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MYCOBACTERIOLOGY AND AEROBIC ACTINOMYCETES

Fluorescent Hybridization of *Mycobacterium leprae* in Skin Samples Collected in Burkina Faso

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ABSTRACT Leprosy is caused by *Mycobacterium leprae*, and it remains underdiagnosed in Burkina Faso. We investigated the use of fluorescent *in situ* hybridization (FISH) for detecting *M. leprae* in 27 skin samples (skin biopsy samples, slit skin samples, and skin lesion swabs) collected from 21 patients from Burkina Faso and three from Côte d'Ivoire who were suspected of having cutaneous leprosy. In all seven Ziehl-Neelsen-positive skin samples (four skin biopsy samples and three skin swabs collected from the same patient), FISH specifically identified *M. leprae*, including one FISH-positive skin biopsy sample that remained negative after testing with PCR targeting the *rpoB* gene and with the GenoType LepraeDR assay. Twenty other skin samples and three negative controls all remained negative for Ziehl-Neelsen staining, FISH, and *rpoB* PCR. These data indicate the usefulness of a microscopic examination of skin samples after FISH for first-line diagnosis of cutaneous leprosy. Accordingly, FISH represents a potentially useful point-of-care test for the diagnosis of cutaneous leprosy.

KEYWORDS fluorescent hybridization, *Mycobacterium*, *Mycobacterium leprae*, leprosy, skin

Leprosy caused by *Mycobacterium leprae* is endemic in some developing countries, including Burkina Faso (192 new cases in 2017), where 31% of newly diagnosed patients present with grade 2 disabilities. Burkina Faso has the highest prevalence in this region of Africa (1), partially due to difficulties in early diagnosis, as routine laboratory diagnosis is limited to the microscopic observation of acid-fast bacilli in skin biopsy and slit-skin samples, which has an acknowledged poor sensitivity of 10^4 bacilli/g of tissue (2). Fluorescence microscopy is known to offer benefits in terms of sensitivity compared to optical microscopy and Ziehl-Neelsen staining (3, 4).

In a previous study, we established that rpoBMTC probe-based fluorescent *in situ* hybridization (FISH) alone or FISH combined with Ziehl-Neelsen staining specifically "FISHes out" *M. tuberculosis* complex mycobacteria in sputum samples (5). Here, we investigated FISH as a confirmatory technique for detecting *M. leprae* in skin biopsy specimens to diagnose leprosy.

MATERIALS AND METHODS

Clinical samples. This prospective, cross-sectional study was authorized by the Hauts Bassins Regional Health Authority (no. 0014, 2017) and the Ethics Committee of the Burkina Faso National Institute of Public Health (no. 23, 2019). After the merits of the study were explained to patients with cutaneous lesions suggestive of leprosy, informed consent was collected before skin sampling was

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TABLE 1 Characteristics of the 24 patients suspected of having cutaneous leprosy[a]

Patient code	Sex	Age (yrs)	Location of the patient	Antileprosy treatment before sampling	Clinical form (WHO criteria)
P1	M	64	Bobo-Dioulasso (BF)	No	PB
P2	M	26	Dogoma (BF)	No	MB
P3	F	57	Bobo-Dioulasso (BF)	No	PB
P4	M	60	Vavoua (CI)	No	MB
P5	M	46	Koundougou (BF)	Yes	PB
P6	F	65	Bobo-Dioulasso (BF)	No	PB
P7	F	20	Karangasso (BF)	No	PB
P8	F	60	Deguelin (BF)	No	PB
P9	F	57	Bobo-Dioulasso (BF)	No	PB
P10	M	30	Dano (BF)	No	PB
P11	M	21	Bobo-Dioulasso (BF)	No	PB
P12	F	29	Bobo-Dioulasso (BF)	No	PB
P13	M	45	Bobo-Dioulasso (BF)	No	PB
P14	M	64	Bobo-Dioulasso (BF)	No	PB
P15	M	24	Bobo-Dioulasso (BF)	No	PB
P16	M	21	Bobo-Dioulasso (BF)	No	PB
P17	M	77	Vavoua (CI)	No	PB
P18	M	15	Boundiali (CI)	No	PB
P19	F	29	Baré (BF)	Yes	PB
P20	F	41	Bobo-Dioulasso (BF)	No	PB
P21	M	20	Bama (BF)	No	MB
P22	F	35	Bobo-Dioulasso (BF)	No	PB
P23	M	28	Bobo-Dioulasso (BF)	No	PB
P24	M	33	Bobo-Dioulasso (BF)	No	PB

[a]F, female; M, male; BF, Burkina Faso; CI, Côte d'Ivoire; MB, multibacillary; PB, paucibacillary.

performed at the Hospital University Centre Souro Sanou of Bobo-Dioulasso, Burkina Faso, by a dermatologist as part of the routine medical investigation of patients. No biopsy specimens and slit skin smears were collated specifically for the present study. Written authorization for the use of photos for scientific or academic purposes was obtained from the patients. Samples were collected in two time periods: the first from January to March 2018 and the second in August 2019. Fifteen males and nine

TABLE 2 *In silico* and experimental tests of the specificity of the Alexa Fluor 555-labeled fluorescent oligonucleotide probe[a]

Test and bacterium	GenBank accession no. or source	*In silico* hybridization (%) or exptl hybridization result[b]
In silico		
Mycobacterium leprae	CP029543.1	100
Mycobacterium lepromatosis	EU203594.2	85
Mycobacterium haemophilum	CP011883.2	65
Mycobacterium tuberculosis	CP023640.1	65
Mycobacterium bovis BCG	CP033311.1	65
Mycobacterium ulcerans	LR135168.1	60
Mycobacterium marinum	CP024190.1	60
Mycobacterium abscessus	CP022234.1	60
Mycobacterium fortuitum	CP014258.1	65
Staphylococcus aureus	LR134267.1	65
Streptococcus pyogenes	AY583221.1	65
Pseudomonas aeruginosa	LR130534.1	60
Total	12	
Experimental specificity		
Mycobacterium ulcerans	Our laboratory	ND
Mycobacterium marinum	Our laboratory	ND
Mycobacterium tuberculosis complex	Our laboratory	ND
Staphylococcus aureus	Our laboratory	ND
Streptococcus pyogenes	Our laboratory	ND
Pseudomonas aeruginosa	Our laboratory	ND
Klebsiella pneumoniae	Our laboratory	ND
Total	7	

[a]5′-CAACTCCTCAGGCAAGTTGA-3′.
[b]ND, hybridization was not detected by fluorescence microscopy.

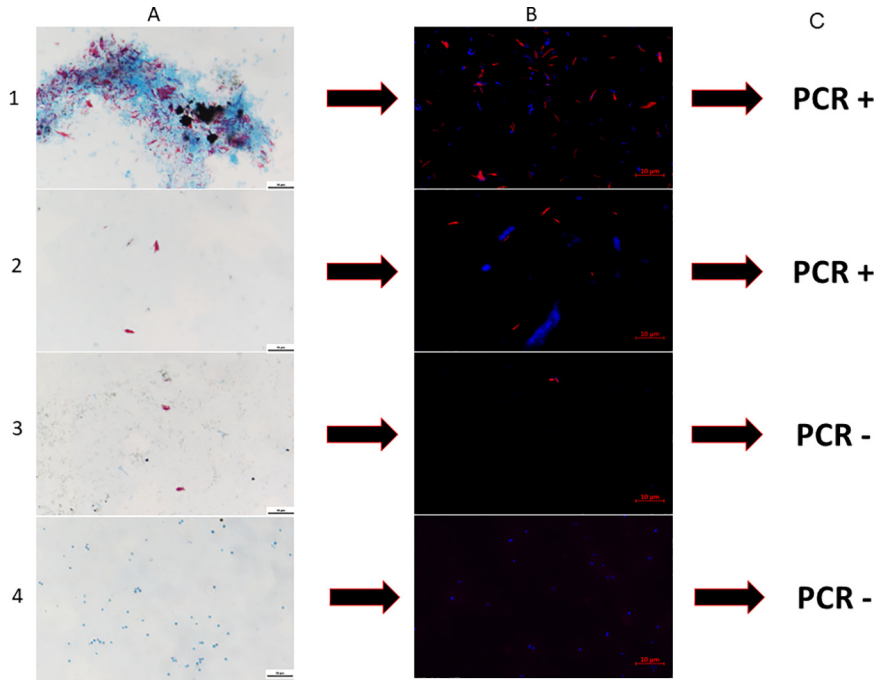

FIG 1 Fluorescent *in situ* hybridization (FISH) of *Mycobacterium leprae* in skin biopsy specimens collected from patients suspected of having cutaneous leprosy in Burkina Faso. Rows 1, 2, and 3 show positive skin biopsy specimens from patients P2, P4, and P5, respectively (Table 1); row 4 shows a negative-control skin biopsy specimen from patient 8111270308 (Table 1). (A) Ziehl-Neelsen staining; (B) FISH-DAPI staining; (C) results of the GenoType LepraeDR test and PCR amplification of the *rpoB* gene. +, positive; −, negative. Bar, 10 μm.

females (age range, 15 to 77 years; mean, 40 ± 18 years) were enrolled. Twenty-one patients, including two with the multibacillary (MB) form of clinical leprosy (more than five lesions), were from Burkina Faso, and three patients, including one with the MB leprosy form, were from Côte d'Ivoire (Table 1). Skin biopsy specimens, slit-skin samples, and skin lesion swabs were affixed to slides that had previously been disinfected with 70% alcohol, as previously described (6). A first Ziehl-Neelsen heat stain performed at the Hospital University Centre Souro Sanou mycobacteriology laboratory for diagnostic purposes was repeated at the Institut Hospitalier Universitaire Méditerranée Infection, Marseille, France, using the Quick-TB kit (cold staining) (RAL Diagnostics, Martillac, France).

Molecular analyses. DNA was extracted through a combination of chemical lysis (Qiagen, Hilden, Germany), treatment with glass powder, heating at 56°C for 2 h, sonication for 30 min, and automatic elution with an EZ1 DNA tissue kit (Qiagen, Hilden, Germany). The extracted DNA was used for partial PCR amplification and sequencing of the *rpoB* gene, as previously described (7). In these experiments, sterile water was used as the negative control, and the beta-actin gene was amplified to assess the quality of DNA extraction. The GenoType LepraeDR test (Hain Lifescience GmbH, Nehren, Germany) was performed in the Laboratory of Bacteriology of Hospital Arnaud de Villeneuve in Montpellier, France, for the molecular detection of *M. leprae* resistant to antileprosy drugs (8).

FISH analysis. SVARAP software (9) was used for the *in silico* design of a *M. leprae rpoB* gene probe after alignment of 100 *rpoB* sequences from different mycobacteria available in GenBank with MEGA-X (www.megasoftware.net/dload_win_gui). A highly conserved region in *M. leprae* was used to design a probe with Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/). We verified that the nucleotide sequence of the designed probe did not target regions harboring missense mutations for rifampin resistance, as previously described (10). The specificity of the probe was ensured *in silico* by conducting a BLAST search of the probe sequence against the GenBank database and aligning it with 12 bacterial genomes (Table 2). Then, the specificity of this Alexa Fluor 555-labeled fluorescent oligonucleotide probe (5′-CAACTCCTCAGGCAAGTTGA-3′) was experimentally tested in a series of microorganisms known to be associated with chronic cutaneous lesions in West African patients, including *Mycobacterium ulcerans*, *Mycobacterium marinum*, *Mycobacterium tuberculosis* complex, *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Klebsiella pneumoniae*. The FISH detection of *M. leprae* in the skin samples was performed using previously described methods (5). Briefly, smears were fixed with 4% paraformaldehyde and treated with 10 mg/ml lysozyme and 10 μg/ml proteinase K. After an overnight incubation with a 10-μl suspension containing the specific probe, smears were serially washed and mounted with ProLong Diamond Antifade containing 4,6-diamidino-2-phenylindole (DAPI) (Fisher Scientific, Illkirch, France). Microscopic observations using the 100× lens objective of a Leica DMI3000 microscope (Leica Microsystèmes, Nanterre, France) were confirmed using a Zeiss LSM 800 confocal microscope (with a 63×, 1.4-numerical-aperture [NA] oil immersion objective and a 568-nm excitation laser) (Zeiss, Marly-le-Roi,

TABLE 3 Results of the laboratory investigation of skin samples[a]

Patient code	Sample	Result of:			GenoType LepraeDR result			
		Ziehl-Neelsen microscopy (BI)	Fluorescent microscopy	PCR for *rpoB*	Detection of *M. leprae*	Rifampin resistance	Dapsone resistance	Ofloxacin resistance
P1	Skin biopsy	Neg	Neg	Neg	NA	NA	NA	NA
P2	Skin biopsy	Pos (4+)	Pos	ML	ML	Sensitive	Sensitive	Sensitive
P3	Skin biopsy	Neg	Neg	Neg	NA	NA	NA	NA
P4	Skin biopsy	Pos (3+)	Pos	ML	ML	Sensitive	Sensitive	Sensitive
P5	Skin biopsy	Pos (1+)	Pos	Neg	Neg	NA	NA	NA
P6	Skin biopsy	Neg	Neg	Neg	NA	NA	NA	NA
P7	Skin biopsy	Neg	Neg	Neg	NA	NA	NA	NA
P8	Skin biopsy	Neg	Neg	Neg	NA	NA	NA	NA
P9	Skin biopsy	Neg	Neg	Neg	NA	NA	NA	NA
P10	Skin biopsy	Neg	Neg	Neg	NA	NA	NA	NA
P11	Skin biopsy	Neg	Neg	Neg	NA	NA	NA	NA
P12	Skin biopsy	Neg	Neg	Neg	NA	NA	NA	NA
P13	Skin biopsy	Neg	Neg	Neg	NA	NA	NA	NA
P14	Skin biopsy	Neg	Neg	Neg	NA	NA	NA	NA
P15	Slit skin	Neg	Neg	Neg	NA	NA	NA	NA
P16	Slit skin	Neg	Neg	Neg	NA	NA	NA	NA
P17	Slit skin	Neg	Neg	Neg	NA	NA	NA	NA
P18	Slit skin	Neg	Neg	Neg	NA	NA	NA	NA
P19	Slit skin	Neg	Neg	Neg	NA	NA	NA	NA
P20	Slit skin	Neg	Neg	Neg	NA	NA	NA	NA
P21	Skin biopsy	Pos (5+)	Pos	ML	NT	NT	NT	NT
	Skin swab (3)	Pos (3+)	Pos	ML	NT	NT	NT	NT
P22	Swab of ulcerative skin	Neg	Neg	Neg	NA	NA	NA	NA
P23	Swab of ulcerative skin	Neg	Neg	Neg	NA	NA	NA	NA
P24	Skin biopsy	Neg	Neg	Neg	NA	NA	NA	NA
8111270308*	Skin biopsy	Neg	Neg	Neg	NA	NA	NA	NA
8121367852*	Skin biopsy	Neg	Neg	Neg	NA	NA	NA	NA
9011404509*	Skin biopsy	Neg	Neg	Neg	NA	NA	NA	NA

[a]NA, not applicable; Neg, negative; Pos, positive; *, negative-control sample collected from patient admitted to the Institut Hospitalier Universitaire Méditerranée Infection, Marseille, France, and presenting skin lesions devoid of any evidence of mycobacteria; NT, not tested; 3, three swabs collected from three types of skin lesions (skin wound, scaly skin evoking cutaneous leishmaniasis, and skin papules with raised extremities) in patient P21; BI, bacterial index determined using Ridley's logarithmic scale (22).

France). Images were acquired using ZEN software (Zeiss). Three skin biopsy specimens devoid of any evidence of mycobacteria were included as negative controls in Ziehl-Neelsen staining and FISH experiments.

RESULTS

Four skin biopsy specimens and three skin lesion swabs (collected from the same patient, P21) exhibited acid-fast bacilli, as confirmed by two independent rounds of Ziehl-Neelsen staining, in the presence of three negative controls that remained free of acid-fast bacilli. After we ensured the *in silico* and experimental specificity of the *rpoB* gene sequence-based FISH probe, FISH highlighted Ziehl-Neelsen-positive mycobacteria as red-fluorescent bacilli (Fig. 1) in these four skin biopsy specimens and the three skin lesion swab smears by both fluorescence microscopy and confocal microscopy, whereas no FISH-positive mycobacteria were detected in the remaining samples. Two of the four FISH-positive skin biopsy specimens were further confirmed to contain *M. leprae* based on the positive results of the GenoType LepraeDR assay, which indicated the absence of detectable antileprosy drug resistance. Three of four FISH-positive skin biopsy specimens were also positive for the *rpoB* gene in the PCR assay, and sequencing indicated 99% gene sequence similarity with the reference *M. leprae* sequence (GenBank accession no. CP029543.1). The fourth (P5) skin biopsy specimen remained negative for *M. leprae*, despite positivity for the beta-actin gene (in all skin samples) and the positivity of Ziehl-Neelsen staining and FISH. In these experiments, the three negative controls remained negative for Ziehl-Neelsen staining, FISH, and *rpoB*-PCR. The three FISH-positive skin lesion swabs were also positive for the *rpoB* gene in the PCR assay, and sequencing confirmed the presence of *M. leprae* (Table 3).

DISCUSSION

We report the utility of FISH for determining the diagnosis of cutaneous leprosy when applied to smears prepared from skin samples collected from patients suspected of having cutaneous leprosy. Indeed, the observations reported here were authenticated by the negativity of negative controls analyzed in the experiments, the agreement of observations obtained using different techniques, and the reproducibility of Ziehl-Neelsen staining. The report confirms the usefulness of microscopic examination of skin sample smears after FISH for the diagnosis of cutaneous leprosy, as only two similar reports have been published previously (11, 12). FISH is increasing the specificity of microscopy in a context where other cutaneous mycobacterioses prevail, such as in Burkina Faso (13). In addition, we observed one FISH-positive sample with a clinical paucibacillary leprosy form that remained negative for the detection of *M. leprae* DNA using two different molecular assays. This discrepancy might be explained by several factors, including the small size of the tissue section extracted, which contained a low number of bacilli (14). DNA amplification inhibitors from human surgical tissue also may explain the false-negative PCR result (15). Another potential explanation is the low sensitivity of the PCR techniques targeting a single copy of a gene in the genome (16–18) in a paucibacillary specimen (19).

We propose that FISH is an efficient method for the first-line diagnosis of cutaneous leprosy. FISH provides greater specificity than Ziehl-Neelsen staining, and a single observation is sufficient for the precise diagnosis of leprosy. Based on a previous estimate of a cost of $5 (U.S.) per FISH test (20), we anticipate the implementation of FISH as a new diagnosis tool for point-of-care testing in one of the Burkina Faso health care centers that is already using fluorescence microscopy, including the possibility of a remote interpretation of smartphone photos (21).

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We have no conflicts of interest to declare.

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