-patient	51 (91)	7 (30)	58 (73)
Type of lesion, *N* (%)			
Nodule	8 (14)	4 (17)	12 (15)
Plaque	8 (14)	5 (22)	13 (16)
Ulcer	35 (63)	14 (61)	49 (62)
Edema	5 (9)	0 (0)	5 (6)
Localization of lesion, *N* (%)			
Upper extremity	30 (54)	10 (43)	40 (51)
Lower extremity	16 (29)	9 (39)	25 (32)
Trunk	10 (18)	4 (17)	14 (18)
Head/neck	0 (0)	0 (0)	0 (0)
Size of lesion, *N* (%)			
Category I (< 5 cm maximal diameter)	30 (54)	11 (48)	41 (52)
Category II (5–15 cm maximal diameter)	18 (32)	8 (35)	26 (33)
Category III (> 15 cm maximal diameter)	8 (14)	4 (17)	12 (15)
Wound closure technique, *N* (%)			
Suturing	5 (9)	7 (30)	12 (15)
Skin grafting	48 (86)	3 (13)	51 (65)
Spontaneous healing	3 (5)	13 (57)	16 (20)
Antimycobacterial therapy, *N* (%)			
Not known	3 (5)	10 (43)	13 (16)
No	1 (2)	0 (0)	1 (1)
Yes	52 (93)	13 (57)	65 (82)
With rifampicin*	13	0	13
With streptomycin*	0	1	1
With combination streptomycin/rifampicin*	38	2	40

* If documented in hospital record and/or BU 01 forms ($N = 54$).

apy was 39.2 ± 32.4 (SD) days for streptomycin and 26.2 ± 21.4 days for rifampicin. In 70% ($N = 38$) of these patients, antimycobacterial treatment was started on the day of excision of the primary lesion, in 24% ($N = 13$) treatment was started before surgery (minimum, 3 days; maximum, 24 days), and in 6% ($N = 3$) of the cases, treatment was started after surgery (minimum, 2 days; maximum, 21 days). Concerning the use of drug treatment, there is less precise information of the patients in the Dunkwa region, with 43% ($N = 10$) missing entries in the BU 01 forms on this topic. Records with missing data were not included in the analysis. There was no significant difference between the two treatment centers.

The time interval between the first BUD treatment and the follow-up assessment ranged between 4 and 29 months (mean, 18 months; median, 20 months). In 25% ($N = 20$) of the patients, the time interval between the first BUD treatment and the follow-up assessment was < 12 months, in 38% ($N = 30$) was 12–23 months, and in 37% ($N = 29$) was > 23 months (range, 4–29 months).

Characteristics of the recurrent cases. Of the 79 patients available for follow-up, there were 11 possible recurrent cases (Table 2). According to the definitions of this study, seven (9%) patients with recurrences were detected.

During the physical examination at follow-up, five patients presented with skin lesions. In four cases, small excoriations with a maximum diameter of 2 cm were located in the scar area of the previous BUD lesion. None of these lesions showed the typical clinical features of a BUD lesion, and the reported duration of the lesions was in no case > 4 weeks. The laboratory examinations (microscopy, culture, and PCR) were negative for *M. ulcerans*.

One patient stated at follow-up that the initial wound never fully closed after surgery 7 months earlier. On inspection, we found a 2 × 13-cm purulent wound without undermined edges on the left flank. In the Ziehl-Neelsen–stained smear of the swab sample, acid-fast bacilli were detected; culture and *IS2404* PCR were negative. Therefore, a superinfection with other mycobacteria, as well as the possibility of a persistent, slowly healing lesion with dead bacilli, can not be excluded. In this patient, no antimycobacterial treatment had been documented.

Six patients reported interim recurrences (i.e., they had developed a recurrent lesion that was clinically diagnosed as BUD and surgically removed in the period between excision of the primary lesion and follow-up). Two of these patients came from Dunkwa and four patients were from Agogo. The median time period of the occurrence of the new lesions after excision of the primary lesion was 3 months (range, 0.5–5 months). In four cases, the patients received antimycobacterial treatment in addition to surgery for at least 14 days starting on the date of excision; in two cases, there was no written documentation of drug treatment, yet the patients remembered receiving oral and intramuscularly medication for treatment. At the time of follow-up, the patients with interim recurrences had no acute medical complaints.

In the analysis of factors possibly influencing the probability of recurrence, no significant difference was found between recurrence and size or type of lesions, use of antimycobacterial treatment, duration of disease before treatment, and place of treatment.

In eight patients from the follow-up group, an assessment of the margins of the excised tissue was conducted after surgical resection of the primary lesion.[20] In all of these specimens, *M. ulcerans* infection was detected even in the macroscopically unaffected tissue at the periphery. However, none of these patients showed a recurrence at follow-up. All had received antibiotic therapy started on the day of excision either as monotherapy with rifampicin ($N = 5$) or as combination therapy with rifampicin and streptomycin ($N = 3$) over at least 30 days.

Treatment outcome. Reduced ROM of one or more joints as a consequence of the BUD infection and treatment was detected in 21 (27%) patients. In nine of these (43%), a disability caused by the primary lesion was already documented before surgical excision. The wrist was most often affected (7.6%, $N = 6$), followed by the joints of the hand and the ankle (6.3%, $N = 5$), the elbow (3.8%, $N = 3$), and the knee (2.5%, $N = 2$). Clinical features and their association to reduced ROM are summarized in Table 3.

In 30 patients, the primary lesion was located over one or more joints, resulting in a reduced ROM of the involved joints in 60% ($N = 18$) of these patients. The association between these two variables is highly significant ($P < 0.001$).

TABLE 2
Characteristics of possible recurrent cases

Sex	Age	Hospital site*	Inclusion†	Size, type and location of primary lesion	Technique‡	Type and location of new lesion	Laboratory confirmation of new lesion§	Time period¶	Antibiotic treatment**
Lesions at follow-up									
M	7	A	No	Medium-sized ulcer, left knee	Grafting at second intervention	Ulcer, left knee (same site)	–	8	RMP (31) SM (19)
M	14	D	No	Small ulcer, right arm	Initial grafting	Ulcer, left leg (different site)	–	29	Not documented
F	12	D	No	Small ulcer, right leg	Spontaneous healing	Ulcer, right leg (different site)	–	28	Not documented
M	4	D	No	Small plaque, left leg	Spontaneous healing	Ulcer, left leg (same site)	–	25	Not documented
M	14	D	Yes	Medium-sized ulcer, abdomen	Spontaneous healing	Ulcer, abdomen (same site)	+/– (MIC/PCR)	0	Not documented
Interim recurrences									
F	4	A	Yes	Medium-sized nodule, trunk	Suturing	Nodule, trunk (same site)	+ (PCR)	2	RMP (37)
M	12	A	Yes	Medium-sized edema, right arm	Grafting at second intervention	Nodule, right arm (same site)	No testing	5	RMP (35) SM (30)
F	2	A	Yes	Small ulcer, right arm	Grafting at second intervention	Nodule, right arm (different site)	No testing	0.5	RMP (14) SM (14)
M	3	A	Yes	Medium-sized ulcer, left leg	Grafting at second intervention	Nodule, right arm (different site)	–	1	RMP (30) SM (75)
M	16	D	Yes	Small ulcer, right arm	Suturing	Ulcer, left arm (different site)	+ (HIS)	3	Not documented
F	12	D	Yes	Medium-sized plaque, right arm	Spontaneous healing	Nodule, right arm (same site)	No testing	3	Not documented

Small < 5 cm diameter (category I); medium 5–15 cm diameter (category II); large > 15 cm diameter (category III).
*A = Agogo; D = Dunkwa.
†Inclusion as recurrent case.
‡Technique of wound closure of primary lesion.
§MIC = microscopy; HIS = histopathological analysis; PCR = polymerase chain reaction.
¶Time period between excision of primary lesion and recurrence (in months).
**Antibiotic treatment with RMP (=rifampicin) and SM (=streptomycin) (duration in days).

In the factor analysis, no correlation between size of the primary BUD lesion and a reduced ROM was found ($P = 0.064$). There was, however, a significant association between the type of primary BUD lesion and the occurrence of a reduced ROM ($P = 0.025$). In contrast to other types of lesions, nodules did not result in a reduced ROM.

There was also a significant association ($P < 0.01$) between the wound closure technique and the risk of a reduced ROM. From the 28 patients who received suturing after surgical intervention or where the lesion was left to heal spontaneously, 2 (7%) presented with a reduced ROM. Of the 51 patients where skin grafting was used, 19 (37%) showed a reduced ROM at follow-up.

From 21 patients at increased risk of a reduced range of motion (lesion other than nodule, joint involvement, skin graft for wound closure), 76% ($N = 16$) presented a reduced ROM at follow-up. Multiple logistic regression models including the four above-mentioned independent variables have shown that the size of the lesion ($P = 0.71$) and the type of lesion ($P = 0.93$) were not significantly associated with the dependent variable reduced ROM. On the other hand, the involvement of joints ($P < 0.001$) and the methods of wound closure ($P = 0.011$) were significantly associated with the dependent variable. Multiple logistic regression detected that the association between the dependent variable and the size of lesion was confounded by involvement of joints. These two independent variables were highly associated ($P = 0.010$), because larger lesions were significantly more often situated over joints and *vice versa*. Other confounding was not found.

A BUD-related functional limitation in daily activities was perceived by 22% ($N = 17$) of the patients or their guardians. Of the 21 patients with a reduced ROM, 52% ($N = 11$) stated that they had difficulties in their daily tasks. Another six patients indicated functional restrictions, although objectively reduced ROM could not be measured.

DISCUSSION

Because there is no causative prevention of BUD, early case detection and optimization of treatment and rehabilitation are the major objectives to reduce morbidity and disability. Data on the long-term outcome after treatment are essential to evaluate treatment strategies and to adapt them according to needs identified. Despite being identified as a major public health concern in endemic areas, implementing routine aftercare and follow-up is still a major problem because people affected with BUD live mostly in remote and poorly accessible rural areas.

The recurrence rate of 9% as detected in our study is relatively low compared with the results of most other published studies where recurrence rates vary from 2% to 35%.[3,21–25] However, because of variation in study design, sample sizes, follow-up periods, and diagnostic tools applied for confirmation of cases, the comparability of these studies is limited. In one study from Benin reporting a similar low recurrence rate of 6% ($N = 4$), in total 15% ($N = 10$) of the followed-up patients including two of the recurrent cases had received antimycobacterial treatment with rifampicin and streptomycin for up to 14 days in addition to surgical treatment.[22] A study conducted in Ghana detected a recurrence rate of 35% ($N = 27$) among patients treated between 1994 and 2001 in the same two treatment centers as in our study. Fifty-eight percent of the patients

TABLE 3
Clinical aspects and reduced range of motion (ROM)

Type and size of lesion*	Reduced ROM ($N = 21$)			ROM not reduced ($N = 58$) total		
	Suturing	Skin graft	Spontaneous healing	Suturing	Skin graft	Spontaneous healing
Nodule, $N = 12$	0	0	0	6	3	3
Joint involvement:						
Yes	0	0	0	1	0	0
No	0	0	0	5	3	3
By size						
Category I	0	0	0	5	3	2
Category II	0	0	0	1	0	1
Category III	0	0	0	0	0	0
Ulcer, $N = 49$	0	13	2	3	22	9
Joint involvement						
Yes	0	11	2	1	4	3
No	0	2	0	2	18	6
By size						
Category I	0	5	0	1	17	4
Category II	0	5	2	1	5	4
Category III	0	3	0	1	0	1
Edema, $N = 5$	0	3	0	0	2	0
Joint involvement						
Yes	0	3	0	0	0	0
No	0	0	0	0	2	0
By size						
Category I	0	0	0	0	0	0
Category II	0	0	0	0	1	0
Category III	0	3	0	0	1	0
Plaque, $N = 13$	0	3	0	3	5	2
Joint involvement						
Yes	0	2	0	2	0	1
No	0	1	0	1	5	1
By size						
Category I	0	2	0	1	0	1
Category II	0	1	0	0	4	1
Category III	0	0	0	2	1	0
Total	0	19	2	12	32	14

*All lesions were single lesions.

treated in Agogo received monotherapy with rifampicin and 9% received it in Dunkwa.[24] A prospective study from Ghana detected a recurrence rate of 20% ($N = 10$) within 1 year after excision of pre-ulcerative lesions.[25] A retrospective study from the Ivory Coast examined hospital records for the frequency of BUD patients returning to the treatment center for a second intervention within 1 year and described 17% ($N = 59$) recurrences.[23] In both cohorts, no antimycobacterial treatment was given.

Different factors could have contributed to the low occurrence of recurrences in our study population. Concerning predisposing factors, no statistically significant influence of size and type of lesion and duration of delay of treatment seeking on the probability of recurrence was observed.

A major factor associated with the incidence of recurrences is the expertise of surgical treatment of the primary lesion. Striking differences in healing rates between treatment centers have previously been described by other authors[24]: whereas there was a recurrence rate of 18% ($N = 6$) after surgical treatment with wide excision margins in Agogo, there was a recurrence rate of 47% ($N = 21$) in Dunkwa, where lesions were excised with no or small excision margins. In our study, however, we observed no statistically significant difference concerning recurrences between the two treatment centers among patients treated between 2003 and 2005. The discrepancy between our and previously reported results may be explained by ongoing training efforts and the implementation of standardized surgical management in both treatment centers.

Another important factor contributing to the low overall recurrence rate and the diminishing difference between the two treatment centers might be the increasing use of antimycobacterial treatment supplementary to surgery. Recent data suggest that antibiotic treatment decreases recurrence rates to 1–4%.[13,17,18,21] Even before the WHO recommendations on antimycobacterial treatment were released,[26] both of our treatment centers introduced the use of a combination of streptomycin and rifampicin in their treatment of BUD. In total, 29% of the patient cohort treated between 1994 and 2001 in Agogo and Dunkwa received antimycobacterial drugs consisting of monotherapy with rifampicin. In contrast, 82% of patients retrieved in our follow-up study were treated with antimycobacterial drugs. Although the analysis of the available records showed that the treatment scheme has not yet been standardized, the majority of patients in both centers received a combination of rifampicin and streptomycin for a variable period of time.

The use of antimycobacterial treatment could also explain why there were no recurrences observed in the eight cases where *M. ulcerans* infection was detected in the macroscopically unaffected tissue of the outer margin of the excised lesions.[20] However, in our study, four of the seven patients that experienced recurrences had also received antimycobacterial treatment. However, considering the lack of precise data on the actual adherence to the prescribed medication and the variable duration of therapy, a positive effect of antimycobacterial treatment on treatment outcome can not be excluded.

Because no single factor proved to have an essential influence on the occurrence of recurrent cases in the presented study, the overall reduced incidence of recurrences in this area is attributed to the multifactorial quality improvement of clinical management of BUD in the field of surgical as well as antimycobacterial treatment.

Although the low incidence of recurrences is promising, adverse sequelae (measurable ROM and/or functional limitations that hampered the daily activities of the patients) were frequent.

The measurement of a reduced ROM of joints can be difficult in the field, and there are no validated normal ranges of motion adjusted to African populations. However, it is a useful tool to assess and document treatment outcomes.[27] In our study, 9 of 21 patients with reduced ROM at follow-up had presented with reduced ROM before the beginning of treatment. No reduced ROM was observed in cases with nodules as primary lesions, emphasizing the importance of early detection and treatment. Whereas lesion over joint and lesion other than nodule and skin grafting for wound closure were identified as risk factors for a reduced ROM, there was no statistically significant association between occurrence of functional limitations and the size of lesion. This is a surprising result because it is obvious that large, far progressed lesions with soft tissue and bone involvement are prone to cause ankylosis and require extensive surgery, leading to significant scarring. A possible explanation for this finding is that the absolute lesion size was not related to actual body surface area. Because most patients are children, the assumed size categories do not apply equally in all patients. Also, the multiple logistic regression

analysis indicated that the size of lesion was confounded by involvement of joints as larger lesions were significantly more often situated over joints.

Although disfigurement and joint deformities as late sequelae of treated BUD patients are described as a common outcome, there is sparse information on its actual incidence and its social and economic impact. It has been emphasized that besides the high direct and indirect treatment costs, the impact of long-term care for disabled and handicapped BUD patients places an enormous burden on the family members.[28]

The frequency of functional impairment after BUD treatment varies from 49% as observed in Ghana, 37% as reported from the Democratic Republic of Congo, to 25% as assumed by WHO.[27,29,30] The detection of a reduced ROM in a joint, however, does not necessarily correspond to the perceived individual functional impairment.[29] In our study, 52% (N = 11) of the patients where a reduced ROM had been recorded stated that they had difficulties fulfilling daily tasks, whereas six patients without objectively reduced ROM indicated functional impairments. A BUD functional limitation score questionnaire (BUFLS) was recently developed to assess the severity of functional limitations.[9–11] After treatment of BUD, 57% (N = 362) of the interviewed patients experienced one or more functional limitations according to the BULFS score.[11] In our study only 22% (N = 17) of the patients indicated a functional impairment in daily activities. However, we did not apply the BULFS score, which may have resulted in a higher proportion of functional impairments.

The relatively high incidence of functional limitations as sequelae after BUD observed in our study sample emphasizes the need to re-evaluate the impact of different wound closure techniques on the long-term outcome and the importance of providing rehabilitative care after primary wound treatment. A main concern of follow-up activities should therefore be the establishment of programs for the prevention of disability (POD) on community level. As advised by WHO, POD activities should have a high priority in national BUD control programs.

This study had several limitations, and some conclusions should be interpreted with caution. Epidemiologic and clinical baseline data were retrieved retrospectively from BU 01 forms and hospital records. This retrospective nature of data collection is prone to errors such as potential interobserver variability and assessment bias. In addition, written information on the duration and sort of drug treatment could only be obtained in 54 of 79 cases (Agogo, N = 51; Dunkwa, N = 3). Only a limited proportion of patients included in the study could be retrieved for follow-up, which might have induced a selection bias. The definition of recurrences that was valid at the time the study was conducted included all patients with a new lesion within 1 year after initial BUD treatment. Especially if the delay between primary lesion and recurrence was < 3 months and the same site was affected, a persistent rather than a recurrent lesion may have to be assumed (non-healer). At the time when our study was conducted, the main focus of the treatment centers was the identification of new cases. Therefore, laboratory confirmation of interim recurrences was not performed on a regular basis. Of the few cases subjected to laboratory analysis, unfortunately, positive culture results were not obtained. As reflected in the revised WHO definition of recurrences, the presence of acid-fast bacilli and/or a positive PCR signal for *IS2404* might equally well reflect the presence of dead bacilli.[17,18] The diagnosis of recurrence in our study patients was always established by clinicians with long-standing experience in BUD. However, we concede that in view of the revised WHO definition, the recurrent cases identified in this study may not fully meet the criteria and may rather be referred to as clinically suspect recurrences.

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Authors' addresses: Mirjam Schunk, Erna Fleischmann, Vera Siegmund, Karl-Heinz Herbinger, Thomas Loscher, and Gisela Bretzel, Department of Infectious Diseases and Tropical Medicine (DITM), Ludwig-Maximilians University of Munich, Leopoldstrasse 5, 80802 Munich, Germany. William Thompson, Agogo Presbyterian Hospital, Agogo, Ghana. Erasmus Klutse, Dunkwa Governmental Hospital, Dunkwa-on-Offin, Ghana. Jörg Nitschke and Bernhard Fleischer, Bernhard Nocht Institute for Tropical Medicine (BNITM), Bernhard-Nocht Strasse 74, 20359 Hamburg, Germany. Kwame Opare-Asamoah, Ruth Thompson, and Ohene Adjei, Kumasi Center for Collaborative Research in Tropical Medicine (KCCR), Kwame Nkrumah University of Science and Technology (KNUST), University Post Office, Kumasi, Ghana.

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