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Mycobacterium ulcerans infection in a child from Angola: diagnosis by direct detection and culture

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Summary Buruli ulcer, caused by *Mycobacterium ulcerans*, is a chronic ulcerative skin disease, found predominantly in central and west Africa and Australia. A boy of 2.5 years of age from Angola was admitted to our hospital with severe kwashiokor and a large ulcer with undermined edges on the left side of the thorax. Further examination revealed anaemia, hypoproteinaemia, bacterial superinfection of the ulcer and intestinal parasites. Histological analysis showed acid-fast bacilli and histopathological changes typical of Buruli ulcer. *M. ulcerans* was detected by PCR and culture. The patient was treated by surgical excision of diseased skin, followed by split-skin grafting. He also received antibiotic therapy (ciprofloxacin, clarithromycin, rifabutin, and dapsone). After six months, the child was discharged from hospital in good condition. This is the first published case of Buruli ulcer from Angola.

keywords Buruli ulcer, Mycobacterium ulcerans, Angola

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Introduction

Mycobacterium ulcerans infections (Buruli ulcer) represent, after tuberculosis and leprosy, the third most common mycobacterial disease of nonimmunocompromised humans (Portaels 1995; Meyers *et al*. 1996). *M. ulcerans* infections have been observed in many tropical areas but most patients have come from central and west Africa and Australia, (Portaels 1989; Hayman 1991). *M. ulcerans* has been isolated from humans and koalas. Epidemiologically, the disease is associated with riverine and swampy terrain (Portaels 1995) and *M. ulcerans* has been isolated from water samples recently (Roberts & Hirst 1997).

Despite the high prevalence of the disease, bacteriological diagnosis is often a problem. *M. ulcerans* infection is frequently diagnosed on histopathological changes and clinical findings, without isolation of the aetiological agent (Meyers *et al*. 1996). In this severely ill patient, we attempted to establish the aetiological diagnosis early by the simultaneous use of histopathological, microbiological and molecular biological methods.

Materials and methods

Case report

The patient, a boy from Caxito (Bengo Province, Angola) was born in August 1993, and admitted to Carl-Thiem-Klinik, Cottbus (Germany), on 21 December 1995. The child presented with severe kwashiokor and a 20 × 25 cm ulcer with undermined edges on the left side of his thorax. The ulcer was clinically compatible with active *M. ulcerans* infection (Figure 1).

Laboratory examination revealed anaemia, hypoproteinaemia, elevated C-reactive protein, and leucocytosis. The patient also had *Hymenolepis nana*, *Trichiuris trichiura* and *Giardia lamblia* infections and otitis media. The ulcer was heavily superinfected with *Pseudomonas aeruginosa*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*.

On day 6 all necrotic tissue was removed, and the child received antibiotics in various combinations according to the

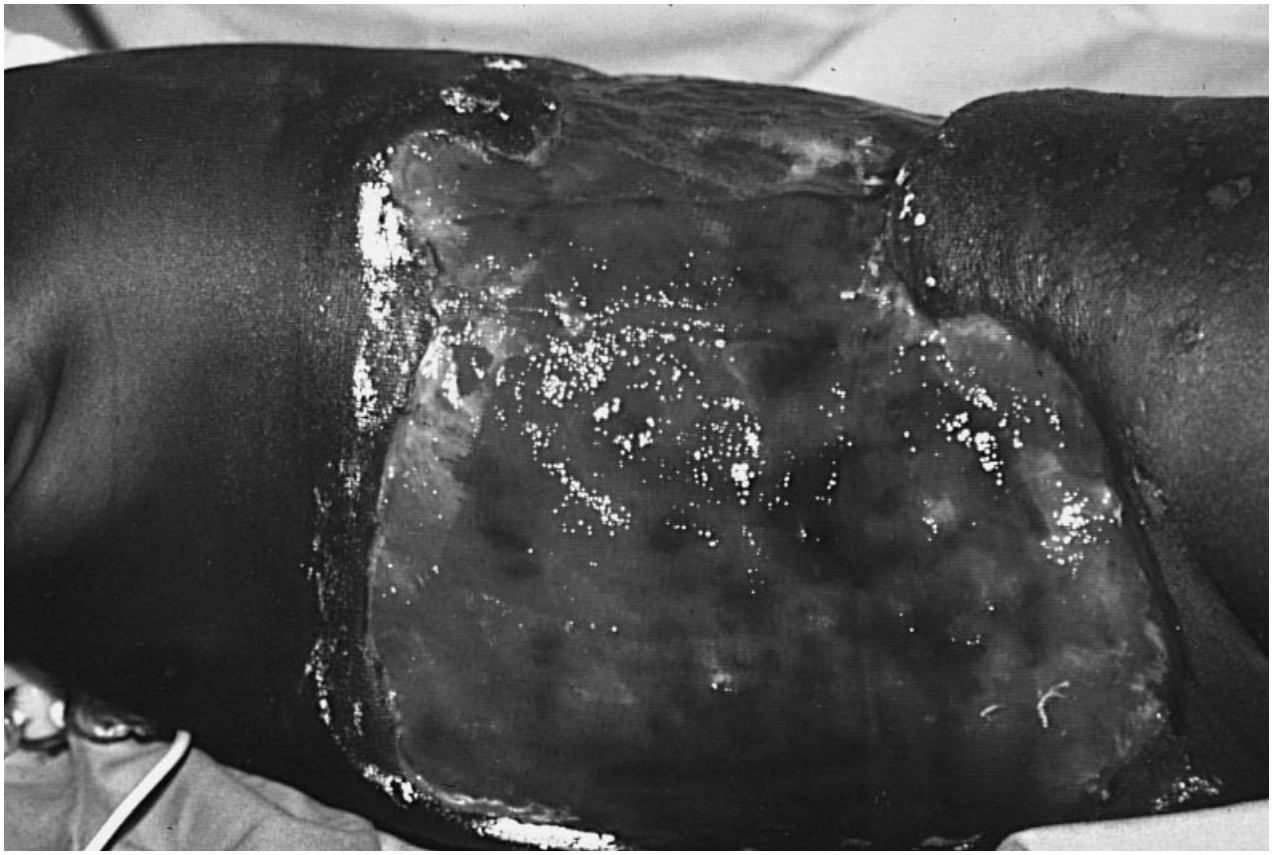

Figure 1 Presentation of the patient on admission with a large ulcer on the left side of this thorax. Note the undermined edges of the ulcer.

bacteriological findings. Autologous split-skin grafting (Meshgrafts) was performed on days 27, 69, 80 and 128 (Table 1). On each of these days, fragments of necrotic tissue were taken for bacteriological, histopathological and molecular biological analyses. Antimycobacterial treatment was started on day 58 with ciprofloxacin (2 × 125 mg p.o.); on day 70, clarithromycin (2 × 125 mg p.o.) and rifabutin (50 mg/daily p.o.) were added. On day 92 ciprofloxacin and clarithromycin were replaced by dapsone (1 × 50 mg p.o.). Dapsone was stopped on day 167 and rifabutin was stopped on day 189. The child was hospitalized for 190 days.

Collection of specimens

Eleven fragments of necrotic tissue were collected (Table 1). Each specimen was processed directly, or (sampling day 69, 80 and 128) placed in 2 ml modified Dubos medium (Saxegaard 1985), transported at ambient temperature, and maintained at 4 °C until analysis. Total elapsed time between collection and analysis did not exceed two weeks. For PCR analysis fragments of tissue were placed in 70% ethanol. Portions of tissue excised on days 6 and 80 were fixed in 10% formalin for histological examination. The tissues were processed routinely, blocked in paraffin, sectioned at 0.004 mm, and stained by haematoxylin-eosin, Ziehl-Neelsen (ZN), Fite-Faraco (FF), Gomori methenamine-silver, and Brown-Hopps methods.

Culture of mycobacteria

Each specimen of tissue was minced and homogenized in saline with a mortar and pestle, and processed by three different methods.

Method I

Homogenized suspensions were decontaminated by the N-acetyl-L-cysteine- NaOH-method (NALC; ASM-Manual) and the resulting sediment used for smear examination (ZN staining) and inoculated onto Löwenstein-Jensen, Middlebrook 7H10 with 0.1 ml mycobactin J and Stonebrink media, and in BACTEC 12 B-vials (0.5 ml on each medium) (Palomino & Portaels 1998). The rest of the sediment was stored at −57°C for nucleic acid amplification.

Table 1 Detection and Identification of *M. ulcerans* in skin biopsies

Date of collection	*Ziehl-Neelsen stain*	*Mycobacterial Culture*			*Direct Detection* PCR and OSCPH	*Histology*
		Liquid culture (BACTEC)	LJ and Ogawa with Mycobactin	LJ and Ogawa		
Day 6	2+	cont		negative		
	(1)*					c/w *M. ulcerans*
		NALC		NALC	N.T.	infection
Day 27	3+	cont		*M. ulcerans***		
				(12 weeks)	N.T.	N.T.
	(1)*	NALC		OA		
Day 69	1+–4+	+	+	+	*M. ulcerans/*	
		(7 weeks)**	(8 weeks)	(12 weeks)	*marinum*	N.T.
	(3)*	NALC	HC1	OA/NALC		
Day 80	1+–4+	negative	negative	+	*M. ulcerans/*	c/w *M. ulcerans*
				(13 weeks)	*marinum*	infection
	(3)*	NALC	HC1	NALC		
Day 128	negative	negative	negative	negative	negative	
						N.T.
	(3)*	NALC	HC1	HC1, OA, NALC		

*Number of samples tested; **Positive after weeks; ***Ogawa not tested; cont, contaminated; N.T., not tested; c/w, compatible with; Decontamination procedure: OA, Oxalic acid; NALC, N-acetyl-L-cysteine; HCL, Hydrochloric acid.

Method II

The homogenized suspensions were decontamined with 0.5 N HCl (final concentration) for 20 min at room temperature followed by neutralization with 1 N NaOH. After centrifugation, the sediment was examinated by ZN staining, and inoculated onto Löwenstein-Jensen, 1% Ogawa egg yolk medium and Ogawa supplemented with mycobactin J (Portaels *et al*. 1988). All inoculated media were incubated in parallel at 28 °C, 33°C and 37 °C for at least 4 months.

Method III

The homogenized suspensions were decontaminated with oxalic acid (ASM-Manual) and the resulting sediment processed as in Method I.

Identification of isolates

Mycobacterial isolates were identified as previously described (Vincent Lévy-Frébault & Portaels 1992; Portaels *et al*. 1996).

PCR analysis

Extraction method

DNA was extracted from the suspensions using a previously described method (Dumonceau *et al*. 1995). Briefly, each tissue (1–3 mm3) was rinsed with UV-treated distilled water, minced with a scalpel, and resuspended in 1 ml TE-SDS (SDS 0,5% Tris 10 mM, EDTA 1 mM). One half of each suspension (500 µl) was extracted as follows: 40 U proteinase K (20 mg/ml solution) was added to the suspension and incubated overnight at 65 °C, then treated for 5 min at room temperature in a waterbath-sonicator, 47 kHz (Branson 1200, Branson Ultrasonics Corporation, Danbury, Connecticut, USA). These suspensions were placed in a boiling water bath for 20 min and then extracted twice with phenol/chloroform. DNA was precipitated overnight at −20 °C with 10% v/v. sodium acetate 3 M (pH 5.2) and 2 volumes of 70% ethanol, and centrifuged at 10000 g at 4 °C for 30 min. The sediment was washed with 70% ethanol, dried and resuspended in 50 µl TE.

For sequencing, the DNA was extracted from the tissue as described above with minor modifications: the lysis buffer contained 50 mM TRIS (pH 7.5), 1% SDS and 1 mg/ml proteinase K. The samples were incubated for 3–4 h at 37 °C and additionally heat/cold shocks were performed four times. After precipitation and washing the dried DNA was resuspended in 10 mM TRIS.

Mycobacterial DNA was amplified by a previously described nested PCR procedure, based on the amplification of the DNA coding for the ribosomal 16S rRNA (Richter *et al*. 1996). Species identification was achieved by sequencing the PCR product.

Detection and identification of mycobacteria by PCR-OSCPH

Detection of mycobacteria was based on the amplification of a partial sequence in the 16S rRNA gene at the 5′ side of the noncoding RNA-like strand. DNA was amplified by a nested PCR specific for the genus Mycobacterium (De Beenhouwer *et al*. 1995). Identification of the amplified DNA was performed by OSCPH (Oligonucleotide-specific capture plate hybridization) as previously described (Portaels *et al*. 1997).

Identification of mycobacteria by DNA sequencing

The amplified fragments were sequenced on a fluorescent DNA sequencer (ABI PRISM 377 DNA sequencer, Perkin Elmer, Weiterstadt, Germany). PCR products were purified with the QIA quick spin PCR purification kit (Diagen, Hilden, Germany) to remove excess primers and nucleotides. Three to five microliters of this preparation were used for cycle sequencing. Cycle sequencing of 300 bp (coordinates 77–377 according to *E. coli* 16S rRNA (Brosius *et al*. 1978) was performed with the RR DyeDeoxy Terminator cycle sequencing kit (Perkin Elmer). The sequencing primer was one of the primers used for the nested PCR (name and location according to Richter *et al*. 1996).

Results

Histopathological analyses

Histopathological analyses (Figures 2 and 3) revealed ulceration of the epidermis with partial re-epithelialization of the margin of the ulcer. The dermis and subcutaneous tissue showed extensive contiguous coagulation necrosis with marked thickening of the interlobular septae in the panniculus. Fat cells appeared nonviable. The walls of many blood vessels were necrotic, and the lumina were often occluded. Mineralization was prominent in the necrotic areas. Multiple ZN and FF stained sections did not reveal acid-fast bacilli. No other aetiological agents were seen. In a few foci there were Langhans' giant cells and a few epitheloid cells, but no organized granulomas. These histopathological changes are typical of *M. ulcerans* infection.

Culture results

A total of 21 biopsies were investigated between day 6 and day 128. As shown in Table 1, *M. ulcerans* was cultured on solid media from specimens collected on days 27, 69 and 80

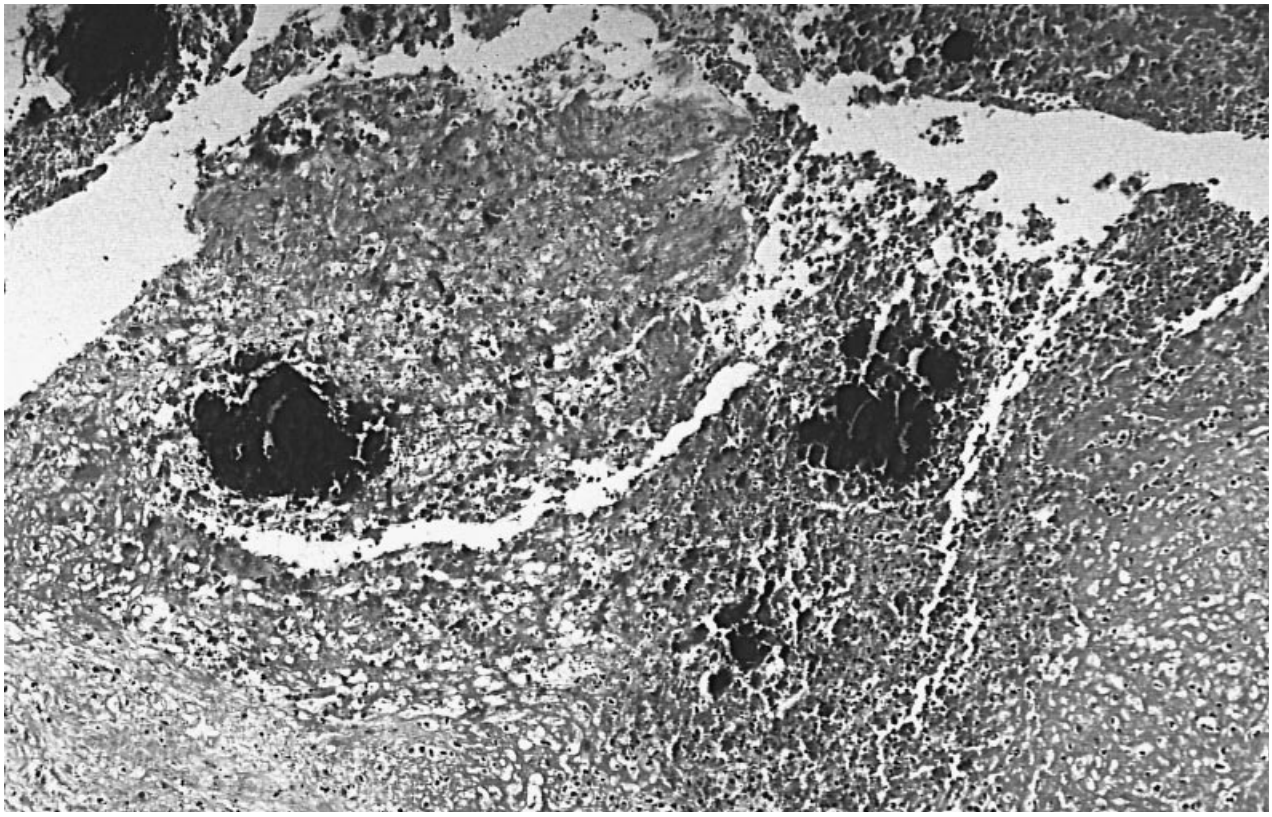

Figure 2 *Mycobacterium ulcerans* infection in the skin of the left hemithorax. There is marked contiguous coagulation necrosis with relatively few inflammatory cells. Dark foci represent dense mineralization. (H & E stain, × 75)

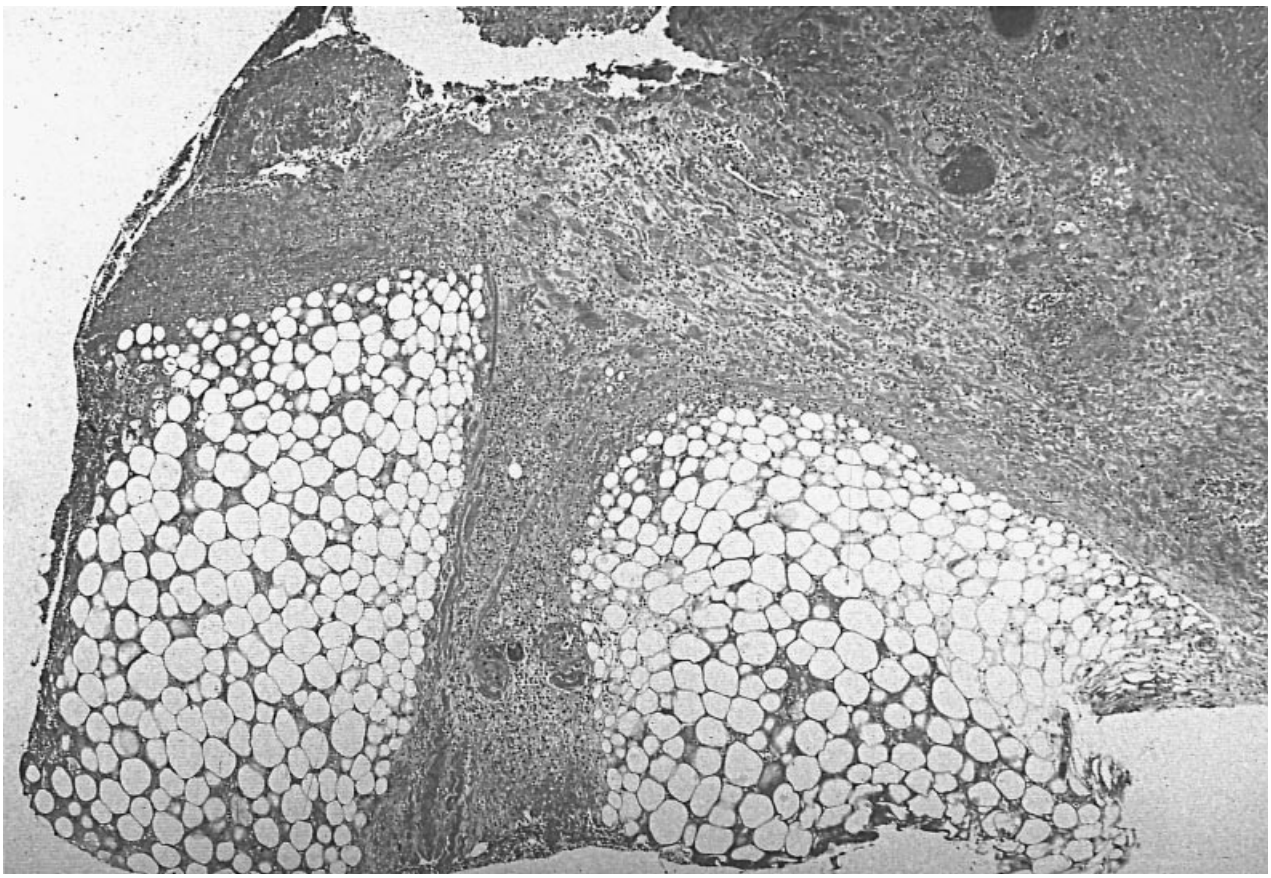

Figure 3 *Mycobacterium ulcerans* infection in skin of left hemithorax, showing fat cell 'ghosts' and thickened necrotic interlobular septum in the panniculus. (Fite-Faraco stain, × 30)

(after 8–20 weeks of incubation at 33 °C) and in liquid media for the specimen taken on day 69 (after 7 weeks of incubation at 28 °C). Decontamination with HCl (Method II) and culture on LJ or Ogawa medium supplemented with mycobactin gave the best results.

Growth was observed only at 28 °C and 33 °C incubation. No other mycobacteria were cultured from the patient. The phenotypic characteristics of the isolate from Angola are similar to those of *M. ulcerans* strains from Africa (Table 2).

Detection and identification of mycobacteria by PCR

As shown in Table 1, PCR was positive in biopsies taken on day 69 and 80; however, no mycobacterial DNA could be detected in the follow-up investigations.Partial sequence of the PCR-product was identical to the corresponding sequence of *M. ulcerans* and *M. marinum*, which cannot be discriminated by 16 S rRNA. Sequenzing of organisms cultured on LJ medium gave the same results. OSCPH hybridization with the *M. ulcerans-marinum* probe was positive for the specimens collected on day 69 and 80, and negative for the specimens collected on day 128.

Discussion

Many *M. ulcerans* infections have been described in west and central Africa (for reviews see Portaels 1989; Portaels 1995; Meyers 1995), but no isolates of *M. ulcerans* have ever been reported from Angola. The presence of the disease in Angola was mentioned by Barker (1973), Connor *et al*. (1976) and Ziefer *et al*. 1981). The authors based their statements on observations of WM Meyers, who, while working in Lower Zaire, saw Buruli ulcer in newly arrived patients from Angola beginning in 1965. These observations were never published. Thus, this is the first published case with Buruli ulcer and bacteriological confirmation from Angola. Since the diagnosis of this boy, more patients from Angola have been identified with lesions characteristic of *M. ulcerans* infection (W. Bär, personal observation).

Table 2 Phenotypic characteristics of *M. ulcerans* strains from Africa and from the isolates from Angola.

	Origin of strains	
	Africa*	Angola
Pigmentation in the dark	−	−
Pigmentation in the light	−	−
Growth at 37 °C	−	−
Growth on peptone agar	−	−
Growth in presence of:		
Isoniazid (10 μg/ml)	M	−
Thiophene – 2 – carboxylic hydrazide	+	+
Hydroxylamine (250 μg/ml)	M	−
p-Nitrobenzoate (500 μg/ml)	M	−
NaCl 5%	−	−
Enzymatic properties		
Catalase, > 45 mm of foam	−	−
Tween 80 hydrolysis (10 days)	−	−
Urease activity	F	−
Niacin production	F	−
Nitrate reduction	−	−
Acid phosphatase activity	M	+
Mycolate types		
Alpha – Mycolates	+	−
Alpha′– Mycolates	−	−
Methoxymycolates	+	+
Ketomycolates	+	+
Omega-Carboxymycolate	−	−

*Data from Portaels *et al.* (1996); +, > 85% of the strains were positive; − , < 15% of the strains were positive; M, 50–85% of strains positive; F, 15–49% of strains positive.

There was excellent concordance between the results obtained by culture and PCR. Indeed, the culture was positive in samples taken on day 27 to day 80. Decontamination by HCl seems preferable to that by NALC. When HCl was used, positive cultures appeared on solid media after 8 weeks (on day 69), while bacterial growth appeared only after 12–13 weeks following decontamination by NALC or by oxalic acid (see Table 1). Palomino & Portaels (1998) observed the high sensitivity of *M. ulcerans* to decontamination methods, especially to 1N NaOH (used in NALC). On the other hand it is known that decontamination by oxalic acid is efficient in destroying nonmycobacterial contaminants but can also kill mycobacteria. Portaels *et al.* (1988) have shown that soil samples decontaminated by HCl yielded more positive mycobacterial cultures than those treated with oxalic acid. The best results are obtained with BACTEC after NALC decontamination (growth after 7 weeks) and on solid medium after HCl decontamination (growth after 8 weeks).

Phenotypic analysis of cultures and PCR analysis of the 3′ end both confirm that the isolates are *M. ulcerans* and not M. marinum and that they belong to the African subgroup recently described by Portaels *et al.* (1996) (Table 2).

We also intended to determine the antibiotic susceptibility of the isolate. For that purpose, the strain was incubated at 31 °C and 37 °C, with liquid (BACTEC) and solid media (Löwenstein-Jensen), containing tuberculostatic substances. However, due to the slow growing nature of the isolate, antibiotic resistance was not evaluable. This phenomenon has been reported previously (Woods & Washington 1987).

Currently the recommended treatment of Buruli ulcer is surgery in combination with antimycobacterial therapy (Goutzamanis. & Gilbert 1995; Marston *et al.* 1995; Aguiar & Stenou 1997). Results of chemotherapy alone are disappointing, (Marston *et al.* 1995). Our experience was similar. Transplanted skin grew well in the centre of the ulcer, but because of persisting foci of *M. ulcerans* infections at the edges, the new skin became necrotic. Skin grafting at these sites was successful only after debridement of these foci and antimycobacterial treatment. The antimycobacterial regimen consisted of ciprofloxacin, rifampicin, dapsone and clarithromycin. This was partially based on the report of Goutzamanis & Gilbert 1995) suggesting that rifampicin and dapsone were effective against *M. ulcerans*, and on the

synergistic antimycobacterial activity between clarithromycin and rifampicin against atypical mycobacteria as shown by Mor *et al*. (1994). Ciprofloxacin alone may have only weak activity against *M. ulcerans*, because after 22 days of treatment, the ulcer remained positive with *M. ulcerans*.

Because of the location of the ulcer on the thorax, we did not attempt adjunct thermotherapy as proposed previously (Meyers *et al*. 1974; Goutzamanis & Gilbert 1995). In conclusion, our report confirms the existence of a focus of Buruli ulcer in Bengo province in Angola; however, surveys need to be conducted to determine the prevalence of the disease in south-west Africa.

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