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Comparison of Two PCRs for Detection of *Mycobacterium ulcerans*

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Two nested PCRs for the detection of *Mycobacterium ulcerans* were compared by using a collection of 65 clinical specimens. The first method amplifies the gene coding for 16S rRNA, and the second method amplifies a repetitive DNA sequence. The sensitivities of bacterioscopy, culture, 16S rRNA gene PCR, and repetitive-sequence PCR were 29, 34, 80, and 85%, respectively. Compared to the 16S rRNA gene PCR, the repetitive-sequence PCR was faster, easier to perform, and less expensive.

Mycobacterium ulcerans is the causative agent of Buruli ulcer, an infection of the subcutaneous fat that results in a chronic indolent ulcer. The disease has been reported since 1948 in several, mostly tropical, regions (2), with a recent dramatic increase of incidence in West Africa (1, 3).

Histological examination of biopsy material reveals extensive necrosis of the dermis and hypodermis, with acid-fast bacilli (AFB) present either in large clumps or as a few, irregularly dispersed organisms, which results in a low sensitivity of microscopic examination. Culture of *M. ulcerans* is also insensitive and has a long lag phase in primary culture, i.e., from 6 to 24 weeks (5), due to the poor viability of *M. ulcerans*, especially after the decontamination procedure (4).

PCR assays offer a welcome alternative to culture for difficult-to-grow organisms. Different targets have been used for the molecular detection of *M. ulcerans*: the gene coding for 16S rRNA (7), the repetitive DNA sequence IS2404 (9), and the gene coding for a 65-kDa heat shock protein (8). In the 16S rRNA gene PCR, specificity depends on postamplification hybridization with a DNA probe in an oligonucleotide-specific capture plate hybridization (OSCPH) assay. In the IS2404 target PCR, a post-PCR hybridization step was applied to increase sensitivity (9). The main advantage of the IS2404 target of PCR is its specificity, since it produces a positive result only with *M. ulcerans*, whereas the assay targeting the 16S rRNA gene does not distinguish between *M. ulcerans* and *M. marinum* (7). The IS2404 PCR has been tested on Australian samples only (9, 10).

The objectives of the present work were (i) to develop a nested IS2404 PCR without hybridization for the detection of *M. ulcerans*, (ii) to compare the sensitivities and specificities of the IS2404 PCR and the 16S rRNA gene PCR with a selection of mycobacterial strains, and (iii) to compare both amplification tests with microscopy and culture for the diagnosis of *M. ulcerans* infection in a collection of clinical specimens.

Table 1 shows the mycobacterial strains studied: 31 reference strains and patient isolates, 9 strains representative of the diversity of *M. ulcerans*, including the closely related *M. shinshuense* strain, and 8 nonulcerans isolates from the environment in regions of endemicity in Benin and Ghana. Bacterial suspensions were prepared from fresh subcultures on Löwenstein-Jensen medium (L-J). The number of AFB in the suspension were counted according to the method of Shepard and McRae (12). Briefly, 5-μl samples of the suspensions were delivered to circular areas marked on microscope slides. Fixation was by formaldehyde vapor, and heat and staining were by the Ziehl-Neelsen procedure. AFB in three circular areas were counted.

Sixty-five tissue fragments of about 5 to 10 g each from 26 patients with clinically active and histologically confirmed *M. ulcerans* infection were selected. Upon collection, the fragments were placed in 2 ml of modified Dubos medium (11) and transported to the Institute of Tropical Medicine at room temperature.

From these fragments, about 1 g was prepared with the help of disposable scalpels and homogenized in 1 ml of phosphate-buffered saline, decontaminated with 1 N hydrochloric acid for 20 min, and neutralized with 1 N sodium hydroxide. After centrifugation, the sediment was inoculated onto L-J, 1% Ogawa egg yolk medium, and Ogawa egg yolk medium supplemented with mycobactin J (6). Media were incubated at 33°C for up to 12 months and examined once weekly. Smears of the suspension were stained by the Ziehl-Neelsen method. The mycobacterial isolates were identified as previously described (14).

Separate fragments of about 1 g were minced with disposable scalpels and homogenized in 500 μl of digestion buffer (30 mM Tris-HCl, 30 mM EDTA, 5% Tween 20, 0.5% Triton X-100, 800 mM guanidine hydrochloride). Twenty microliters of proteinase K (from a 20-mg/ml stock solution) was added, and the suspensions were incubated for 30 min at 60°C in a rotating incubator. Samples were sonicated for 8 min at room temperature in a water bath sonicator. Forty microliters of diatomaceous earth was added, and the samples were incubated for 1 h at 37°C and washed twice in 70% ethanol and then once in acetone. After drying at 50°C, sediments were resuspended in 100 μl of Tris-EDTA and incubated at 65°C for 20 min. Ten microliters of the fresh solution was used for PCR.

The PCR in a nested format was directed at the repetitive sequence IS2404 of *M. ulcerans* (GenBank accession no. U38540), with the primers MU1 and MU2, which amplify a 549-bp sequence (9). DNA recovered was reamplified with the new primers PGP3 (5′-GGCGCAGATCAACTTCGCGGT-3′, positions 547 to 563 in the sequence U38540) and PGP4 (5′-CT GCGTGGTGCTTTACGCGC-3′, positions 764 to 745).

For the first PCR, 10 μl of the sample was amplified in a 50-μl reaction mixture containing 20 pmol of each primer (MU1 and MU2), 1 U of Ampli Taq DNA polymerase (Roche Molecular Systems), 200 μM concentrations of each deoxyribonu-

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TABLE 1. Mycobacterial strains used for assessing specificities of molecular tests for detection of *M. ulcerans*[a]

ITM no.	Species identification	Source or strain, origin	OSCPH result	IS2404 PCR result
96-961	*M. tuberculosis*	ITM, Belgium	Neg[b]	Neg
96-1635	*M. malmoense*	ITM, Australia	Neg	Neg
9403	*M. paratuberculosis*	ITM, Burundi	Neg	Neg
9664	*M. avium*	ITM, Belgium	Neg	Neg
8736	*M. intracellulare*	ITM, Australia	Neg	Neg
4988	*M. scrofulaceum*	ATCC 19981	Neg	Neg
3098	*M. parafinicum*	IPP, 831128	Neg	Neg
96-51	*M. simiae*	ITM, Peru	Neg	Neg
96-109	*M. genavense*	ITM, Belgium	Neg	Neg
4980	*M. nonchromogenicum*	ATCC 19530	Neg	Neg
7735	*M. chitae*	ATCC 19267	Neg	Neg
96-268	*M. flavescens*	ITM, United States	Neg	Neg
8181	*M. asiaticum*	RIVM, 661/76	Neg	Neg
7369	*M. terrae*	ITM, United States	Neg	Neg
5077	*M. gordonae*	ITM, Belgium	Neg	Neg
4978	*M. vaccae*	ATCC 15483	Neg	Neg
7736	*M. triviale*	ATCC 23292	Neg	Neg
9741	*M. xenopi*	ITM, Belgium	Neg	Neg
94-122	*M. duvalii*	ITM, Burundi	Neg	Neg
4981	*M. szulgai*	ATCC 25932	Neg	Neg
96-1182	*M. kansasii*	ITM, Belgium	Neg	Neg
8223	*M. chelonae*	ITM, Belgium	Neg	Neg
97-461	*M. fortuitum*	ATCC 12790	Neg	Neg
94-462	*M. peregrinum*	ATCC 14467	Neg	Neg
97-996	*M. triplex*	ATCC 700071	Neg	Neg
98-269	*M. bovis BCG*	IPB, 1173P2	Neg	Neg
96-1701	*M. lentiflavum*	ITM, Belgium	Neg	Neg
96-117	*M. intermedium*	ITM, Belgium	Neg	Neg
8231	*M. marinum*	ITM, Belgium	Pos[c]	Neg
8756	*M. shinshuense*[d]	ATCC 33728	Pos	Pos
7832	*M. ulcerans*	CIPT 141090018, French Guyana	Pos	Pos
842	*M. ulcerans*	H. J. van Keulen 701357, Suriname	Pos	Pos
5114	*M. ulcerans*	Lavalle, Mexico	Pos	Pos
8849	*M. ulcerans*	ITM, Australia	Pos	Pos
5153	*M. ulcerans*	ITM, Congo	Pos	Pos
1837	*M. ulcerans*	ITM, Ghana	Pos	Pos
9146	*M. ulcerans*	ITM, Benin	Pos	Pos
94-886	*M. ulcerans*	ITM, Benin	Pos	Pos
97-457	*M. nonchromogenicum*	ITM, Benin	Neg	Neg
97-456	*Mycobacterium* sp.	ITM, Benin	Neg	Neg
97-450	*Mycobacterium* sp.	ITM, Benin	Neg	Neg
97-449	*M. gordonae*	ITM, Ghana	Neg	Neg
97-1035	*Mycobacterium* sp.	ITM, Benin	Neg	Neg
97-1033	*Mycobacterium* sp.	ITM, Benin	Neg	Neg
97-1029	*Mycobacterium* sp.	ITM, Benin	Neg	Neg
97-1023	*Mycobacterium* sp.	ITM, Benin	Neg	Neg

[a] ITM, Institute of Tropical Medicine; RIVM, Rijksinstituut voor Volksgezondheid en milieu; IPB, Institut Pasteur du Brabant, Brussels, Belgium; IPP, Institut Pasteur de Paris. Countries where samples have been collected are identified.

[b] Neg, negative.

[c] Pos, positive.

[d] *M. shinshuense* has been isolated from the skin ulcer of a patient in Japan (13).

cleotide triphosphate, 1.5 mM $MgCl_2$, 0.1% Triton X-100, and 10 mM Tris-HCl (pH 8.4) and overlaid with mineral oil. Cycling was as follows: denaturation at 94°C for 5 min; amplification for 40 cycles of 94°C for 1 min, 66°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 7 min. For the second PCR, 1 μl from the first-run product was amplified in a 25-μl reaction mixture with primers PGP3 and PGP4. The annealing temperature was 64°C. Five microliters of amplified DNA was submitted to electrophoresis through a 2% agarose gel and detected by ethidium bromide staining and UV transillumination.

OSCPH was performed as described previously (7). Briefly, part of the DNA coding for 16S rRNA was amplified in a nested PCR specific for the genus *Mycobacterium*. During PCR, a label (digoxigenin-11-dUTP) was incorporated into the amplicons, which are detected by hybridization in microtiter plates coated with streptavidin and a biotinylated oligonucleotide probe specific for *M. ulcerans* and *M. marinum* only.

As shown in Table 1, the IS2404 PCR was positive only with *M. ulcerans* isolates and the closely related *M. shinshuense*. Both tests identified *M. ulcerans* isolates regardless of the geographic origin. Both PCRs were reproducibly positive with fewer than 10 bacteria in the PCR specimen.

Bacterioscopy, culture, 16S rRNA gene PCR, and IS2404 PCR with the 65 clinical specimens were positive with 21 (32%), 22 (34%), 52 (80%), and 55 (85%) specimens, respectively. All culture-positive samples were positive by both molecular tests. All 16S rRNA-positive specimens were confirmed by the IS2404 PCR. Among the 39 Ziehl-Neelsen-negative and culture-negative specimens, 33 were positive by the IS2404 PCR. The difference between the positive results of both molecular tests was not significant ($P = 0.5$). Correlation between results of both molecular tests was excellent (degree of nonrandom agreement [kappa] = 0.8). As shown in Table 2, bacterioscopy, culture, the 16S rRNA gene PCR, and the IS2404 PCR were able to confirm the diagnoses for, respectively, 11 (42%), 11 (42%), 22 (85%), and 22 (85%) patients of the 26 histologically confirmed cases.

In their original paper claiming that the IS2404 PCR is

TABLE 2. Comparison of results of microscopy, culture, and the two PCRs for confirmation of the diagnoses of 26 patients with clinically active and histologically confirmed *M. ulcerans* infection

Patient code	No. of specimens positive by Ziehl-Neelsen test/total no. of specimens	No. of cultures identified as *M. ulcerans*/ total no. of cultures	No. of specimens positive by 16S rRNA gene PCR/total no. of specimens	No. of specimens positive by IS2404 PCR/ total no. of specimens
96-150	0/3	2/2	3/3	3/3
96-162	0/2	0/2	2/2	2/2
96-187	1/3	2/3	3/3	3/3
97-10	2/3	0/3	3/3	3/3
97-72	0/4	0/4	3/4	3/4
97-102	0/3	1/3	2/3	3/3
97-104	1/2	1/2	2/2	2/2
97-105	0/2	0/2	1/2	1/2
97-107	0/2	0/2	2/2	2/2
97-108	0/2	0/2	2/2	2/2
97-117	0/1	0/1	0/1	0/1
97-119	1/2	0/2	1/2	1/2
97-120	0/2	0/2	0/2	0/2
97-121	2/2	2/2	2/2	2/2
97-122	0/2	0/2	2/2	2/2
97-124	2/3	2/3	3/3	3/3
97-125	1/3	2/3	3/3	3/3
97-126	0/2	0/2	1/2	1/2
97-127	1/2	2/2	1/2	2/2
97-129	2/2	2/2	2/2	2/2
97-130	5/7	5/7	7/7	7/7
97-128	2/4	1/4	4/4	4/4
97-187	0/3	0/3	2/3	3/3
97-188	0/1	0/1	1/1	1/1
DED	0/1	0/1	0/1	0/1
GNA	0/2	0/2	0/2	0/2
Total	19/65	22/65	52/65	55/65

specific for *M. ulcerans*, Ross et al. (9) tested a limited number of strains representing 17 different species of mycobacteria and a number of Australian strains of *M. ulcerans*. We confirmed the sensitivity of the IS2404 PCR by including *M. ulcerans* isolates from widely different geographical origins and its specificity by testing 30 different mycobacterial species. The poor sensitivity of microscopy was confirmed by the study of clinical specimens from clinically diagnosed and histologically confirmed Buruli ulcer patients.

Compared to those of the molecular methods, the sensitivity of culture of *M. ulcerans* on L-J, considered until now the reference method, is very low. Loss of viability of the organism resulting from transport and decontamination of the samples may be responsible for the great difference in detection rates. In the previous paper (7), detection rates obtained with culture and 16S rRNA gene PCR were similar. However, in the present study, a different DNA extraction method using diatomaceous earth was employed. This procedure, in contrast to ethanol precipitation, concentrates the DNA and eliminates PCR inhibitors.

The difference in detection rates between the IS2404 PCR and the 16S rRNA gene PCR is not significant, suggesting that availability of target DNA for amplification rather than the technique used determines the detection rate. The high positivity rates produced by the molecular methods with negative samples both by microscopy and culture illustrate the usefulness of these techniques for analysis of specimens transported at room temperature.

In conclusion, the use of molecular methods for the detection of *M. ulcerans* is justified by the gain of time and sensitivity. Compared with the 16S rRNA gene PCR, the IS2404 PCR is faster, less expensive, and easier to perform.

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