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Molecular Method for Typing *Mycobacterium ulcerans*

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A cluster of *Mycobacterium ulcerans* infections has recently occurred on Phillip Island, Victoria, Australia. Previous cases of infection have generally been located around Bairnsdale in southeast Gippsland. The aim of this study was to determine the epidemiological relationship between these strains and other strains originating in Australia and Africa. The previously described plasmid pTBN12 was used as a probe with restriction enzyme-digested chromosomal DNA to differentiate the strains of *M. ulcerans*. The probe was able to distinguish 11 restriction fragment length polymorphisms (RFLPs). Forty-three strains originating in Victoria were divided into three types, i.e., V1, V2, and V3. The majority of strains (40) yielded a type V1 pattern, including strains from southeast Gippsland. Fourteen strains from Queensland yielded three additional RFLP types, i.e., Q1, Q2, and Q3. Five strains from Benin and seven strains from Zaire yielded five additional RFLP types. It is envisaged that molecular typing of *M. ulcerans* strains from around the world may have a great impact on understanding of the epidemiology of infection with this organism.

Mycobacterium ulcerans is a slowly growing acid-fast bacillus which causes chronic necrotizing skin ulcers. The disease was first observed in Australia in Bairnsdale, Victoria, in 1948 (10). Since then many cases have from other countries been reported, particularly from areas in Uganda and Zaire where *M. ulcerans* infection is endemic (1, 9, 14). The source of *M. ulcerans* is thought to be environmental; however, the organism has never been cultured from environmental specimens (6, 11). Efforts to culture the organism from soil, vegetation, and water are probably hampered by the slow rate of growth of *M. ulcerans* (6 to 12 weeks) and contamination by more rapidly growing acid-fast and non-acid-fast bacteria. *M. ulcerans* also prefers a slightly acidic medium and may grow poorly on conventional Lowenstein-Jensen medium. Over the last 10 years or so, there have been 43 isolations of *M. ulcerans* from clinical specimens in Victoria. Over one-third of these cases have occurred since March 1993 and have been associated with Phillip Island and neighboring towns in southern Victoria (3). This relatively large cluster of cases in a localized area prompted us to examine the epidemiological relationship between these strains and those isolated from around Bairnsdale in east Gippsland and from other parts of Australia and Africa. Until now, there have been no reported methods for reliably differentiating strains of *M. ulcerans*. This paper describes a method for typing strains of *M. ulcerans* on the basis of restriction fragment length polymorphisms (RFLPs). The probe used is plasmid pTBN12, which has been successfully used to differentiate strains of the *Mycobacterium tuberculosis* complex and *Mycobacterium kansasii* (2, 12, 13). The use of this method for differentiating strains of *M. ulcerans* may provide valuable information on the epidemiology of infection with this organism.

MATERIALS AND METHODS

Clinical isolates. The 43 Victorian strains were obtained from the culture collection of the Mycobacterium Reference Laboratory, Fairfield Hospital. Fourteen strains were kindly supplied by the tuberculosis section of the Laboratory of Microbiology and Pathology, State Health Laboratory, Brisbane, Queensland, Australia. Twelve African strains were obtained from the Belgian Institute of Tropical Medicine, Antwerpen, Belgium. The American Type Culture Collection strain ATCC 19423, which originated in Bairnsdale, was also tested.

Preparation of mycobacterial DNA. *M. ulcerans* strains were grown on egg yolk agar at 31°C until confluent. The growth was swabbed into Middlebrook 7H9 broth and incubated at 31°C for 1 week. D-Cycloserine (Sigma Chemical Co., St. Louis, Mo.) was then added to a final concentration of 1 mg/ml, and the cultures were then incubated for an additional 3 to 7 days. The DNA was extracted as previously described (12). The concentration of DNA in each sample was estimated by electrophoresis through 0.7% agarose gels stained with ethidium bromide.

Southern blotting. Approximately 1 μg of chromosomal DNA was digested with 10 U of *Alu*I for 5 h at 37°C in the incubation buffer supplied by the enzyme manufacturer (Boehringer Mannheim, Mannheim, Germany). The digested samples were electrophoresed through 0.7% agarose gels at 70 V for 17 h (20-cm gel) in 90 mM Tris base–90 mM boric acid–2 mM EDTA and then Southern transferred onto positively charged nylon membranes (Boehringer Mannheim) by using a vacuum transfer apparatus (Hybaid) at a negative pressure of 80 cm of H_2O for 1 h with 0.5 M NaOH and 1.5 M NaCl. The DNA was fixed to the membrane by baking at 120°C for 30 min. Prehybridization, hybridization, and detection were performed as previously reported (13). An RNA probe was prepared from plasmid pTBN12 with the nonradioactive digoxigenin nucleic acid labelling and detection system supplied by Boehringer Mannheim.

RESULTS

The use of the pTBN12 probe resulted in 11 RFLPs. Figure 1 shows the results for one or more representative strains of each pattern obtained, except for type Z3. The probable source of infection and number of strains in each RFLP group are summarized in Table 1.

When multiple isolates were obtained from a single patient, the same RFLP pattern was produced. Similarly, when a single isolate was tested several times, the pattern remained the same, indicating the stability of the pattern over time (results not shown).

There were three banding patterns seen with the Victorian isolates; these patterns have been named V1, V2, and V3 and contain 40 strains, 3 strains, and 1 strain, respectively. A second strain with type V3 was isolated in Queensland from a patient who is believed to have acquired *M. ulcerans* infection in Darwin. Two of the strains of type V1 were also isolated in Queensland. The V1 and V2 patterns differed by the migration of only one band (Fig. 1). Strain ATCC 19423 yielded a V2 RFLP. The V3 strains, however, showed a unique banding pattern. The isolates from Phillip Island demonstrated banding

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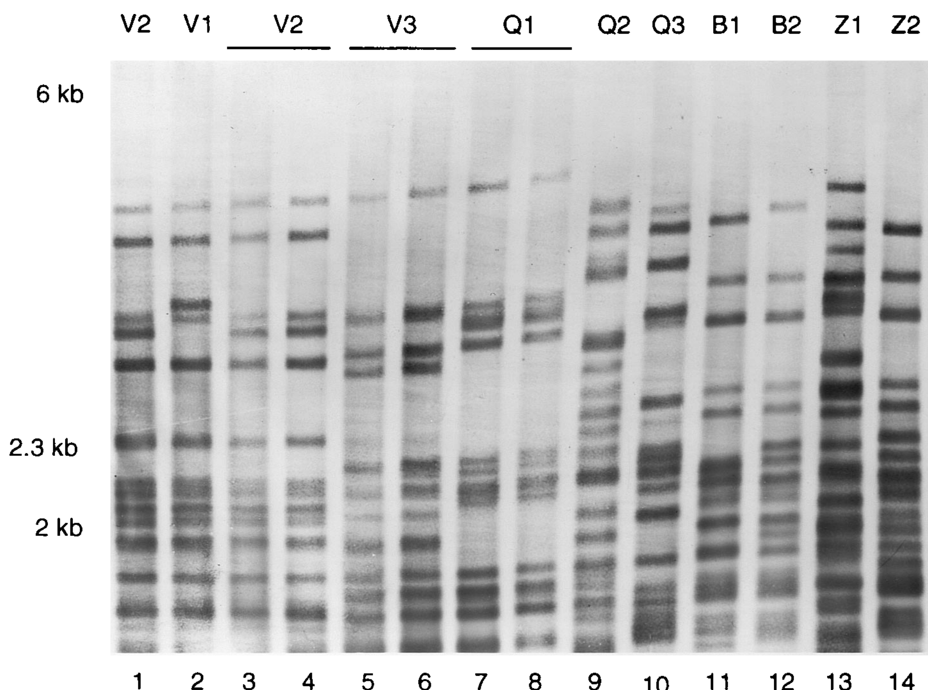

FIG. 1. Southern blot analysis of representative *M. ulcerans* strains of different RFLP types after digestion of chromosomal DNA with *Alu*I and hybridization with a digoxigenin-labelled RNA probe made from plasmid pTBN12. Lane 1, type strain ATCC 19423. The locations of the molecular size standards are shown on the left.

patterns identical to those from Bairnsdale and East Gippsland (type V1). There were three RFLP patterns obtained for the strains isolated in Queensland, i.e., Q1, Q2, and Q3. Two of the 11 strains with type Q1 were isolated in Victoria; these included one from an ulcer excised in Mossman, north Queensland, and sent to Victoria for examination and one isolated from a Papua New Guinea patient who was visiting Victoria.

The Benin strains yielded two RFLP types, B1 and B2, which differ from each other by the migrations of two bands. The seven strains from Zaire yielded an additional three banding patterns called Z1, Z2, and Z3. The Z3 profile differs from the Z2 profile by the minor migrations of only two bands (results not shown).

Figure 2 shows a map of Victoria depicting the areas where *M. ulcerans* infection has been acquired.

TABLE 1. Summary of the sources and numbers of strains in each RFLP type

RFLP type	Probable source of infection	No. of strains
V1	East Gippsland	15
	Phillip Island	17
	Frankston/Langwarrin	5
	Warneet	1
	Unknown	2
V2	East Gippsland	3[a]
V3	Malaysia	1
	Darwin	1
Q1	North Queensland	10
	Papua New Guinea	1
Q2	North Queensland	1
Q3	Papua New Guinea	1
B1	Benin	3
B2	Benin	2
Z1	Zaire	3
Z2	Zaire	2
Z3	Zaire	2
Total		70

[a] Includes strain ATCC 19423, which originated in Bairnsdale, Victoria.

DISCUSSION

In the past, the majority of *M. ulcerans* strains isolated in Victoria were obtained from patients who acquired their infections in the east Gippsland area approximately 250 km east of Melbourne. This includes places such as Bairnsdale, Lochsport, Paradise Beach, and surrounding towns (Fig. 2) (6, 7). Two smaller foci of infection have been observed, in the Frankston/Langwarrin region (about 40 km south of Melbourne) (Fig. 2) and the Warneet/Tooradin area on Western Port (5). The occurrence of *M. ulcerans* infection in areas of rain forest such as Daintree and pockets of residual rain forest around Bairnsdale have been previously described (6). However, the development of a relatively large number of cases over the last 2 years in the locality of Phillip Island, 75 km south of Melbourne (Fig. 2), was unexpected and unexplained. It was initially postulated that the creation of a shallow swamp as a result of the construction of two earth embankments may be the source of infection in these patients (3). This event occurred simultaneously with the construction of roads in the epicenter of the outbreak, which involved the importation of sand and topsoil from an area above Western Port where previous cases have been reported. It has also been suggested that the recycling of sewage water for spray irrigation of the football field and golf course may also be responsible for local dissemination of *M. ulcerans*, since many of the cases occurred in this area (8). The epidemiological relationship between the strains from Phillip Island and those from other parts of Victoria was thus sought to determine whether this sudden cluster of infections was due to the introduction of a new strain into the area or to the reemergence of an existing strain.

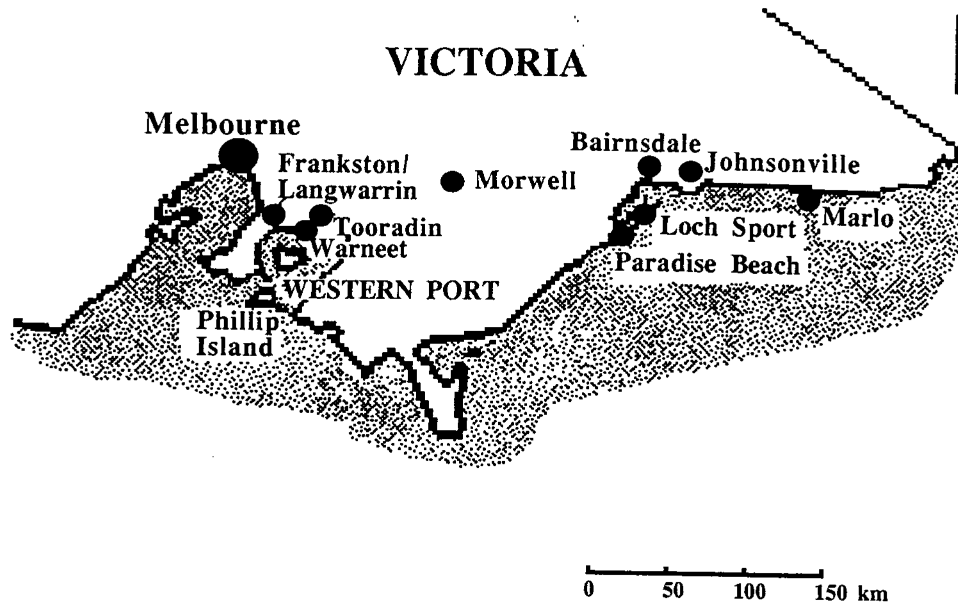

FIG. 2. Map of eastern Victoria, showing places mentioned in the text where *M. ulcerans* infection has occurred.

The plasmid pTBN12, which hybridizes to areas of repeated sequence in many mycobacterial species, has been successfully employed as a probe for typing strains of *M. tuberculosis*, *M. bovis*, and *M. kansasii* (2, 12, 13). The same technique was applied in this study to differentiate strains of *M. ulcerans*. The results of this study demonstrate that it is possible to genetically distinguish *M. ulcerans* strains from at least two different states in Australia and from two countries in Africa (Benin and Zaire).

The strains from Phillip Island yielded patterns (RFLP type V1) identical to those of strains acquired in east Gippsland, suggesting that they do in fact have a common ancestral origin. It is perhaps in retrospect not surprising that we were unable to differentiate the strains from these two areas and from the other foci of infection in Victoria. *M. ulcerans* is a very slowly growing organism and probably remains dormant in its natural environment for long periods. The natural mutation rate should therefore be very low.

There were three strains with RFLP type V2, which differed from the V1 pattern in the migration of only one band. This occurrence probably represents a rare point mutation in each strain. One of these strains was the American Type Culture Collection strain, which was first isolated in east Gippsland in the 1940s. The other two strains were isolated from two unrelated cases. One strain was from a 7-year-old boy who lives in Melbourne but holidays in Marlo, a coastal town 85 km east of Bairnsdale (Fig. 2). The second strain was isolated from a patient who lives in Morwell, which is midway between Phillip Island and Bairnsdale (Fig. 2). Although this patient has not visited either of these places, he may have acquired the infection after contact with ferns obtained from Johnsonville, which is east of Bairnsdale.

The two strains with RFLP type V3 do not appear to have originated in Victoria. One patient is believed to have acquired the infection in Darwin. The other patient may have acquired the infection in latent form in Malaysia, 6 years before onset of symptoms; this patient is not known to have been in an area where the infection is endemic since that time.

The majority of the Queensland strains demonstrated RFLP type Q1 patterns (Table 1). Most of these strains were isolated from patients who resided or had visited Cairns, the Daintree rain forest, or Townsville in north Queensland. One strain was obtained from a Papua New Guinea patient who recently visited Victoria for 3 months before the onset of symptoms. The occurrence of an RFLP type Q1 pattern in both Queensland and Papua New Guinea may in part be explained by the geographical closeness of these two places. This lends support to the hypothesis that *M. ulcerans* has occurred since the Jurassic period, when northern Australia and New Guinea were conjoined (4). However, another strain isolated from a patient from Papua New Guinea yielded a completely different RFLP pattern (type Q3).

The strain with type Q2 yielded a unique banding pattern, with several more bands than the patterns for the other strains. This strain was isolated from a patient who, ironically, was born in Bairnsdale, Victoria, but has lived in Daintree, Queensland, since 1958. This strain was morphologically unusual, and the histology of the patient's leg ulcer was also atypical in that there was excessive granulation tissue at the site. Sequencing of the hypervariable region of the 16S rRNA genes of this isolate (results not shown), however, confirmed that this organism was indeed *M. ulcerans* or a very closely related variant.

The Benin and Zaire strains yielded five additional RFLP types, which supports the discriminatory ability of the pTBN12 probe to differentiate strains of *M. ulcerans*. It is envisaged that continuing molecular typing of *M. ulcerans* strains from around the world may have great importance in understanding the epidemiology of infection, particularly since attempts to culture the organism from the environment have repeatedly failed.

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