JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 2005, p. 271–276 Vol. 43, No. 1
0095-1137/05/$08.00+0 doi:10.1128/JCM.43.1.271–276.2005
Copyright © 2005, American Society for Microbiology. All Rights Reserved.

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*

Received 22 June 2004/Returned for modification 20 August 2004/Accepted 21 September 2004

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.

ACKNOWLEDGMENTS

We are grateful to Christian Drosten (BNITM) for stimulating discussion and technical advice. We thank Serwaa Akosua (KCCR), Sabine Köhler, Monika Picker, Petra Plähn, Julia Polansky, Anja Schörle, Petra Meyer, and Birgit Raschdorff (BNITM) for excellent technical assistance. We thank Jörg Nitschke for providing his surgical expertise. We are especially grateful to Christel Schmetz (BNITM) for her continuous support.

REFERENCES

1. **Amofah, G., F. Bonsu, C. Tetteh, J. Okrah, K. Asamoa, K. Asiedu, and J. Addy.** 2002. Buruli ulcer in Ghana: results of a national case search. Emerg. Infect. Dis. **8:**167–170.
2. **Asiedu, K., and S. Etuaful.** 1998. Socioeconomic implications of Buruli ulcer in Ghana: a three-year review. Am. J. Trop. Med. Hyg. **59:**1015–1022.
3. **Asiedu, K., M. Raviglione, and R. Scherpbier (ed.).** 2000. Buruli ulcer. *Mycobacterium ulcerans* infection. World Health Organization, Geneva, Switzerland.
4. **Guarner, J., J. Bartlett, E. A. Whitney, P. L. Raghunathan, Y. Stienstra, K. Asamoa, S. Etuaful, E. Klutse, E. Quarshie, T. S. van der Werf, W. T. A. van der Graaf, C. H. King, and D. A. Ashford.** June 2003, posting date. Histopathologic features of *Mycobacterium ulcerans* infection. Emerg. Infect. Dis. [Online.] http:/www.cdc.gov/ncidod/EID/vol9no6/02–0485.htm.
5. **Guimaraes-Peres, A., F. Portaels, P. de Rijk, K. Fisette, S. R. Pattyn, J. P. van Vooren, and P. A. Fonteyne.** 1999. Comparison of two PCRs for detection of *Mycobacterium ulcerans*. J. Clin. Microbiol. **37:**2006–2008.

6. **King, C. H., D. A. Ashford, K. Dobos, E. A. S. Whitney, P. L. Raghunathan, J. Guarner, and J. W. Tappero.** 2001. *Mycobacterium ulcerans* infection and Buruli ulcer disease: emergence of a public health dilemma, p. 137–152. *In* W. M. Sheld, W. A. Craig, and J. M. Hughes (ed.), Emerging infections 5. ASM Press, Washington, D.C.
7. **Meyers, W. M., N. Tignokpa., G. B. Priuli., and F. Portaels.** 1996. *Mycobacterium ulcerans* infection (Buruli ulcer): first reported patients in Togo. Br. J. Dermatol. **134:**1116–1121.
8. **Mwanatambe, M., M. Yajima, S. Etuaful, K. Suzuki, K. Nishigai, K. Asiedu, N. Yamada, and G. Asano.** 2002. Malignant melanoma of the buttock presenting as a Buruli ulcer. J. Dermatol. **29:**357–361.
9. **N′Guessan, K., Y. Kouassi, S. Bouzid, P. Ehuie, K. Koffi, C. Oniangue, N. Aka, and M. Dosso.** 2001. Value and limits of microscopy of exudates in *Mycobacterium ulcerans* cutaneous infection in Cote d'Ivoire. Bull. Soc. Pathol. Exot. **94:**9–10.
10. **Portaels, F., J. Agular, K. Fisette, P. A. Fonteyne, H. De Beenhouver, P. de Rijk, A. Guedenon, R. Lemans, C. Steunou, C. Zinsou, J. M. Dumonceau, and W. M. Meyers.** 1997. Direct detection and identification of *Mycobacterium ulcerans* in clinical specimens by PCR and oligonucleotide specific capture plate hybridizytion. J. Clin. Microbiol. **35:**1097–1100.
11. **Portaels, F., P. Elsen, A. Guimares-Peres, P. Fonteyne, and W. M. Meyers.** 1999. Insects in the transmission of *Mycobacterium ulcerans* infection. Lancet **353:**986.
12. **Portaels, F., P. Johnson, and W. M. Meyers (ed.).** 2001. Buruli ulcer. Diagnosis of *Mycobacterium ulcerans* disease. A manual for health care providers. World Health Organization, Geneva, Switzerland.
13. **Ross, B. C., L. Marino, F. Oppedisano, R. Edwards, R. M. Robins-Browne, and P. D. Johnson.** 1997. Development of a PCR assay for rapid diagnosis of *Mycobacterium ulcerans* infection. J. Clin. Microbiol. **35:**1696–1700.
14. **Stinear, T., B. C. Ross, J. K. Davies, L. Marino, R. M. Robins-Browne, F. Oppedisano, A. Sievers, and P. D. Johnson.** 1999. Identification and characterization of IS*2404* and IS*2606*: two distinct repeated sequences for detection of *Mycobacterium ulcerans* by PCR. J. Clin. Microbiol. **37:**1018–1023.
15. **Whitney, E. A. S., M. Phelan, P. L. Raghunathan, Y. Stienstra, K. Dobos, J. Guarner, S. Kilhstrom, A. Ablordey, S. Etuaful, E. Klutse, E. Quarshie, D. Ofori-Adjei, T. S. van der Werf, W. T. A. van der Graaf, K. Asamoah, G. Amofah, C. H. King, and D. A. Ashford.** 2002. Latent class analysis (LCA) evaluation of four diagnostic tests for Buruli ulcer disease. Presented at the 5th W.H.O. Advisory Group Meeting on Buruli Ulcer, Geneva, Switzerland.
16. **World Health Organization.** 2002. Weekly epidemiological record, vol. 77, p. 271–272.