Am. J. Trop. Med. Hyg., 64(5, 6), 2001, pp. 270–273

THE USE OF IS*2404* RESTRICTION FRAGMENT LENGTH POLYMORPHISMS SUGGESTS THE DIVERSITY OF *MYCOBACTERIUM ULCERANS* FROM DIFFERENT GEOGRAPHICAL AREAS

KARIM CHEMLAL, KARIN DE RIDDER, PIERRE-ALAIN FONTEYNE, WAYNE M. MEYERS, JEAN SWINGS, AND F. PORTAELS

Department of Microbiology, Division of Mycobacteriology, Institute of Tropical Medicine, Antwerp, Belgium; Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium; Armed Forces Institute of Pathology, Washington, District of Columbia

Abstract. Buruli ulcer, caused by *Mycobacterium ulcerans,* has been reported in five continents: Africa, Asia, Australia, and North and South America. In the present study, restriction fragment length polymorphism with the recently described *M. ulcerans* specific insertion sequence IS*2404* as a probe, was applied to *Mycobacterium shinshuense, Mycobacterium marinum,* and 14 clinical *M. ulcerans* isolates originating from six geographic areas: Africa (n = 6), Australia (n = 2), Mexico (n = 1), south Asia (n = 2), Asia (n = 1), and South America (n = 2). Using this probe, six subtypes of *M. ulcerans,* related to the six geographic origins of the isolates were distinguished, confirming that *M. ulcerans* can be divided into subgroups corresponding to different geographic variants of the same species.

INTRODUCTION

Mycobacterium ulcerans infection, or Buruli ulcer (BU), is the third most common mycobacterial disease of humans.[1] Infections with *M.ulcerans* classically present as advancing necrotizing skin ulcers, but other clinical forms are becoming increasingly common (Portaels F and others, unpublished data). Only in very few cases do lesions respond favorably to antimicrobial therapy,[2] making wide surgical excision and skin grafting the treatment of choice.

Mycobacterium ulcerans was first described in Bairnsdale Australia, in 1948,[3] and was subsequently reported in Africa, the Americas, southeast Asia, and the central Pacific.[4] Recent reports mention increased incidence of BU in Benin,[5] Australia,[6] and Côte d'Ivoire.[7] These observations have revived interest in the microbiology of *M. ulcerans*.

The epidemiology of BU is poorly understood, but most foci are associated with slow-flowing or stagnant water. Recent studies using polymerase chain reaction (PCR) have identified *M. ulcerans* in water samples from Phillip Island, Australia.[8] Portaels and others[9] have implicated aquatic insects in the transmission of *M. ulcerans* infection; however, after continuous attempts, *M. ulcerans* has never been isolated from the environment.

Information on the molecular characterization of *M. ulcerans* is scarce. Jackson and others[10] used pTBN12 as a probe to differentiate *M. ulcerans* strains on the basis of restriction fragment length polymorphism. Portaels and others[11] demonstrated that variability in the 3′ end of 16S rRNA sequence of *M. ulcerans* is related to the geographic origin of the isolates. Stinear and others[12] detected a specific *M. ulcerans* insertion sequence: IS*2404* with more than 50 copies per genome. Primers based on this repetitive DNA sequence allow detection of *M. ulcerans* in clinical samples with high sensitivity and specificity.[12, 13]

In the current study we investigated the usefulness of restriction fragment polymorphism based on the IS*2404* probe to differentiate *M. ulcerans* strains coming from different geographic areas.

MATERIALS AND METHODS

Strains. Fourteen isolates of *M. ulcerans,* including the reference strain ATCC 19423, one *Mycobacterium shinshuense* (ATCC 33728) and one *Mycobacterium marinum* are described in Table 1. The six African strains were isolated at the Institute of Tropical Medicine (ITM) from tissue fragments of patients. Two strains were from Australia, one from Mexico and one *Mycobacterium* sp. strain ITM842, phenotypically related to *M. ulcerans,* was obtained from a granulomatous lesion of a patient from Surinam living in the Netherlands (unpublished data). The strain from Guyana was contributed by the Institut Pasteur, Paris. The *M. shinshuense* strain, isolated from a skin ulcer of a Japanese patient, is phenotypically related to *M. ulcerans.*[14] The strain from China was isolated by Faber and others from a patient with an ulcer of the skin.[15] Two strains from Malaysia and Papua New Guinea were isolated in Australia. The only *M. marinum* strain in the study was isolated at ITM from an axolotl salamander in the Antwerp Zoo.

Preparation of the probe. Probe IS*2404* was prepared by chemical labeling of a polymerase chain reaction (PCR) product as described in the protocol of Van Embden and others for the preparation of the IS6110 probe.[16]

PCR. The primers used were: PGP3 (5′-GGCGCAGATCAACTTCGCGGT-3′, positions 547 to 563 in the sequence U38540) and PGP4 (5′-CTGCGTGGTGCTTTACGCGC-3′, position 764 to 745). The *M. ulcerans* lysate was obtained by re-suspending a loopful of mycobacteria in 100 μl of TE (10 mM Tris, 1 mM EDTA [pH 8]) containing 1% (vol/vol) Triton X-100, and heating at 100°C for 15 min. Ten microliters of lysate was added to 50 μl PCR reaction mixture containing 50 pmol of each primer (PGP3 and PGP4), 1 U of Ampli Taq DNA polymerase (Roche Molecular System, Belgium), 200 μM concentrations of each deoxyribonucleotide triphosphate, 1.5 mM MgCl2, 0,1% TritonX-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 30 cycles at 94°C for 45 sec, 64°C for 45 sec, 72°C for 45 sec, and a final extension at 72°C for 7 min. Five microliters of amplified DNA was electrophoresed through a 2% agarose gel; bands were detected by ethidium bromide staining and UV transillumination.

Probe cloning. The PCR product (219 base pairs [bp]) was then purified (Wizard PCR preps, Promega, Madison,

TABLE 1
Source and origin of mycobacterial strains

ITM no.	Species identification	Origin	Source
96-657	*Mycobacterium ulcerans*	Angola	ITM
94-511	*M. ulcerans*	Ivory Coast	ITM
5151	*M. ulcerans*	Congo	ITM
97-483	*M. ulcerans*	Ghana	ITM
97-680	*M. ulcerans*	Togo	ITM
97-104	*M. ulcerans*	Benin	ITM
5147	*M. ulcerans*	Australia	J. L. Stanford
5142	*M. ulcerans*	Australia	ATCC 19423
94-1328	*M. ulcerans*	Malaysia	K. Jackson no. 186510
94-1331	*M. ulcerans*	Papua New Guinea	K. Jackson no. 186395
8756	*Mycobacterium shinshuense*	Japan	ATCC 33728
98-912	*M. ulcerans*	China	W. R. Faber
842	*Mycobacterium* sp.	Surinam	P. H. J. van Keulen, strain 701357
7922	*M. ulcerans*	French Guyana	V. Vincent IPP IPT 141090018
5114	*M. ulcerans*	Mexico	P. Lavalle
97-1320	*Mycobacterium marinum*	Belgium	ITM

* IPP = Institut Pasteur de Paris.
ITM = Institute of Tropical Medicine, Antwerp, Belgium.

WI) ligated in a plasmid vector (pCR-XL-TOPO) of TOPO XL PCR Cloning Kit (Invitrogen Corp, Belgium) and transformed into TOP10 *Escherichia coli* cells. The DNA of transformed colonies was purified using the High Pure Plasmid Isolation Kit (Boerhinger Mannheim, Germany).

Probe labeling. The probe was labeled using the ECL system (Amersham Life Science, Belgium) which involves direct labeling of probe DNA with the enzyme horseradish peroxidase (HRP).[16]

Southern blot analysis. *Mycobacterium ulcerans* genomic DNA was digested with the appropriate restriction enzyme (PvuII) (Roche, Belgium) and separated overnight by electrophoresis in a 0.8% agarose gel.[16] DNA was transferred to Hybond N+ nylon membrane (Amersham) over a 1 hour period in 0.4 M NaOH using a Vacuum Blotter system (Appligene-oncor, France). Hybridizations were performed at 42°C with high stringency post-hybridization washes.[16.] Detection of DNA was achieved with the enhanced chemiluminesence (ECL) direct system following the protocol of the manufacturer (Amersham).

RESULTS

The use of IS*2404* probe resulted in 6 restriction fragment length polymorphism (RFLP) profiles. Figure 1 shows the results for representative strains of each geographical origin. The banding patterns can be separated into two main regions; an upper part that is difficult to interpret (molecular size higher than 2 kb), and a lower section which allows detectable genomic polymorphism. Table 2 summarizes the

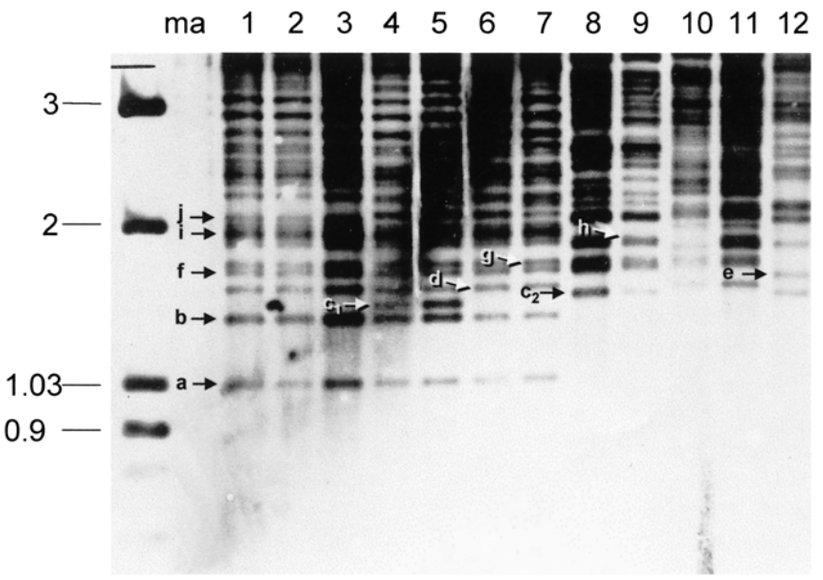

FIGURE 1. Southern blot analysis of one *Mycobacterium marinum* strain and representative *Mycobacterium ulcerans* strains originating from six geographical areas. Lanes 1 to 3: Africa; lane 4: reference strain ATCC 19423; lane 5: Australia; lanes 6 and 7: south Asia; lanes 8 and 9: Asia; lanes 10 and 11: South America; and lane 12: Mexico. Molecular size (kb) is shown on the left and alphabetical letters (a–j) are assigned to specific bands.

TABLE 2
IS2404 restriction fragment length polymorphism (RFLP) profiles

Country	a	b	c1	c2	d	e	f	g	h	i	j	Subtype
Africa*	+	+	−	−	+	−	+	+	−	+	+	I
Australia	+	+	+	−	+	−	+	+	−	+	−	II
South Asia†	+	+	−	−	+	−	+	+	−	+	−	IV
Asia‡	−	−	−	+	−	−	+	+	+	+	−	V
South America§	−	−	−	−	+	−	−	+	+	+	−	VI
Mexico	−	−	−	+	−	+	−	−	+	−	+	III

* Angola, Benin, Congo, Ghana, Ivory Coast, and Togo.
† Malaysia and Papua New Guinea.
‡ Japan and China.
§ Surinam and French Guyana.

comparison between different profiles by noting the presence or absence of particular bands. Alphabetical letters, a–j, were assigned to each band (Figure 1). One band was named c2 because its size was very close to c1. On initial inspection, the African strains present the same profile except for the one from Ghana where the band j was absent (Subtype I). The two Australian strains exhibited the same banding pattern (Subtype II) which was identical to the patterns of the Malaysian and the Papua New Guinean isolates (Subtype IV) except for the absence of band c1 in the two south Asian isolates. The two RFLP patterns obtained from the strains isolated in China and Japan gave the same genotypic information (Subtype V). The isolates from Surinam and French Guyana had an identical RFLP pattern (Subtype VI). The only Mexican strain in the study had a banding pattern (Subtype III) quite different from the others. The *M. marinum* genome displayed no band when probed with IS*2404*.

DISCUSSION

Analysis of all the profiles reveals that the genomes of isolates from Africa, Australia, and southeast Asia contain more IS*2404* copies than the strains from Asia, South America, and Mexico. A detailed comparison of the banding patterns further reveals that Subtypes I, II, and IV exhibit only a few differences with a close genotypic relationship that may be related to the close evolutionary link of those strains as suggested by Stinear and others.[17]

Molecular typing of *M. ulcerans* may provide valuable information on the epidemiology of infection with this organism. Several techniques have been used for fingerprinting studies. In our laboratory, a direct comparison of DNA sequences has permitted the differentiation of four *M. ulcerans* subtypes, and a fifth subtype (ITM842) which was identical to *M. marinum*.[11] Other results based on the triple restriction (Rsa I, DraI, and EcoNI) of the 16S rRNA gene PCR product showed 3 different geographic profiles: Australia, Mexico, and Africa (Chemlal and others, unpublished data). Using an RFLP technique based on the pTBN12 plasmid, which hybridizes to repeat sequences in many mycobacterial species, Jackson and others[10] demonstrated genetic differences between *M. ulcerans* strains from Benin and Democratic Republic of Congo, and from at least two states in Australia. The specificity of IS*2404* for *M. ulcerans* has been previously corroborated by Guimaraes-Peres and others[13] and by Stinear and others,[12] thereby providing confirmed molecular identification of the strains. Figure 2 shows the presence of the disease in six geographic areas, Australia, Southeast Asia, Asia, Africa, South America, and Mexico. The distribution of the IS*2404* in the genome of the strains isolated in those regions reveals six geographic types. Our study also shows that the distribution of IS*2404* in the six African isolates is stable except for the strain from Ghana where the band j is absent. Hence, this probe is unable to differentiate between most strains from the same continent. Similarly, strains from closely related geographic regions gave the same profiles. These included Japan and China, and Papua New Guinea and Malaysia. The Mexican strain showed the smallest number of copies of IS*2404* and has exhibited different phenotypic properties (e.g., rapid growth rate, and inhibition by the antimicrobial combination polymyxin-amphotericin B, nalidixic acid, trimethoprim, azlocillin [PANTA]) compared to the Australian and the African isolates.[19] These differences may also relate to the distribution of the IS in the genome, which may induce mutations in functional genes.

The variation in the distribution of the IS*2404* in the genome of *M. ulcerans* may be explained by the ability of this repeat element to actively jump or duplicate in the genome. The presence of ''inert'' or ''fossil'' IS has been suspected after a sequence comparison of the African and the Australian IS (Chemlal and others, unpublished data), which suggests that the numerous copies of IS*2404* from a single genome are not identical.

In conclusion, this study differentiated *M. ulcerans* into six subtypes related to the six geographic areas of the disease. In addition, IS*2404* is a good fingerprinting tool to determine relatedness of strains isolated from closely related geographic areas. However, the probe was unable to discriminate isolates within the same region (Africa and Australia). On the other hand, this study showed that the Surinam strain (ITM 842), can be placed with the same subtype as the French Guyana *M. ulcerans* isolate. It would be desirable to analyze a large number of isolates from those regions; however, such strains are difficult to obtain.

RFLP or other fingerprinting systems have been developed for the *M. tuberculosis* complex and have contributed valuable information on many facets of tuberculosis including epidemiology, treatment outcome, reinfection versus reactivation, mixed infections, and disease control. Development of similar systems for *M. ulcerans* should provide important information on the epidemiology, pathogenesis and control of Buruli ulcer, especially if more discriminating analyses are developed that could differentiate among strains from the same geographic area. While IS*2404* based fingerprinting appears to be useful, other fingerprinting approaches for strains of *M. ulcerans* need to be investigated.

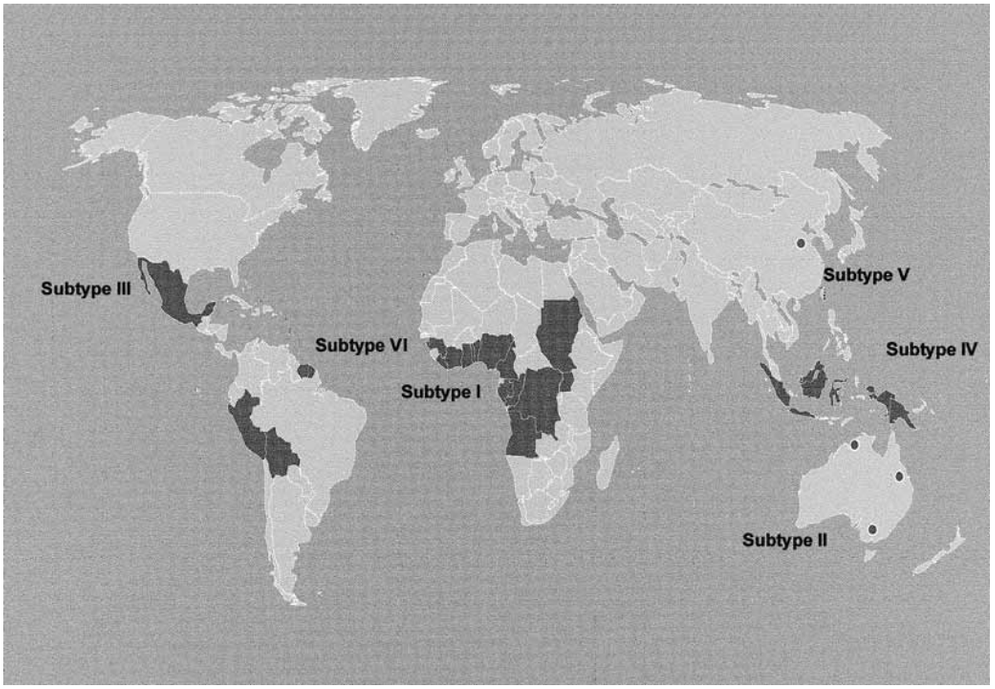

FIGURE 2. World map showing areas where Buruli ulcer was reported and the related subtypes.

Acknowledgments: We thank D. Dawson, K. Jackson, V. Vincent, P. Lavalle, P. H. J. Van Keulen, J. L. Stanford, and W. R. Faber for providing *M. ulcerans* isolates. The authors thank also J. C. Palomino, S. R. Pattyn, and I. Bastian for assistance and advice.

Financial support: This work was generously supported by the Damien Foundation (Brussels) and the Belgian Agency for Development (Project: Buruli ulcer in Benin).

Authors' addresses: Karim Chemlal, Françoise Portaels, Pierre Alain Fonteyne, and Karin De Ridder, Department of Microbiology, Division of Mycobacteriology, Institute of Tropical Medicine, B-2000 Antwerp, Belgium. Jean Swings, Laboratorium voor Microbiologie, Universiteit Gent, B-9000 Gent, Belgium. Wayne Meyers, Armed Forces Institute of Pathology, Washington, DC 20306.

REFERENCES

1. Portaels F, 1995. Epidemiology of mycobacterial diseases. *Clinics Dermatol 13:* 207–222.
2. Pettit JHS, Marchette NJ, Rees RJW, 1966. *Mycobacterium ulcerans* infection. Clinical and bacteriological study of the first cases recognized in South East Asia. *Brit J Dermatol 78:* 187–197.
3. MacCallum P, Tholhurst JC, Buckle G, Sissons HA, 1948. A new mycobacterial infection in man. I. Clinical. aspects. *J Pathol Bacteriol 60:* 93–101.
4. Christie M, 1987. Suspected *Mycobacterium ulcerans* in Kiribati. *Med J Aust 146:* 600–609.
5. Josse R, Guédénon A, Aguiar J, Anagonou S, Zinsou C, Porst C, Foundohou J, Touze JE, 1994. L'ulcère de Buruli, une pathologie peu connue au Bénin. A propos de 227 cas. *Bull Soc Pathol Exot 87:* 170–175.
6. Johnson PDR, Veitch MGK, Flood PE, Hayman JA, 1995. *Mycobacterium ulcerans* infection on Phillip Island, Victoria. *Med J Aust 162:* 221–222.
7. Marston BJ, Diallo MO, Horsburgh CR, Diomande JrI, Saki MZ, Kanga JM, Patrice G, Lipman HB, Ostroff SM, Good R, 1995. Emergence of Buruli ulcer disease in the Daloa region of Côte d'Ivoire. *Am J Trop Med Hyg 52:* 219–224.
8. Ross BC, Johnson PD, Oppedisano F, Marino L, Sievers A, Stinear T, Hayman JA, Veitch MG, Robins-Browne RM, 1997. Detection of *Mycobacterium ulcerans* in environmental samples during an outbreak of ulcerative disease. *Appl Environ Microbiol 63(10):* 4135–4138.
9. Portaels F, Elsen P, Guimaraes-Peres A, Fonteyne P-A, Meyers WM, 1999. Insects in the transmission of *Mycobacterium ulcerans* infection. *The Lancet 353:* 986.
10. Jackson K, Edwards R, Leslie DE, Hayman J, 1995. Molecular method for typing *Mycobacterium ulcerans. J Clin Microbiol 33:* 2250–2253.
11. Portaels F, Fonteyne P-A, De Beenhouwer H, De Rijk P, Guédénon A, Hayman J, Meyers WM, 1996. Variability in the 3′ end of 16S rRNA sequence of *Mycobacterium ulcerans* is related to geographic origins of isolates. *J Clin Microbiol 34:* 962–965.
12. Stinear T, Ross PBC, Johnson PDR, Marino L, Robins-Browne RM, Oppedisano F, Sievers A, Davies JK, 1999. Identification and characterization of IS2404 and IS2606: two distinct repeated sequences for detection of *Mycobacterium ulcerans. J Clin Microbiol 37:* 1018–1023.
13. Guimaraes-Peres A, Portaels F, De Rijk P, Fissette K, Pattyn SR, Van Vooren P, Fonteyne P-A, 1999. Comparison of two PCRs for detection of *Mycobacterium ulcerans. J Clin Microbiol 37:* 206–208.
14. Tsukamura M, Mikoshiba H, 1982. A new *Mycobacterium* which caused skin infection. *Microbiol Immunol 26:* 951–955.
15. Faber WR, Bouda LM, Zeegelaar JE, Kolk AJ, Fonteyne P-A, Landerberg W, Toonstra J, Portaels F. First case of *Mycobacterium ulcerans* infection in the Peoples Republic of China 2000. *Trans Roy Soc Trop Med Hyg 94:* 277–279.
16. Van Embden JDA, Cave MD, Crawford JT, Dale JW, Eisenach KD, Gicquel B, Hermans PWM, Martin C, Shinnick TM, Small PM, 1993. Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. *J Clin Microbiol 31:* 406–409.
17. Stinear T, Davies JK, Jenkin GA, Portaels F, Ross BC, Oppidesano F, Purcell M, Hayman JA, Johnson DR, 2000. A simple PCR method for rapid genotype analysis of *Mycobacterium ulcerans. J Clin Microbiol 38:* 1482–1487.
18. Palomino JC, Obiang AM, Realini L, Meyers WM, Portaels F, 1998. Effect of oxygen on *Mycobacterium ulcerans* growth in the BACTEC system. *J Clin Microbiol 36:* 3420–3422.