JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 2000, p. 1482–1487 Vol. 38, No. 4
0095-1137/00/$04.00+0

A Simple PCR Method for Rapid Genotype Analysis of *Mycobacterium ulcerans*

TIMOTHY STINEAR,[1]* JOHN K. DAVIES,[1] GRANT A. JENKIN,[1] FRANÇOISE PORTAELS,[2] BRUCE C. ROSS,[3] FRANCES OPPEDISANO,[4] MARIA PURCELL,[5] JOHN A. HAYMAN,[6] AND PAUL D. R. JOHNSON[1,4,7]

Department of Microbiology, Monash University,[1] and Department of Infectious Diseases and Clinical Epidemiology, Monash Medical Centre,[7] Clayton, Research and Development, CSL Limited,[3] and Microbiology Research Unit, Royal Children's Hospital,[4] Parkville, Victorian Infectious Diseases Reference Laboratory, North Melbourne,[5] and Department of Pathology, Box Hill Hospital, Box Hill,[6] Victoria, Australia, and Institute of Tropical Medicine, Antwerp, Belgium[2]

Received 3 November 1999/Accepted 23 January 2000

Two high-copy-number insertion sequences, IS*2404* and IS*2606*, were recently identified in *Mycobacterium ulcerans* and were shown by Southern hybridization to possess restriction fragment length polymorphism between strains from different geographic origins. We have designed a simple genotyping method that captures these differences by PCR amplification of the region between adjacent copies of IS*2404* and IS*2606*. We have called this system 2426 PCR. The method is rapid, reproducible, sensitive, and specific for *M. ulcerans*, and it has confirmed previous studies suggesting a clonal population structure of *M. ulcerans* within a geographic region. *M. ulcerans* isolates from Australia, Papua New Guinea, Malaysia, Surinam, Mexico, Japan, China, and several countries in Africa were easily differentiated based on an array of 4 to 14 PCR products ranging in size from 200 to 900 bp. Numerical analysis of the banding patterns suggested a close evolutionary link between *M. ulcerans* isolates from Africa and southeast Asia. The application of 2426 PCR to total DNA, extracted directly from *M. ulcerans*-infected tissue specimens without culture, demonstrated the sensitivity and specificity of this method and confirmed for the first time that both animal and human isolates from areas of endemicity in southeast Australia have the same genotype.

Mycobacterium ulcerans is an environmental mycobacterium with worldwide distribution (Fig. 1) and causes chronic, necrotizing skin lesions in otherwise healthy humans. The incidence of *M. ulcerans* disease has been increasing worldwide, particularly throughout rural west Africa (11, 22). The reasons for this increase are unknown. Epidemiological and PCR-based evidence suggests that swamps and slow-flowing water are sources of the organism (1, 6, 8, 17, 25), and recent PCR data from Benin and Ghana have identified *M. ulcerans* DNA in aquatic insects (12). So far *M. ulcerans* has never been isolated by culture from any of these environments. It has therefore been difficult to clearly identify reservoirs or modes of transmission. One approach to improving our understanding of the ecology of this organism has been to study the molecular epidemiology of *M. ulcerans*, for which two methods have been reported. The first of these identified restriction fragment length polymorphism (RFLP) between isolates by probing with the polymorphic GC-rich repeat sequence contained in plasmid pTBN12 (7). This approach distinguished 11 distinct RFLP types among isolates from four countries and suggested a clonal population structure within a geographic region. Supporting this conclusion, nucleotide sequence analysis of the 3′ region of the 16S rRNA gene identified three alleles that correlated with the geographic origin of an isolate (13). However, the methods used in these studies were limited by requiring high-concentration, purified DNA or by offering limited discriminative capability.

PCR genotyping of mycobacteria by targeting insertion sequences (IS) has been well described (10, 16). The recent discovery of two high-copy-number IS elements in *M. ulcerans* that displayed RFLP between strains (21) suggested that PCR amplification between adjacent copies of these elements (IS*2404* and IS*2606*) may be suitable for genotyping this species.

The aims of this study were twofold. The first was to develop a method that would offer strain discrimination in a simple, reproducible format. Second, using this method, we hoped to improve our understanding of the molecular epidemiology of *M. ulcerans* by analyzing a selection of isolates from disease foci around the world.

MATERIALS AND METHODS

Mycobacterial strains, clinical specimens, and culture conditions. The origins of the *M. ulcerans* strains used in this study are listed in Table 1. The origins of the other species of mycobacteria that were used and general culture conditions have been described previously (21).

DNA preparation. An approximately 10-μl loopful of cells was scraped from an egg yolk agar slope and resuspended in 500 μl of 1% Triton X-100. This cell suspension was added to a 2-ml skirted, screw cap tube containing 200 μl of washed 100-μ-m-diameter glass beads and 500 μl of chloroform-isoamylalcohol (24:1). The tube was placed in a Fastprep cell disrupter (Savant Instruments, Holbrook, N.Y.) at speed setting 6 for 40 s. After cell disruption, the tube was cooled on ice for 5 min and then centrifuged at 17,000 × *g* for 5 min. In some instances DNA was also prepared by resuspending cells in 300 μl of water, heating cells to 100°C for 20 min, and then centrifuging as described above. The aqueous phases were retained and stored at −20°C. A 2-μl volume of DNA from either preparation was used as a template for the PCR.

For primer specificity testing, DNA was extracted from a panel of 23 species of mycobacteria and pooled into five groups. Each pool of DNA was then tested by 2426 PCR. The composition of each group was as follows: pool 1, *Mycobacterium heidelbergense*, *M. intermedium*, *M. lentiflavum*, *M. obuense*, and *M. parafortuitum*; pool 2, *M. pulveris*, *M. rhodesiae*, *M. shimoidei*, *M. tokaiense*, and *M. triplex*; pool 3, *M. vaccae*, *M. xenopi*, *M. gordonae*, *M. simiae*, and *M. flavescens*; pool 4, *M. terrae*, *M. aurum*, *M. chelonae*, and *M. smegmatis*; pool 5, *M. fortuitum*, *M. marinum*, *M. tuberculosis*, and *M. haemophilum*.

* Corresponding author. Mailing address: Department of Microbiology, Monash University, Wellington Rd., Clayton 3168, Australia. Phone: 61 3 9905 4809. Fax: 61 3 9905 4811. E-mail: tim.stinear@med.monash.edu.au.

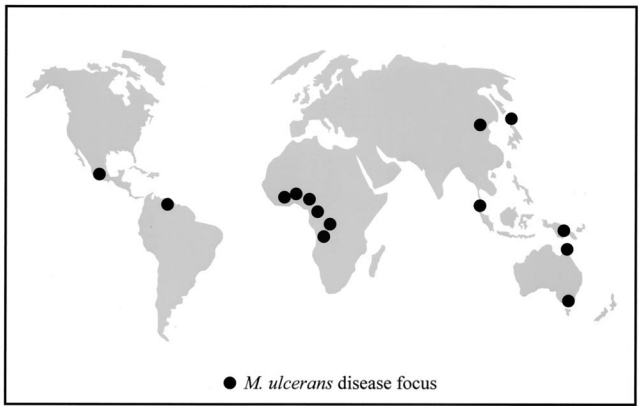

FIG. 1. World map showing regions where the isolates used in the study were obtained.

For method sensitivity experiments, purified DNA was extracted by the method of Boddinghaus et al. (2) from 50 mg (wet weight) of *M. ulcerans* cells harvested from egg yolk agar slopes. The DNA was diluted as previously described to represent *M. ulcerans* genome equivalents, where 4 to 5 fg of DNA approximates one mycobacterial genome (9).

2426 PCR. Reaction conditions used for 2426 PCR were as follows: each PCR mixture (20 μl) contained 1× PCR buffer II (10× PCR buffer II contained 500 mM KCl and 100 mM Tris-HCl [pH 8.3]), 2.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates (0.2 mM [each] dATP, dTTP, dCTP, and dGTP), an 0.5 μM concentration of each primer (MU4, 5′ ATCGCCGAAGCCTGCCGGAT 3′, positions 1119 to 1138, and MU9, 5′ TCTTCGTGGTTTTGTGATGGC 3′, positions 1306 to 1326), 1 U of Ampli-*Taq* DNA polymerase (Perkin-Elmer, Melbourne, Australia), and 2 μl of DNA. PCR was performed in an FTS-960 thermal sequencer (Corbett Research, Sydney, Australia) with the following protocol, optimized for this application: 5 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min and 25 cycles (30 cycles for tissue specimens) of 95°C for 20 s, 58°C for 30 s, and 72°C for 40 s, followed by a final extension step at 72°C for 5 min. The PCR products were held at 4°C until analyzed and detected with 7.5% (0.75 mM) native polyacrylamide gel electrophoresis at 200 V for 38 min in a Mini-Protean II apparatus (Bio-Rad) with rapid silver staining. A 100-bp ladder was used as a size marker (Gibco, BRL). The silver stain procedure was adapted from Caetano-Anoelles and Breshoff (Promega Notes **45:**13–18, 1994) as follows. After electrophoresis, gels were fixed in 7.5% acetic acid for 6 min, rinsed three times in distilled water, and then stained for 8 min with 50 ml of a 0.075% silver nitrate solution containing 75 μl of formaldehyde. Gels were rinsed twice in distilled water and developed in 50 ml of developing solution, which contained 1.5 g of sodium carbonate, 0.2 μg of sodium thiosulfate, and 75 μl of formaldehyde. The developing reaction was terminated with 7.5% acetic acid.

The banding patterns from all strains were compared visually. A binary matrix was constructed by scoring each pattern for the presence or absence of the 29 PCR products that constituted the total pool of fragments between 200 and 900 bp. It was assumed that comigrating bands were of identical sequence and therefore an indication of genetic relatedness. The patterns were compared by the method of unweighted pair group average linkage and the simple matching coefficient (19). The goodness of fit of the cluster analysis was tested with the cophenetic correlation coefficient, where *r* values greater than 0.9 suggest a very good fit (18). All analyses were performed with NTSYS, version 1.8, software (Applied Biostatistics Inc., Setauket, N.Y.).

RESULTS

A test panel of *M. ulcerans* clinical isolates was assembled from many of the known *M. ulcerans* disease foci in the world (Fig. 1), a collection that represented both temporal and spatial diversity. All isolates were found to contain IS*2404* and IS*2606* as determined by PCR, and a selection of these results is shown in Fig. 2A.

Several combinations of outward-priming oligonucleotides for IS*2404* and IS*2606* were then evaluated in order to obtain reproducible and discriminatory patterns of bands for different strains of *M. ulcerans*. Primers MU4 and MU9 gave the largest number of clearly resolvable bands when PCR products were separated by nondenaturing polyacrylamide gel electrophoresis and detected by silver staining (Fig. 2B). The use of each primer alone in a PCR did not produce a useful banding pattern. Nine different 2426 PCR genotypes were observed (Fig. 2B). All isolates were tested at least twice with the same Triton X-100 DNA preparation, and identical profiles were produced. For the strains from Africa, Queensland, Malaysia, and Papua New Guinea and for two of the Victorian isolates, DNA was also extracted by boiling cells in water. The crude DNA preparations from this method produced banding patterns identical to that obtained from the detergent-extracted DNA (data not shown). A single pattern for all seven African isolates was obtained despite the large distances and long times separating some of these isolates (Table 1; Fig. 1 and 3). The 35 Australian isolates produced two profiles, representing one genotype from Queensland (northern Australia) and one from Victoria (southeastern Australia). No variation was detected among the 32 isolates from Victoria (Fig. 4), but there were two distinct genotypes among the 3 isolates from Papua New Guinea (PNG I and PNG II). The Malaysian genotype was nearly identical to PNG II, differing only by the positions of two bands between 200 and 900 bp. Distinct profiles were observed for the isolates from Surinam and Mexico; however, the patterns for isolates from Japan and China were identical.

To investigate primer specificity 24 other species of mycobacteria were tested by 2426 PCR. Some PCR products from other mycobacteria were observed, but these were generally faint, and none produced profiles similar to any of the patterns obtained from the different *M. ulcerans* strains (Fig. 5).

A dendrogram was constructed to display the relatedness between strains based on banding pattern similarity (Fig. 6). The clustering of the southeast Asian genotypes and the African genotype is suggestive of an evolutionary link among these strains; however, the PNG I genotype was an interesting exception to this cluster. The genotypes representing the other regions appeared less related to each other, displaying 30 to 60% similarity ($r = 0.91$) (Fig. 6).

The detection sensitivity of 2426 PCR was tested with a dilution series of purified *M. ulcerans* genomic DNA from an African isolate. This demonstrated a detection limit of 100 genomes (Fig. 7).

The relatively low concentration of cells required to produce a profile and the specificity of the target sequences to *M. ulcerans* suggested that this method may be useful for genotyping directly from clinical samples. To investigate this, DNA extracted from 50 patient specimens was subjected to 2426 PCR. These samples included swabs and formalin-fixed and paraffin-embedded tissue specimens that had been screened previously by IS*2404* PCR for *M. ulcerans* diagnosis (unpublished data). Of these 50 samples, 40 were IS*2404* positive and the remainder were IS*2404* negative. All 50 samples were then tested by 2426 PCR. An example of some profiles obtained is shown in Fig. 8. A recognizable genotype was obtained for 20 of the 40 IS*2404*-positive specimens. All samples that were IS*2404* negative were also 2426 PCR negative. The specificity of the 2426 PCR was 100% (calculated as the number of true negatives divided by the number of true negatives plus false positives), and the sensitivity was 66.7% (calculated as the number of true positives divided by the number of true positives plus false negatives). From the 20 recognizable profiles, 13 were identified as the Victorian genotype and 7 as the Queensland genotype. These profiles also correctly predicted the geographic origin of the specimen. Analysis of tissue samples from an *M. ulcerans*-infected alpaca (*Quechua alpako*) and possum (*Trichosurus vulpecula*) clearly demonstrated that these animals were infected with the same genotype as the human cases from these areas (Fig. 8). Both these animals

TABLE 1. Origins of the *M. ulcerans* isolates used in this study

Isolate	Origin	Year isolated	Source[a]	2426 type
96-658	Angola	1996	ITM	African
94-856	Benin	1994	ITM	African
97-111	Benin	1997	ITM	African
5152	Congo	1976	ITM	African
5155	Congo	1976	ITM	African
97-610	Ghana	1997	ITM	African
97-680	Togo	1997	ITM	African
98-912	China	1997	ITM	Asian
ATCC 33728	Japan	1980		Asian
186510	Malaysia	1992	VIDRL	Malaysian
5114	Mexico	1953	ITM	Mexican
5143	Mexico	1967	ITM	Mexican
11878/70	Papua New Guinea	1971	QDRLMD	PNG I
94-1331	Papua New Guinea	1994	ITM	PNG II
186463	Papua New Guinea	1992	VIDRL	PNG II
13822/70	North Queensland, Australia	1971	QDRLMD	Queensland
6537/78	North Queensland, Australia	1978	QDRLMD	Queensland
95016102	North Queensland, Australia	1994	VIDRL	Queensland
842	Surinam	1986	ITM	Suriname
95023625	East Gippsland, Victoria, Australia	1994	VIDRL	Victorian
ATCC 19423	East Gippsland, Victoria, Australia	1948		Victorian
95045548	East Gippsland, Victoria, Australia	1995	VIDRL	Victorian
144727	East Gippsland, Victoria, Australia	1989	VIDRL	Victorian
186453	Langwarrin, Victoria, Australia	1992	VIDRL	Victorian
187636	Langwarrin, Victoria, Australia	1992	VIDRL	Victorian
189182	Langwarrin, Victoria, Australia	1992	VIDRL	Victorian
93112352	Langwarrin, Victoria, Australia	1992	VIDRL	Victorian
95046437	Langwarrin, Victoria, Australia	1995	VIDRL	Victorian
95063471	Langwarrin, Victoria, Australia	1995	VIDRL	Victorian
97029961	Langwarrin, Victoria, Australia	1997	VIDRL	Victorian
93133170	Phillip Island, Victoria, Australia	1993	VIDRL	Victorian
93138796	Phillip Island, Victoria, Australia	1993	VIDRL	Victorian
93147172	Phillip Island, Victoria, Australia	1993	VIDRL	Victorian
93160339	Phillip Island, Victoria, Australia	1993	VIDRL	Victorian
93170008	Phillip Island, Victoria, Australia	1993	VIDRL	Victorian
93171525	Phillip Island, Victoria, Australia	1993	VIDRL	Victorian
94100241	Phillip Island, Victoria, Australia	1993	VIDRL	Victorian
94114510	Phillip Island, Victoria, Australia	1994	VIDRL	Victorian
94128817	Phillip Island, Victoria, Australia	1994	VIDRL	Victorian
94151667	Phillip Island, Victoria, Australia	1994	VIDRL	Victorian
94155376	Phillip Island, Victoria, Australia	1994	VIDRL	Victorian
94161099	Phillip Island, Victoria, Australia	1994	VIDRL	Victorian
94162101	Phillip Island, Victoria, Australia	1994	VIDRL	Victorian
94171428	Phillip Island, Victoria, Australia	1994	VIDRL	Victorian
95001022	Phillip Island, Victoria, Australia	1995	VIDRL	Victorian
95067597	Phillip Island, Victoria, Australia	1995	VIDRL	Victorian
96042434	Phillip Island, Victoria, Australia	1996	VIDRL	Victorian
94179419	Victoria, Australia	1994	VIDRL	Victorian
96035870	Victoria, Australia	1996	VIDRL	Victorian
94112961	Westernport, Victoria, Australia	1993	VIDRL	Victorian
95060263	Westernport, Victoria, Australia	1995	VIDRL	Victorian

[a] ITM, Institute for Tropical Medicine; VIDRL, Victorian Infectious Diseases Reference Laboratory; QDRLMD, Queensland Diagnostic and Reference Laboratory for Mycobacterial Diseases.

inhabited regions in southeast Australia where *M. ulcerans* disease is known to be endemic.

DISCUSSION

In mycobacteria, genetic diversity is driven by the activity of mobile DNA such as IS rather than nucleotide sequence drift (20). Thus IS elements are potentially ideal targets for indexing rapidly evolving change in mycobacterial populations (10, 26). *M. ulcerans* has at least two high-copy-number IS elements, IS*2404* and IS*2606*, that demonstrate RFLP between strains (21) and that appear to be present in isolates of diverse geographic origin (Fig. 2A) (4). However, *M. ulcerans* is a slow-growing organism, and extraction of a sufficient quantity and quality of DNA to perform a Southern analysis is a time-consuming and impractical process for routine analysis.

The PCR typing method we have developed is a simple and robust tool for quickly determining the origin of an isolate, requiring a minimum of 100 genomic equivalents of DNA (Fig. 7). The high detection sensitivity and specificity permitted typing directly from patient specimens without requiring culture. Other mycobacteria tested by 2426 PCR did produce some bands, but none reproduced any of the profiles seen with *M. ulcerans* (Fig. 5). The sensitivity of 2426 PCR for genotyping

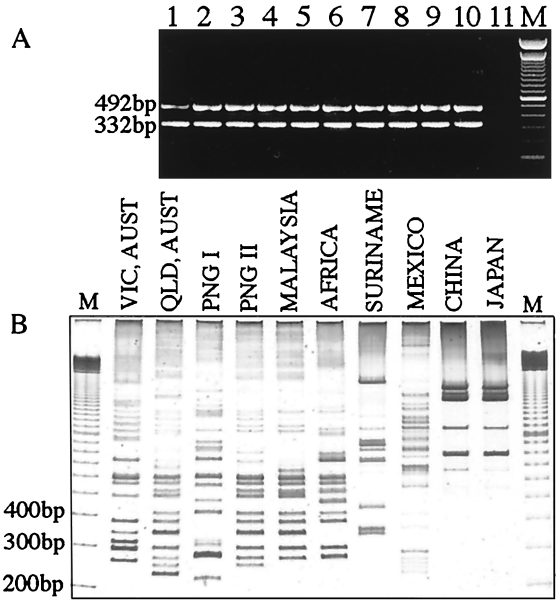

FIG. 2. (A) PCR detection of IS*2404* (492 bp) and IS*2606* (332 bp) in *M. ulcerans* isolates from different geographic regions. (B) 2426 PCR genotype analysis of *M. ulcerans* isolates from different geographic regions. Lanes (A and B), 1, ATCC 19423; 2, 13822/70; 3, 11878/70; 4, 94-1331; 5, 186510; 6, 5155; 7, 842; 8, 5114; 9, 98-912; 10, ATCC 33728; lane 11, no-template control; lane M, 100-bp size ladder. Vic., Victoria; QLD, Queensland; Aust., Australia.

directly from tissue specimens was, however, found to be somewhat low (66.7%). This was most likely due to the low numbers of target cells in some samples; however, DNA extracted from swabs or tissue specimens served equally well as a template. The detection sensitivity of 2426 PCR in this application may be improved by ensuring that specimens are taken from areas of ulceration likely to contain large numbers of bacilli.

Analysis by 2426 PCR of IS*2404*-positive swab and tissue specimens, obtained from animals and humans in areas of southeast Australia where *M. ulcerans* disease is endemic identified the same genotype in all specimens, suggesting that animals are infected from the same environmental source as humans (Fig. 8). Genotyping directly from tissue specimens will be particularly useful for studying the epidemiology of *M. ulcerans*, as culture of this organism is slow and relatively insensitive (4).

To investigate the discriminating capability of 2426 PCR, 55 *M. ulcerans* isolates were tested (Table 1). The combination of

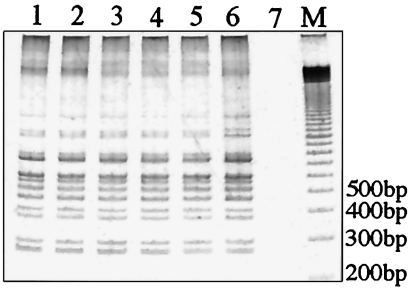

FIG. 3. 2426 PCR genotype analysis of *M. ulcerans* isolates from five countries in Africa. Lane 1, 96-658; lane 2, 94-856; lane 3, 97-111; lane 4, 5155; lane 5, 97-610; lane 6, 97-680; lane 7, no-template control; lane 8, 100-bp ladder size marker.

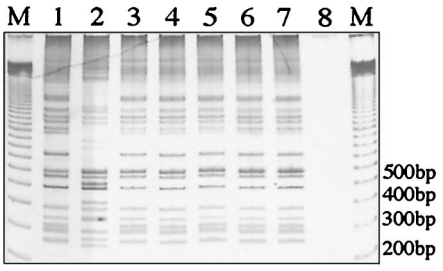

FIG. 4. 2426 PCR genotype analysis of *M. ulcerans* isolates from southeast Australia and Papua New Guinea. Lanes 1 and 3 to 7, strains 94114510, 95046437, 95067597, 94161099, 94171428, 94151667, respectively; lane 2, 186463; lane 8, no-template control; lane M, 100-bp ladder size marker.

primers MU4 and MU9 produced nine distinct profiles that correlated absolutely with the geographic source of the isolates, i.e., the origin of an isolate could be unambiguously assigned based on its banding pattern. This result is somewhat surprising as other mycobacteria, such as *Mycobacterium avium*, exhibit considerable IS diversity even within a geographic region when analyzed by a similar genotyping technique (26). The lack of genotype variation in *M. ulcerans* within a region may be a reflection of its slow generation time and perhaps a low population density within its environmental niche. PCR-based incidence data, gathered during a disease outbreak, suggested a very low abundance of bacteria in the environment (17).

A lack of IS mobility as a cause of the limited genotype diversity is possible but unlikely, as the different 2426 PCR genotypes are indicative of ongoing transposition events or IS-associated genome rearrangements. Furthermore, these events appear to have occurred relatively recently, considering (i) the absence of IS*2404* and IS*2606* from the closely related *M. marinum* and (ii) the very high nucleotide sequence similarity between the 16S rRNA and *groEL* genes of *M. ulcerans* and *M. marinum* (13–15, 23), which is indicative of the recent evolutionary divergence of these species. The inferred close genetic relationship among the five genotypes obtained from the southeast Asian isolates (Fig. 6) suggests that *M. ulcerans* is introduced into an area and then is isolated or sequestered, undergoing occasional transposition or genome rearrangement events to produce a unique genotype in a particular region. However, rapid dispersal over large distances also seems possible considering that only one genotype was observed among the isolates from five countries throughout central and west Africa (Fig. 3). This analysis suggests that the spread of disease

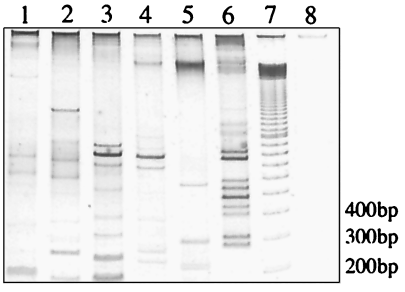

FIG. 5. Determination of primer specificity for *M. ulcerans* by 2426 PCR of 23 other species of mycobacteria as described in Materials and Methods. Lane 1, mycobacterial DNA pool 1; lane 2, pool 2; lane 3, pool 3; lane 4, pool 4; lane 5, pool 5; lane 6, *M. ulcerans* Malaysian strain 186510; lane 7, 100-bp ladder size marker; lane 8, no-template control.

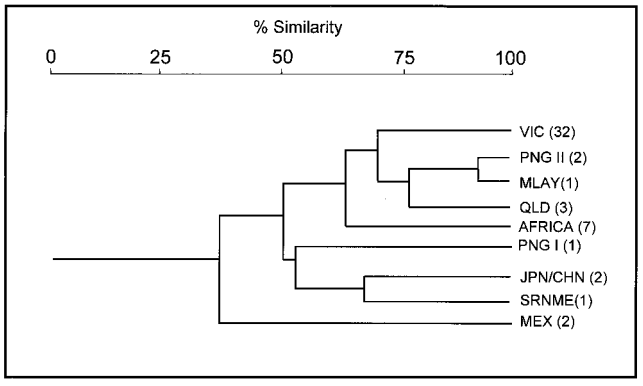

FIG. 6. Cluster analysis of the genetic relationships among 51 isolates based on banding pattern similarity displayed by 2426 PCR. Percentage similarity is indicated by a scale at the top. Genotypes codes are given on the right and reflect the origin of the isolates. Codes are as follows: VIC; Victoria, Australia; PNG I and PNG II, Papua New Guinea; MLAY, Malaysia; QLD, Queensland, Australia; JPN/CHN, Japan and China; SRNME, Surinam, South America; MEX, Mexico. The numbers in parentheses denote the numbers of isolates displaying the genotypes.

across Africa has occurred after its spread throughout southeast Asia and Australia if the rate of genome rearrangements displayed by 2426 PCR is assumed to be the same for all strains.

The 2426 PCR method is highly reproducible. The DNA from all isolates was tested at least twice, and each set of tests produced identical profiles. In addition, 2426 PCR performed with DNA extracted from cells in crude form by boiling produced profiles identical to those produced by PCR performed with purified DNA. The reproducibility of this method is further reinforced by the ability to produce the same pattern from DNA over a 5-log-unit dilution range of template DNA (Fig. 7) and by the fact that the same pattern was obtained for the 32 Victorian isolates and 7 African isolates (Fig. 4).

The findings of previous genotype studies of *M. ulcerans* by Southern hybridization with the pTBN12 probe (7) and nucleotide sequence analysis of the 16SrRNA gene (13) generally concurred with the results from 2426 PCR analysis, where identical genotypes were detected within a region. However the pTBN12 probe method also identified some additional RFLP among African isolates. The apparent increased discriminatory capability of the pTBN12 probe is countered by the length of time taken to perform a Southern hybridization

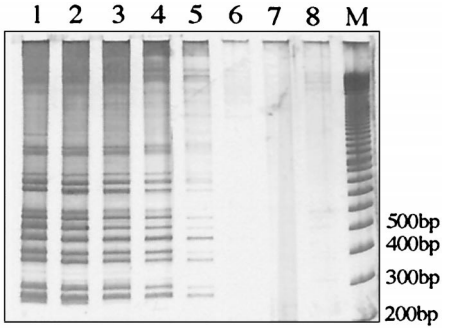

FIG. 7. Detection sensitivity of 2426 PCR for genotyping *M. ulcerans* (strain 5152) with a dilution series of DNA. Lane 1, 10^6 genomes; lane 2, 10^5 genomes; lane 3, 10^4 genomes; lane 4, 10^3 genomes; lane 5, 10^2 genomes; lane 6, 10 genomes; lane 7, 1 genome; lane 8, no-template control; lane M, 100-bp ladder size marker.

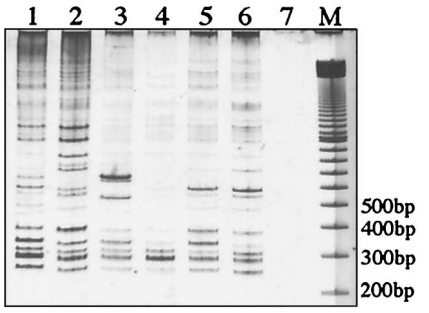

FIG. 8. 2426 PCR genotype analysis of *M. ulcerans* from DNA extracted directly from human and animal specimens. Lanes 1 to 3, dry swab, paraffin-embedded tissue, and formaldehyde-preserved tissue specimens, respectively, from a possum captured on Phillip Island; lane 4, formaldehyde-preserved human tissue specimen from the Langwarrin focus; lane 5, formaldehyde-preserved alpaca tissue from the east Gippsland focus; lane 6, Victorian isolate ATCC 19423; lane 7, no-template control; lane M, 100-bp ladder size marker.

(3 to 4 days) compared with that taken by 2426 PCR (4 to 6 h) and the ability of 2426 PCR to obtain a genotype directly from a clinical specimen.

Interestingly the pTBN12 probe offered no additional discrimination among Victorian isolates, thus supporting the suggestion of clonality of *M. ulcerans* within a region. Analysis of additional African isolates with the pTBN12 probe and 2426 PCR is warranted to more thoroughly examine evidence for clonality between endemic foci and to better compare the capabilities of each method.

It is of interest that two genotypes were detected in Papua New Guinea (Fig. 1B). The PNG II pattern appears closely related those of the Malaysian and Australian strains. By comparison, the PNG I pattern is quite distinct and appears, at least by this method, less related to those of the other southeast Asian strains than to those of the African isolates (Fig. 6). Unfortunately further details regarding the origin of this strain were unavailable but, it is likely to represent an endemic focus distant to the PNG II isolates. We are currently sequencing multiple gene loci from *M. ulcerans* to better establish the genetic relatedness between all strains.

It has been proposed that the global distribution of *M. ulcerans* may be linked to the breakup of the supercontinents at the end of the Jurassic period (5). However, the data presented in this study, previous 16S rRNA sequence comparisons of *M. ulcerans* and *M. marinum*, and the discovery of *M. ulcerans* in Japan and China (3, 24) argue against this hypothesis and suggest a far more recent mechanism of dispersal. More-extensive nucleotide sequence analyses of *M. ulcerans* strains are now required to investigate intraspecies and interspecies evolution and to further our understanding of the molecular epidemiology of *M. ulcerans*.

ACKNOWLEDGMENTS

We thank David Dawson for the provision of mycobacterial isolates.

This work was supported in part by funding from the Government of Victoria through the Department of Human Services.

REFERENCES

1. **Anonymous.** 1971. Epidemiology of *Mycobacterium ulcerans* infection (Buruli ulcer) at Kinyara, Uganda. Trans R. Soc. Trop. Med. Hyg. **65:**763–775.
2. **Boddinghaus, B., T. Rogall, T. Flohr, H. Blocker, and E. C. Bottger.** 1990. Detection and identification of mycobacteria by amplification of rRNA. J. Clin. Microbiol. **28:**1751–1759.
3. **Faber, W. R., L. M. Pereira Arias-Bouda, J. E. Zeegelaar, A. H. J. Kolk, P. A. Fonteyne, J. Toonstra, and F. Portaels.** 1999. First case of *Mycobacterium ulcerans* infection in the Peoples Republic of China. Trans. R. Soc. Trop. Med. Hyg., in press.

4. **Guimaraes-Peres, A., F. Portaels, P. de Rijk, K. Fissette, S. R. Pattyn, J. van Vooren, and P. Fonteyne.** 1999. Comparison of two PCRs for detection of *Mycobacterium ulcerans*. J. Clin. Microbiol. **37:**206–208.
5. **Hayman, J.** 1984. *Mycobacterium ulcerans*: an infection from Jurassic time? Lancet **ii:**1015–1016.
6. **Hayman, J.** 1991. Postulated epidemiology of *Mycobacterium ulcerans* infection. Int. J. Epidemiol. **20:**1093–1098.
7. **Jackson, K., R. Edwards, D. E. Leslie, and J. Hayman.** 1995. Molecular method for typing *Mycobacterium ulcerans*. J. Clin. Microbiol. **33:**2250–2253.
8. **Johnson, P. D., M. G. Veitch, D. E. Leslie, P. E. Flood, and J. A. Hayman.** 1996. The emergence of *Mycobacterium ulcerans* infection near Melbourne. Med. J. Aust. **164:**76–78.
9. **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.
10. **Picardeau, M., and V. Vincent.** 1996. Typing of *Mycobacterium avium* isolates by PCR. J. Clin. Microbiol. **34:**389–392.
11. **Portaels, F.** 1998. Historical overview of the Buruli ulcer, p. 17. *In* K. Asiedu, M. Raviglione, and R. Scherpbier (ed.), Proceedings of the International Conference on Buruli Ulcer Control and Research. World Health Organization, Geneva, Switzerland.
12. **Portaels, F., P. Elsen, A. Guimares-Peres, P. A. Fonteyne, and W. M. Meyers.** 1999. Insects in the transmission of *Mycobacterium ulcerans* infection. Lancet **353:**986. (Letter.)
13. **Portaels, F., P. A. Fonteyne, H. de Beenhouwer, P. de Rijk, A. Guedenon, J. Hayman, and M. W. Meyers.** 1996. Variability in 3′ end of 16S rRNA sequence of *Mycobacterium ulcerans* is related to geographic origin of isolates. J. Clin. Microbiol. **34:**962–965.
14. **Roberts, B., and R. Hirst.** 1997. Immunomagnetic separation and PCR for detection of *Mycobacterium ulcerans*. J. Clin. Microbiol. **35:**2709–2711.
15. **Rogall, T., J. Wolters, T. Flohr, and E. C. Bottger.** 1990. Towards a phylogeny and definition of species at the molecular level within the genus *Mycobacterium*. Int. J. Syst. Bacteriol. **40:**323–330.
16. **Ross, B. C., and B. Dwyer.** 1993. Rapid, simple method for typing isolates of *Mycobacterium tuberculosis* by using the polymerase chain reaction. J. Clin. Microbiol. **31:**329–334.
17. **Ross, B. C., P. D. Johnson, F. Oppedisano, L. Marino, A. Sievers, T. Stinear, J. A. Hayman, M. G. Veitch, and R. M. Robins-Browne.** 1997. Detection of *Mycobacterium ulcerans* in environmental samples during an outbreak of ulcerative disease. Appl. Environ. Microbiol. **63:**4135–4138.
18. **Sneath, P. H. A.** 1978. Classification of microorganisms, p. 9/1–9/31. *In* J. R. Norris and M. H. Richmond (ed.), Essays in microbiology. John Wiley, Chichester, United Kingdom.
19. **Sneath, P. H. A., and R. R. Sokal.** 1973. Numerical taxonomy. W. H. Freeman & Co., San Francisco, Calif.
20. **Sreevatsan, S., X. Pan, K. E. Stockbauer, N. D. Connell, B. N. Kreiswirth, T. S. Whittam, and J. M. Musser.** 1997. Restricted structural gene polymorphism in the *Mycobacterium tuberculosis* complex indicates evolutionarily recent global dissemination. Proc. Natl. Acad. Sci. USA **94:**9869–9874.
21. **Stinear, T., B. C. Ross, J. K. Davies, L. Marino, R. M. Robins-Browne, F. Oppedisano, A. Sievers, and P. D. R. Johnson.** 1999. Identification and characterization of IS*2404* and IS*2606*: two distinct repeated sequences for detection of *Mycobacterium ulcerans* by PCR. J. Clin. Microbiol. **37:**1018–1023.
22. **Thangaraj, H. S., M. R. W. Evans, and M. H. Wansborough-Jones.** 1999. *Mycobacterium ulcerans* disease; Buruli ulcer. Trans. R. Soc. Trop. Med. Hyg. **93:**337–340.
23. **Tonjum, T., D. B. Welty, E. Jantzen, and P. L. Small.** 1998. Differentiation of *Mycobacterium ulcerans*, *M. marinum*, and *M. haemophilum*: mapping of their relationships to *M. tuberculosis* by fatty acid profile analysis. DNA-DNA hybridization, and 16S rRNA gene sequence analysis. J. Clin. Microbiol. **36:**918–925.
24. **Tsukamura, M., K. Kaneda, T. Imaeda, and H. Mikoshiba.** 1989. A taxonomic study on a mycobacterium which caused a skin ulcer in a Japanese girl that resembled *Mycobacterium ulcerans*. Kekkaku **64:**691–697.
25. **Veitch, M. G., P. D. Johnson, P. E. Flood, D. E. Leslie, A. C. Street, and J. A. Hayman.** 1997. A large localized outbreak of *Mycobacterium ulcerans* infection on a temperate southern Australian island. Epidemiol. Infect. **119:**313–318.
26. **Yoder, S., C. Argueta, A. Holtzman, T. Aronson, O. G. W. Berlin, P. Tomasek, N. Glover, S. Froman, and G. Stelma, Jr.** 1999. PCR comparison of *Mycobacterium avium* isolates obtained from patients and foods. Appl. Environ. Microbiol. **65:**2650–2653.