JOURNAL OF CLINICAL MICROBIOLOGY, May 1997, p. 1097–1100 Vol. 35, No. 5
0095-1137/97/$04.00+0

Direct Detection and Identification of *Mycobacterium ulcerans* in Clinical Specimens by PCR and Oligonucleotide-Specific Capture Plate Hybridization

F. PORTAELS,[1]* J. AGUIAR,[2] K. FISSETTE,[1] P. A. FONTEYNE,[1] H. DE BEENHOUWER,[1] P. DE RIJK,[1] A. GUÉDÉNON,[3] R. LEMANS,[1] C. STEUNOU,[4] C. ZINSOU,[5] J. M. DUMONCEAU,[1,6] AND W. M. MEYERS[7]

Department of Microbiology, Mycobacteriology Unit, Institute of Tropical Medicine, Antwerp,[1] and Department of Gastroenterology and Hepato-Pancreatology, Erasme University Hospital, Brussels,[6] Belgium; Centre Sanitaire et Nutritionnel Gbemoten, Zangnanado,[2] and Hôpital "Terre des Hommes," Sagon,[5] Zou, Lazaret-Akpakpa, Cotonou,[3] and Centre Antilèpre de Davougon,[4] Benin; and Armed Forces Institute of Pathology, Washington, DC 20306-6000[7]

Received 24 September 1996/Returned for modification 10 January 1997/Accepted 10 February 1997

We compared various diagnostic tests for their abilities to detect *Mycobacterium ulcerans* infection in specimens from patients with clinically active disease. Specimens from 10 patients from the area of Zangnanado (Department of Zou, Benin) with advanced, ulcerated active *M. ulcerans* infections were studied by direct smear, histopathology, culture, PCR, and oligonucleotide-specific capture plate hybridization (OSCPH). A total of 27 specimens, including 12 swabs of exudate collected before debridement and 15 fragments of tissue obtained during debridement, were submitted to bacteriologic and histopathologic analysis. The histopathologic evaluation of tissues from all six patients so tested revealed changes typical of those caused by *M. ulcerans* infection. Five specimens were contaminated, and *M. ulcerans* was cultivated on Löwenstein-Jensen medium from 12 of the remaining 22 (54.5%) specimens. Detection of mycobacteria was performed by PCR, and *M. ulcerans* was detected by OSCPH with a new probe (5′-CACGGGATTCATGTCCTGT-3′) reacting with *M. ulcerans* and *Mycobacterium marinum*. In 10 of 22 (45.5%) specimens, *M. ulcerans* was identified by PCR-OSCPH. There was no statistically significant difference between the detection of *M. ulcerans* by culture and by PCR-OSCPH ($P > 0.05$). This is the first demonstration of an amplification system (PCR-OSCPH) with a sensitivity similar to that of culture for the direct and rapid recognition of *M. ulcerans* in clinical specimens. This system is capable of identifying *M. ulcerans*, even in paucibacillary lesions. Our findings suggest that PCR-OSCPH should be used in the quest for the elusive environmental reservoir(s) of *M. ulcerans*.

Cutaneous infection caused by *Mycobacterium ulcerans*, popularly known as "Buruli ulcer," represents, after tuberculosis and leprosy, the third most frequent mycobacterial disease in immunocompetent individuals in intertropical areas (5). Most recent reports on Buruli ulcer concern new foci or microepidemics in West Africa and Australia (5, 7, 9). Quantitative evaluation of the epidemiologic importance and socioeconomic impact of Buruli ulcer remains difficult because health professionals fail to recognize the disease (7), and prevalence studies are available from only a few isolated foci of wide areas where the disease is endemic. We define *M. ulcerans* infection as classic findings that are clinically typical either in the early nodular or in the late ulcerative stage. Detailed descriptions of these lesions have been published by many observers, and widely accepted (5, 8, 9). Buruli ulcer is rarely detected in an early and readily curable stage. The preulcerative lesion, the earliest stage, is often neglected by both the patient and health care workers; thus, bacteriologic confirmation is rarely undertaken. More precise and appropriate techniques for the identification of *M. ulcerans* are needed to improve medical management of the patient and provide more reliable prevalence data.

Cultivation of *M. ulcerans* from tissue remains a difficult task. Primary cultures usually become positive only after several months of incubation, and many clinically and histopathologically typical Buruli ulcers are reported as culture negative (11, 15, 16). Development of rapid identification methods for *M. ulcerans* in clinical specimens would constitute an important advance in the management of patients.

This report describes the first direct method for the rapid detection and identification of *M. ulcerans* in clinical specimens by PCR amplification and oligonucleotide-specific capture plate hybridization (OSCPH) (2).

MATERIALS AND METHODS

Collection of the specimens. Ten patients with clinically typical, active *M. ulcerans* infections were selected for this study in February 1995. All patients were from the Department of Zou (Benin) and were diagnosed and treated at the Centre Sanitaire et Nutrionnel at Zangnanado (Department of Zou, Benin).

A total of 27 specimens were collected from the 10 patients. Before debridement, 12 exudates were collected with sterile cotton swabs from the base of the undermined margins of the ulcers. During surgical debridement, 15 fragments of tissue from the undermined edges of the ulcers were taken for bacteriologic study. Each swab or tissue fragment was placed immediately in 2 ml of modified Dubos medium (17), kept at 4°C for 3 days, transported to Antwerp, Belgium, at room temperature, and maintained again at 4°C until analyses were initiated. The total elapsed time between collection and analysis was 7 to 9 days. Entire surgically excised specimens of skin and subcutaneous tissue from six patients were placed in 10% formalin solution for histopathologic study.

Culture of mycobacteria. (i) Swab specimens. The swabs in transport medium were treated by vortex agitation to suspend the exudates. These suspensions in transport medium were decontaminated with hydrochloric acid at a final concentration of 0.5 N for 20 min at room temperature and were then neutralized with 1 N sodium hydroxide. After centrifugation, the sediment was inoculated onto Löwenstein-Jensen medium, 1% Ogawa egg yolk medium, and Ogawa egg

* Corresponding author. Mailing address: Institute of Tropical Medicine, Nationalestraat 155, B-2000 Antwerp, Belgium. Phone: 32.3.247.63.17. Fax: 32.3.247.63.33.

yolk medium supplemented with mycobactin J (14). All inoculated media were incubated for up to 12 months at 33°C and were observed weekly.

(ii) Tissue specimens. The tissues (weight, ±1 g) were homogenized in 1 ml of saline, decontaminated, and inoculated onto mycobacteriologic media as described above for the specimens from swabs.

Direct smear examination. Smears of the suspensions were stained for acid-fast bacilli (AFB) by the Ziehl-Neelsen method.

Identification of the isolates. The mycobacterial isolates were identified as described previously (18).

PCR analysis. (i) Extraction method. DNA was extracted from the suspensions by a previously described method (3). Briefly, each tissue specimen (1 to 3 mm^3) was washed with UV-treated distilled water, minced with a scalpel, and resuspended in 1 ml of TE (10 mM Tris, 1 mM EDTA) plus 0.5% (wt/vol) sodium dodecyl sulfate. One-half of each suspension (500 μl) was extracted as follows. A total of 40 μl of proteinase K (from a 20-mg/ml stock solution) was added to the suspension and the suspension was incubated overnight at 65°C in a rotating incubator (hybridization incubator 310; Robbens Scientific Corporation, Sunnyvale, Calif.) and then treated for 5 min at room temperature in a water bath sonicator (47 kHz; Branson 1200; Branson Ultrasonics Corporation, Danbury, Conn.). The suspensions were then placed in a heating block (16 500 Dri-Bath; Thermolyne; Merck-Belgolabo SA, Overijse, Belgium) at 95°C for 20 min. The solution was extracted twice with phenol-chloroform (1:1; vol/vol). DNA was precipitated overnight at −20°C with 10% (vol/vol) 3 M sodium acetate (pH 5.2) and 2 volumes of ethanol and was then centrifuged at 10,000 × *g* at 4°C for 30 min. The centrifugate was then washed with 70% (vol/vol) ethanol, dried, and resuspended in 50 μl of TE.

(ii) Detection and identification of mycobacteria by PCR-OSCPH. Detection of mycobacteria in specimens was based on the amplification of a partial sequence in the 16S rRNA gene (5′ side of the noncoding RNA-like strand). DNA was amplified by a nested PCR specific for the genus *Mycobacterium*, and amplified DNA was identified by OSCPH (2). A new probe (5′-CACGGGATTC ATGTCCTGT-3′) has been designed for the detection of *M. ulcerans*. This probe, which also reacts with *Mycobacterium marinum*, was used in the previously described OSCPH assay (2) with the following modifications. Hybridization buffer contained 20% formamide, 6× SSC (0.9 M NaCl plus 0.09 M sodium citrate [pH 7.0]), 0.1% *N*-lauroyl sarcosine, and 0.5% (wt/vol) blocking reagent (Boehringer Mannheim, GmbH, Mannheim, Germany), and the hybridization temperature was 41 ± 0.5°C. The washing series was as follows: a brief wash twice with 20% formamide–6× SSC at room temperature, a wash for 15 min with 20% formamide–6× SSC at 41 ± 0.5°C, and finally, a brief wash with a solution containing 0.1 M maleic acid, 0.15 M NaCl, and 0.3% (vol/vol) Tween 20 (pH 7.5). Immunoenzymatic detection of hybrids was performed as described by De Beenhouwer et al. (2).

The specificity of the *M. ulcerans-M. marinum* probe has been evaluated with all the mycobacterial species described in the report of De Beenhouwer et al. (2). From the 70 mycobacterial strains belonging to 21 different species, the probe reacted only with *M. ulcerans* and *M. marinum*. The specificity of OSCPH was tested on each plate by using *M. ulcerans* as a positive control and *Mycobacterium asiaticum* (1 bp difference from the probe) as a negative control (data not shown).

Histopathologic analysis. The entire surgically excised specimens of skin and subcutaneous tissues from six patients were kept in 10% formalin for 1 to 12 months before histopathologic evaluation. Multiple fragments of each fixed specimen were cut from the base and margin of the ulcer and at various distances (up to 8 cm) from the undermined margin of the ulcer. The fragments were processed routinely and blocked with paraffin, and 4-μm-thick sections were cut. Sections were stained by hematoxylin-eosin, Ziehl-Neelsen, Gomori methenamine-silver, and Brown-Hopps tissue Gram staining techniques and were studied by light microscopy.

Statistical analysis. Comparisons between the culture and PCR-OSCPH results were evaluated statistically by the χ^2 test.

RESULTS

Histopathologic analysis. At least one fragment of the specimen from each of the six patients showed the following typical histopathologic changes as a result of *M. ulcerans* infection: (i) contiguous coagulation necrosis of subcutis and dermis, (ii) necrosis and thickening of the interlobular septae of subcutis, (iii) damage to blood vessels with frequent occlusion, and (iv) mineralization. Three of the six specimens (from patients 3, 5, and 8; Table 1) revealed AFB, primarily in the subcutis or underlying necrotic fascia. The AFB were extracellular and were often present in large clumps. Thus, in all six patients the histopathologic changes were consistent with *M. ulcerans* infection, and in three patients they were confirmed by finding AFB in typical sites. In the three specimens that did not show AFB (from patients 1, 6, and 7; Table 1), there was no necrotic slough from the center of the ulcer. This apparently had been removed by active scrubbing and preliminary debridement prior to surgery. The slough in the base of pristine Buruli ulcers often contains the largest numbers of *M. ulcerans* organisms and histologically is the best hunting ground for AFB.

Culture of mycobacteria. As shown in Table 1, *M. ulcerans* was cultivated from specimens from 5 of the 10 patients investigated. No other mycobacteria were cultivated from these patients.

Of the 13 specimens collected from these 5 patients (4 swab and 9 tissue specimens), 12 were positive by culture for *M. ulcerans* after 6 to 28 weeks of incubation at 33°C (Table 1). The single swab from patient 6 remained negative by culture.

The nine specimens collected from four patients (patients 1, 2, 7, and 10) were all negative for mycobacteria by culture, and five specimens from one patient (patient 5) were all heavily contaminated with gram-negative bacilli.

Detection and identification of mycobacteria by PCR. As shown in Table 1, nested PCR for members of the genus *Mycobacterium* was positive for 10 of the 27 suspensions tested. OSCPH with the *M. ulcerans-M. marinum* probe was positive for the 10 PCR-positive suspensions.

Comparison between culture and PCR results. The results of the comparison between culture and PCR-OSCPH results for the detection of *M. ulcerans* in 22 specimens (the results for the five contaminated specimens were excluded) are presented in Table 2. Ten specimens from five patients (patients 1, 3, 6, 8, and 9; Table 1) were positive by PCR-OSCPH (45.5%), and 12 specimens from five patients (patients 3, 4, 6, 8, and 9; Table 1) were positive for *M. ulcerans* by culture (54.5%). The difference between the sensitivities of the two tests compared to the clinical diagnosis was not statistically significant ($\chi = 0.36$; $P > 0.05$).

Comparison between culture, OSCPH, and staining. Excluding the five contaminated specimens, all of the specimens positive by Ziehl-Neelsen staining were also positive by culture and OSCPH (Table 1). Among the 14 specimens negative by Ziehl-Neelsen staining, 4 were positive by culture and two were positive by OSCPH.

Among the five contaminated specimens collected from the same patient (patient 5), two were positive by Ziehl-Neelsen staining. Mycobacteria were, however, not detected by PCR in these two specimens.

DISCUSSION

Isolation of the etiologic agent by culture is useful in the diagnosis of a putative Buruli ulcer only when *M. ulcerans* is grown from the lesions. Many clinically and histopathologically typical Buruli ulcers are reported as culture negative (9, 11, 15, 16).

The following are some of the reasons why *M. ulcerans* may be difficult to isolate by cultivation: (i) poor sampling (samples should be taken from materials usually rich in bacilli, e.g., the necrotic base of the ulcer); (ii) *M. ulcerans* is a slowly growing organism, and some laboratories do not observe cultures for long periods; (iii) *M. ulcerans* is susceptible to the decontamination methods (10) which are essential for isolation of mycobacteria from tissue or exudates often contaminated by other microorganisms (usually saprophytic bacteria and fungi); and (iv) in many studies, cultures for mycobacteria are not set up until days or weeks after the specimen is obtained; thus, *M. ulcerans* may not survive under the conditions used for preservation and transport of the specimens.

DNA amplification techniques offer interesting alternatives for the direct detection of *M. ulcerans* in tissues. The main

TABLE 1. Comparison between histopathologic and bacteriologic results for specimens collected from 10 patients in Benin

Patient no.	Specimen no.	Location of ulcer	Type of specimen	Histopathology reaction[a]	AFB[b]	Ziehl-Neelsen staining[c]	Mycobacterial culture positivity (time [wk] to positivity)	Nested PCR result	OSCPH detection of *M. ulcerans*
1	95-237	Toe	Swab			Neg[d]	Neg	−	
	95-238	Toe	Swab			Neg	Neg	−	
	95-252	Toe	Tissue	+	−	Neg	Neg	−	
	95-253	Toe	Tissue			Neg	Neg	+	+
2	95-239	Foot	Swab	ND[e]	ND	Neg	Neg	−	
3	95-240	Elbow	Swab			2+	*M. ulcerans* (19)	+	+
	95-250	Elbow	Tissue	+	+	4+	*M. ulcerans* (6)	+	+
	95-251	Elbow	Tissue			4+	*M. ulcerans* (6)	+	+
4	95-241	Navel	Swab	ND	ND	Neg	*M. ulcerans* (28)	−	
5	95-242	Chest	Swab			Neg	Contaminated	−	
	95-243	Chest	Swab			Neg	Contaminated	−	
	95-245	Chest	Tissue	+	+	2 AFB	Contaminated	−	
	95-246	Chest	Tissue			Neg	Contaminated	−	
	95-247	Chest	Tissue			2+	Contaminated	−	
6	95-244	Shoulder	Swab			Neg	Neg	−	
	95-248	Shoulder	Tissue	+	−	1+	*M. ulcerans* (6)	+	+
	95-249	Shoulder	Tissue			4+	*M. ulcerans* (6)	+	+
7	95-254	Arm	Tissue	+	−	Neg	Neg	−	
	95-255	Arm	Tissue			Neg	Neg	−	
8	95-256	Arm	Tissue			Neg	*M. ulcerans* (13)	−	
	95-257	Arm	Tissue	+	+	2 AFB	*M. ulcerans* (14)	+	+
	95-258	Arm	Tissue			2 AFB	*M. ulcerans* (15)	+	+
	95-259	Arm	Tissue			Neg	*M. ulcerans* (21)	+	+
9	95-262	Elbow	Swab	ND	ND	Neg	*M. ulcerans* (27)	−	
	95-263	Elbow	Swab			1+	*M. ulcerans* (6)	+	+
10	95-264	Leg	Swab	ND	ND	Neg	Neg	−	
	95-265	Leg	Swab			Neg	Neg	−	

[a] Histopathologic changes typical of Buruli ulcer.
[b] AFB in histopathologic sections.
[c] Scale of the American Thoracic Society (1).
[d] Neg, negative.
[e] ND, not done.

advantage is that results can be obtained in a few days instead of the few months that it would normally take with culture techniques. The PCR-OSCPH technique was found to be useful for identifying other mycobacteria that are difficult to isolate in culture; for example, DNA of the fastidious organism *Mycobacterium genavense* can be identified by this method in intestinal biopsy specimens from nonimmunocompromised humans (3).

PCR-OSCPH confirmed *M. ulcerans* infection in one of the specimens negative by culture, thus permitting the identification of *M. ulcerans* in 10 of 22 samples (45.5%). Although the detection of *M. ulcerans* by culture and by PCR-OSCPH gave similar results ($P > 0.05$), the rapid availability of results by PCR-OSCPH is a distinct advantage for early diagnosis. Three specimens (from patients 4, 8, and 9), however, were positive by culture but negative by PCR-OSCPH.

Of particular interest, specimens from patient 7 showed histopathologic changes diagnostic of Buruli ulcer, but were negative by Ziehl-Neelsen staining, culture, and PCR-OSCPH.

The negative results obtained by culture or PCR-OSCPH are probably related to sampling. The distribution of bacilli in lesions is not homogeneous (5), and the samples used for culture are not the same as those used for PCR or histopathology. The heterogeneity of the samples is demonstrated by the fact that different samples from the same patient (Table 1) are variably positive for AFB by direct examination (patients 5, 6, 8, and 9) and histopathologic analysis. The volume of the sample analyzed by each method also differs markedly: Com-

TABLE 2. Comparison between culture and PCR-OSCPH for the detection of *M. ulcerans* in 22 specimens from nine patients from Benin with active Buruli ulcers[a]

Result of detection of *M. ulcerans* by PCR-OSCPH	No. of specimens with the following culture result:		Total
	+	−	
+	9	1	10
−	3	9	12
Total	12	10	22

[a] $\chi^2 = 0.36$ ($P > 0.05$).

pared to the amount used for PCR analysis, an estimated 10 to 100 times more material is used for culture, and even much larger amounts are used for histopathologic analysis.

Some negative culture results may also be related to delays in the transport of specimens and by ambient conditions during transport. The specimens were kept at 4°C in a modified Dubos medium for 7 to 9 days before being processed for culture. The results of culture attempts may have been better if it had been possible to inoculate the samples immediately after collection. New transport media are being investigated to increase the probability of successful culture.

The PCR-OSCPH test that we have perfected for the recognition of *M. ulcerans* and *M. marinum* in human tissue confirmed that it is possible to identify *M. ulcerans* even in paucibacillary specimens (i.e., specimens that are negative upon direct examination). These encouraging results suggest that these techniques may be useful not only for rapid diagnosis but also in the quest for environmental reservoirs of *M. ulcerans* which have never been identified bacteriologically (12, 13).

Several problems remain to be resolved before PCR can be used more efficiently to identify *M. ulcerans* directly in the specimens.

(i) Techniques for the extraction of DNA from samples need to be refined. The sensitivity of the currently used PCR-OSCPH technique is no better than that of culture methods. The same problem has been reported for different PCR assays for *M. tuberculosis* (6). The sensitivity of detection of *M. ulcerans* DNA in clinical specimens could be improved by refining the DNA extraction methods. Recent work in our laboratory indicates that the yield and the quality of DNA from biopsy specimens can be improved with a new combined extraction method (proteinase K treatment followed by extraction with guanidium thiocyanate) (15a).

(ii) A system which is specific for the detection of *M. ulcerans* in clinical samples is required. The probe that we have designed has the disadvantage of not being able to distinguish *M. ulcerans* from *M. marinum* by PCR-OSCPH. These two species, although phenotypically different, are genetically closely related (4). Rarely, if ever, would this seem to represent a diagnostic problem, because the diseases caused by *M. ulcerans* and by *M. marinum* differ greatly in their clinical, histopathologic, and epidemiologic aspects (8).

The close genetic relationship between *M. ulcerans* and *M. marinum* may constitute a problem if the PCR-OSCPH technique is used with specimens from the environment. *M. marinum* is ubiquitous in the environment (13) and, unlike *M. ulcerans*, is easily cultivated in primary culture. Therefore, any search for *M. ulcerans* in the environment should include both the isolation of the mycobacterium by culture and detection by PCR-OSCPH or the use of a specific oligonucleotide probe or combination of probes (15). We recently described the potential value of a mismatch at the 3′ end of the 16S rRNA sequence for the distinction between *M. marinum* and *M. ulcerans* (15). The value of that sequence for the specific detection of *M. ulcerans* is being evaluated in our laboratory.

In this study, the diagnosis was confirmed in all six patients studied by histopathology alone. To determine with a greater degree of confidence the specificity and sensitivity of the PCR-OSCPH method for diagnosis, a larger number of samples, including a larger proportion of paucibacillary samples, and samples from a control group should be tested.

In conclusion, we have developed a new method (PCR-OSCPH) for the direct identification of *M. ulcerans*. This method permits the rapid identification of *M. ulcerans* in clinical specimens and could be used in the search for reservoirs of *M. ulcerans* in the environment. While rapid identification of the etiologic agent is helpful for establishing appropriate management of all stages of Buruli ulcer, it is especially important for preulcerative lesions, in which the differential diagnosis is sometimes difficult (e.g., early lesions of *Mycobacterium abscessus* infection or phaemycotic cysts). Finally, because of the potential for contamination in molecular biological procedures under field conditions or where other mycobacteriological work is in progress, we recommend confirmatory histopathologic analysis for all clinical specimens.

ACKNOWLEDGMENTS

We thank K. Janssens and B. Antoine for assistance in the preparation of the manuscript. We also thank M. Boelart for critical comments on the manuscript.

This work was supported by the Damien Foundation (Brussels, Belgium), grant 3.4601.90 from the Funds for Medical Scientific Research (Brussels, Belgium), and the American Registry of Pathology (Washington, D.C.).

REFERENCES

1. **American Thoracic Society.** 1981. Diagnostic standards and classification of tuberculosis and other mycobacterial diseases, 14th ed. Am. Rev. Respir. Dis. **123:**343–358.
2. **De Beenhouwer, H., L. Zhong, P. de Rijk, C. van Eekeren, and F. Portaels.** 1995. Detection and identification of mycobacteria by DNA amplification and oligo specific capture plate hybridization. J. Clin. Microbiol. **33:**2994–2998.
3. **Dumonceau, J. M., P. A. Fonteyne, L. Realini, A. Van Gossum, J. P. Van Vooren, and F. Portaels.** 1995. Species-specific *Mycobacterium genavense* DNA in intestinal tissues of individuals not infected with human immunodeficiency virus. J. Clin. Microbiol. **33:**2514–2515.
4. **Hofer, M., B. Hirschel, P. Kirschner, M. Beghetti, A. Kaelin, C. A. Slegrist, S. Sutter, A. Teske, and E. C. Böttger.** 1993. Brief report: disseminated osteomyelitis from *Mycobacterium ulcerans* after a snakebite. N. Engl. J. Med. **328:**1007–1009.
5. **Josse, R., A. Guédénon, H. Darie, S. Anagonou, F. Portaels, and W. M. Meyers.** 1995. Les infections cutanées à *Mycobacterium ulcerans*: ulcères de Buruli. Med. Trop. **55:**363–373.
6. **Mangiapan, G., M. Vokurka, L. Schouls, J. Cadranel, D. Lecossier, J. van Embden, and A. J. Hance.** 1996. Sequence capture-PCR improves detection of mycobacterial DNA in clinical specimens. J. Clin. Microbiol. **34:**1209–1215.
7. **Marston, B. J., M. O. Diallo, C. R. Horsburgh, I. Diomande, M. Z. Saki, J. M. Kanga, G. Patrice, H. B. Lipman, S. M. Ostroff, and R. Good.** 1995. Emergence of Buruli ulcer disease in the Daloa region of Côte d'Ivoire. Am. J. Trop. Med. Hyg. **52:**219–224.
8. **Meyers, W. M.** 1995. Mycobacterial infections of the skin, p. 291–377. *In* W. Doerr and G. Seifert (ed.), Tropical pathology. Springer-Verlag Berlin, Germany.
9. **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.
10. **Palomino, J. C., and F. Portaels.** 1995. Evaluation of decontamination methods for the recovery of *Mycobacterium ulcerans*, abstr. M-72. *In* Proceedings of the European Conference on Tropical Medicine.
11. **Pettit, J. H. S., N. Marchette, and R. J. W. Rees.** 1966. *Mycobacterium ulcerans* infection: clinical and bacteriological study of the first cases recognized in South East Asia. Br. J. Dermatol. **78:**187–197.
12. **Portaels, F.** 1989. Epidémiologie des ulcères à *Mycobacterium ulcerans*. Ann. Soc. Belg. Med. Trop. **69:**91–103.
13. **Portaels, F.** 1995. Epidemiology of mycobacterial diseases, p. 207–222. *In* M. Schuster (ed.), Clinics in dermatology, vol. 13. Elsevier Science Inc., New York, N.Y.
14. **Portaels, F., A. De Muynck, and P. Sylla.** 1988. Selective isolation of mycobacteria from soil: a statistical analysis approach. J. Gen. Microbiol. **134:**849–855.
15. **Portaels, F., P. A. Fonteyne, H. De Beenhouwer, P. de Rijk, A. Guédénon, J. Hayman, and W. M. Meyers.** 1996. Variability in the 3′ end of the 16S rRNA sequence of the species *Mycobacterium ulcerans* is related to geographic origins of isolates. J. Clin. Microbiol. **34:**962–965.

15a. **Portaels, F., et al.** Unpublished data.

16. **Ravisse, P., M. C. Roques, F. Le Bourthe, J. Tchuembou, C. Jan, and J. C. Menard.** 1975. Une affection méconnue au Cameroun: l'ulcère à mycobactérie. Med. Trop. **35:**471–474.
17. **Saxegaard, F.** 1985. Isolation of *Mycobacterium paratuberculosis* from intestinal mucosa and mesenteric lymph nodes of goats by use of selective Dubos medium. J. Gen. Microbiol. **22:**312–313.
18. **Vincent Lévy-Frébault, V., and F. Portaels.** 1992. Proposed minimal standards for the genus *Mycobacterium* and for description of new slowly growing *Mycobacterium* species. Int. J. Syst. Bacteriol. **42:**315–323.