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Detection of *Mycobacterium ulcerans* in Environmental Samples during an Outbreak of Ulcerative Disease

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Mycobacterium ulcerans **is an environmental bacterium which causes chronic skin ulcers. Despite significant epidemiological evidence to suggest that water is the source of infection, the organism has never been identified in the environment. Environmental water samples were collected from a small town in which an outbreak of 29 cases had occurred in a 3-year period. These were examined by mycobacterial culture and PCR amplification. Similar to previous studies, *M. ulcerans* was not cultured from the water samples. However, five samples were positive for *M. ulcerans* by PCR. These samples were collected from a swamp and a golf course irrigation system within the outbreak area. This is the first time that *M. ulcerans* has been demonstrated to be present in the environment and supports the postulated epidemiology of disease due to this organism.**

Mycobacterium ulcerans is an environmental mycobacterium that causes chronic progressive skin ulcers (4, 11). The ulcers do not respond reliably to antimicrobial therapy and, if left untreated, can result in severe scarring and contractual deformities (1). Epidemiological studies in many parts of the world have shown that cases occur in close proximity to slow-moving or stagnant bodies of water (5, 8, 9), suggesting that water is the primary source of infection. Possibly, *M. ulcerans* is incorporated into aerosols generated by gas bubbles from decomposing organic matter in stagnant water and distributed by wind. The bacteria may then be inoculated into intact, contaminated skin by minor injury, or they may colonize preexisting skin lesions, where they proliferate and produce ulcers (4, 10). Unfortunately, laboratory data to support this epidemiological model have not been able to be obtained due to the failure to isolate *M. ulcerans* from the environment despite numerous attempts (5, 9, 12, 13). The present study describes the adaption of a PCR-based assay developed for clinical diagnosis of *M. ulcerans* infections (15) to detect an environmental source of *M. ulcerans* during an outbreak of ulcerative disease.

Epidemiology. Before 1982, the annual number of cases diagnosed in Victoria, Australia, ranged between zero and four, all of which were associated with the eastern part of the state (7). However, between 1992 and 1995 there was an outbreak of cases near the small Victorian seaside town of Cowes on Phillip Island, 80 km southeast of Melbourne (3, 6, 7). Before 1992, there were no recorded cases of *M. ulcerans* infection on Phillip Island. Between September 1992 and December 1995, there were 29 such cases, of which 28 patients either lived in or close to Cowes or were frequent visitors to the area (7). Almost all of the cases were confined to a sparsely populated region to the east of the town center, and epidemiological investigation of this area revealed a number of possible environmental sources of infection (Fig. 1). These included a golf course (Fig. 1A, site 1), which lay at the center of the outbreak. This golf course was irrigated with water drawn from a storage dam (Fig. 1A, site 2) which contained natural groundwater supplemented by treated wastewater from a sewage plant approximately 10 km to the southwest of Cowes (Fig. 1A, site 4). A water pump (Fig. 1A, site 3) was used to pump water from this dam to a series of large sprinklers on the fairways and greens. This water was also used to irrigate a recreation reserve to the west of the golf course (Fig. 1A, site 5). On the western edge of the golf course was a naturally occurring, permanent, densely wooded swamp (Fig. 1A, site 6), while approximately 500 m east of the course was an area of swampy ground which drained into Western Port (Fig. 1A, site 7). The majority of the area to the east contained no significant permanent bodies of water. However, during 1991 and 1992 road surfaces on the eastern edge of Cowes were sealed and the excavated soil was used to build a fire access track that ran across the swamp (Fig. 1A, site 8). This caused water to accumulate on the western side of the track, resulting in the formation of a permanent swamp (Fig. 1A, site 9).

Water sampling and mycobacterial culture. A series of water samples were collected in October 1994 and 1 year later (Fig. 1B). Samples from the sewage recycling plant were taken before and after chlorination (Fig. 1B, samples f and g). The samples collected from the golf course irrigation pump were taken from a header tank that was used to prime the pump before operation (Fig. 1B, samples b and i). Other test samples included residential water taken from a rainwater tank within the outbreak area and the town drinking water supply (Fig. 1B, samples m and n). A series of control water samples were also collected in 1995 from distant sites around the state of Victoria where no cases of *M. ulcerans* infection had been recorded (Fig. 1B, samples o to v). Water samples collected on the two separate occasions in 1994 and 1995 were processed by different methods. The 1994 samples were concentrated by centrifugation before bacterial pellets were resuspended in one-quarter-strength Ringer's solution. Nonmycobacteria in the samples were inactivated by treatment with either NaOH or hexadecylpyridinium chloride. Bacterial suspensions were then inoculated into Bactec 12B broth (Becton Dickinson Instrument Systems, Sparks, Md.) and onto Brown and Buckle agar

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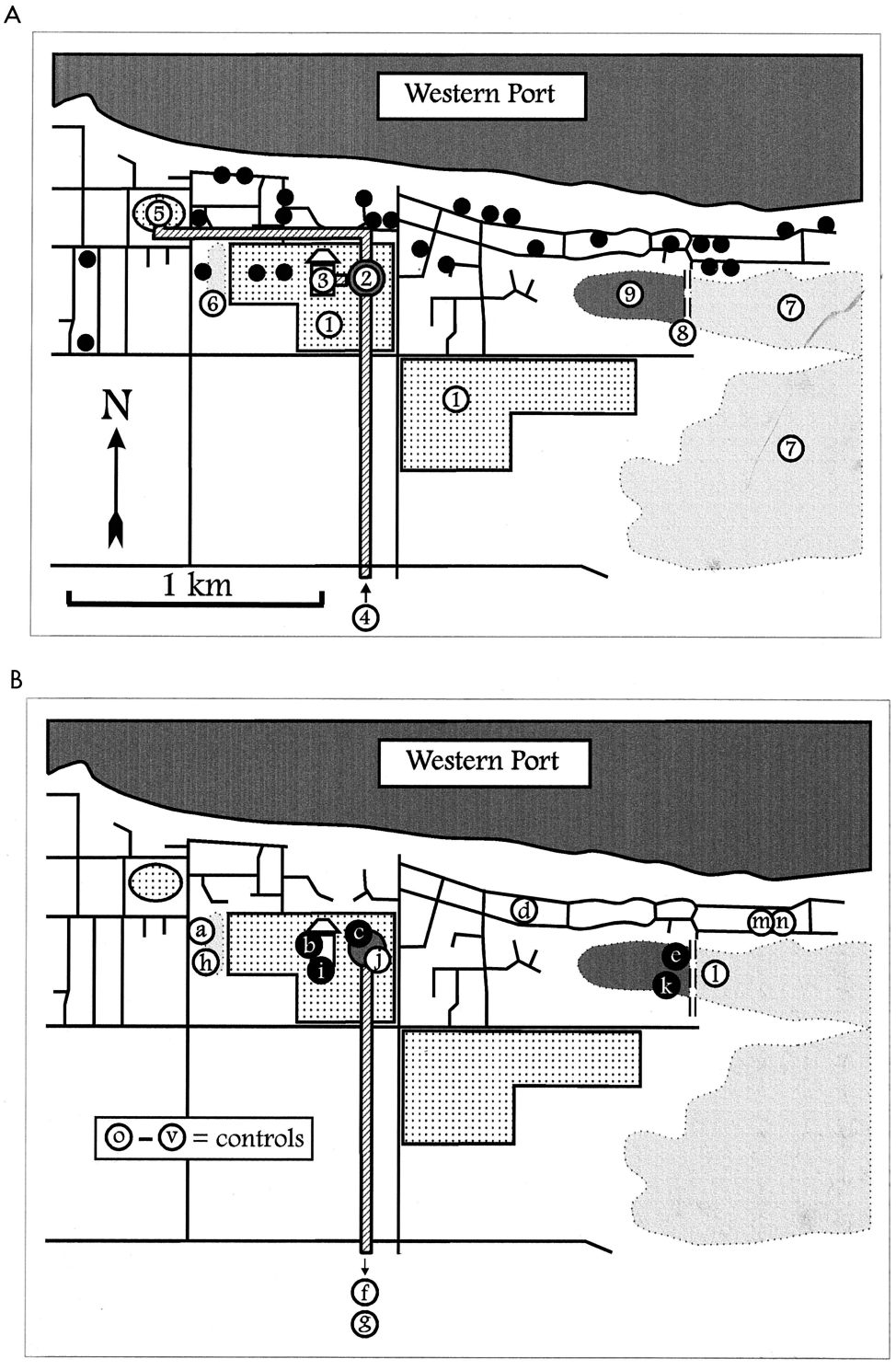

FIG. 1. Schematic diagram of the eastern part of the township of Cowes indicating the locations of cases, pertinent geographical features, and water sampling sites. (A) Locations of 27 cases of *M. ulcerans* are indicated as black circles. The major geographical features of the area are as follows: 1, golf course; 2, water storage dam; 3, water pump for golf course irrigation; 4, water supply from sewage recycling plant; 5, recreation reserve irrigated by water from storage dam; 6, natural swamp at western edge of golf course; 7, tidal swampy ground; 8, road built across swamp in 1992; 9, swamp created by road building. (B) Map indicating the locations from which water samples were collected in 1994 (a to e) and 1995 (f to v). Samples were collected from the swamp to the west of the golf course (a and h), the golf course water pump (b and i), the water storage dam (c and j), groundwater (d), the swampy area to the east of the golf course (e, k, and l), the water recycling plant (f and g), the rainwater tank (m), and residential drinking water (n), and a number of controls were sampled from sites distant from Phillip Island (o to v) where no cases of *M. ulcerans* had been recorded. The samples positive for *M. ulcerans* by PCR are indicated by white lettering in a black circle.

slopes (prepared in the Mycobacterial Reference Laboratory, Victorian Infectious Diseases Reference Laboratory). Cultures were incubated at 31°C and examined for growth each week for 6 months. The 1995 samples were concentrated by filtration through 47-mm-diameter, 0.4-μm-pore-size, sterile cellulose acetate membranes (Millipore HAWG 047 S1). After filtration, bacteria on the membranes were resuspended, and nonmycobacteria were inactivated by treatment with oxalic acid and inoculated into Bactec 12B broth. Cultures were incubated at 30°C and examined weekly for 6 months.

Mycobacteria were isolated from water collected in 1994 (Fig. 1B, samples a to e) from three of five sites. The mycobacteria were identified as *M. scrofulaceum* and *M. nonchromogenicum* (sample a), *M. terrae* complex (sample b), and *M. nonchromogenicum* (sample c). *M. ulcerans* was not isolated from any sample. Cultures of water collected in November 1995 (Fig. 1B, samples f to v) yielded mycobacteria from 4 of 17 sites, namely, f, i, j, and k. Growth was confirmed by staining the culture media for acid-fast bacilli, but the bacteria were not subcultured or identified to species level. PCR analysis of these cultures was negative for *M. ulcerans.*

DNA purification and PCR amplification. For DNA extraction, water concentrates were suspended in 0.2% sodium dodecyl sulfate–0.05 M NaOH and heated at 95°C for 15 min before homogenization with glass beads in a cell homogenizer (model MSK; Braun). After purification of the DNA, potential inhibitors of the PCR were removed by gel filtration chromatography adapted from a previously published method (17). The DNA was passed through a Biogel A-50 column (Bio-Rad Laboratories, Richmond, Calif.) containing 5% insoluble polyvinylpyrrolidone (Polyclar AT; BDH Laboratory Supplies, Poole, England). DNA was precipitated from the fractions and resuspended in water for PCR amplification.

PCR amplification. The target DNA sequence for PCR amplification is a highly repeated insertion sequence (16). The primer sequences and conditions for PCR amplification were as previously described (15). With each batch of samples, a positive and a negative control were included. The positive control was purified genomic *M. ulcerans* DNA with an estimated 10 genome equivalents. Reaction products were analyzed by Southern blot hybridization with a digoxigenin-labelled probe directed against the PCR product (15). Samples were considered positive if they yielded a 568-bp PCR product by Southern hybridization. To ensure that negative samples were free of PCR inhibitors, a further amplification was performed on all samples in the presence of an internal positive control consisting of 10 genome equivalents of *M. ulcerans* DNA.

Water concentrates and cultures from 1994 were stored at −20°C and tested by PCR before analysis of the 1995 specimens. Investigation of all water concentrates and cultures from both collection time periods resulted in five positive results, namely, in samples b, c, e, i, and k (Fig. 1B and 2). Concentrates of water collected from the golf course pump were positive in both 1994 and 1995. Positive results were also obtained from water taken in 1994 and 1995 from the swamp at the eastern end of the area. The 1994 sample was positive only after enrichment in Bactec 12B broth. The golf course dam was positive only once in a 1994 sample which was cultured prior to PCR analysis.

PCR of all positive samples yielded a 568-bp band in fraction 4 only. The Southern hybridization depicted in Fig. 2 was the most positive result obtained and represents a sample from the swamp to the east of the golf course. By comparison with the positive control, this probably represents less than 1,000 organisms per liter of water. No PCR product was obtained from other water samples or from the internal negative controls. PCR of negative samples with an internal positive control demonstrated that inhibitors of the reaction were removed from the samples by the purification procedure (data not shown). To confirm the specificity of the PCR, the mycobacterial isolates obtained from the water in 1994 and 1995 were examined by PCR and found to be negative (data not shown).

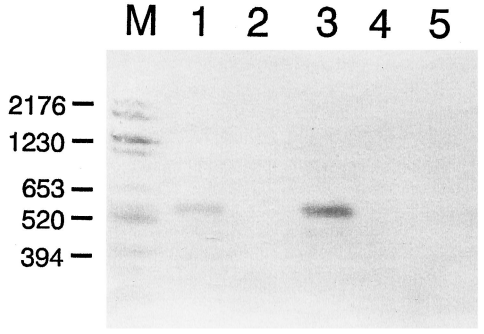

FIG. 2. Southern blot hybridization of PCR amplification products of DNA from an environmental water concentrate (Fig. 1B, sample k). Following electrophoresis through a 1% agarose gel, Southern blot hybridization was performed with an internal fragment of the amplicon as probe. Lanes contain molecular size markers (lane M), *M. ulcerans* DNA-positive control (lane 1), and fractions 3 to 6 of a Biogel-polyvinylpyrrolidone column (lanes 2 to 5). The expected size of the PCR amplicon is 568 bp. Numbers at left are molecular sizes in base pairs.

Discussion. This detection of *M. ulcerans* DNA by PCR is the first time that the presence of this organism has been detected in environmental water samples. However, the organism could not be cultured from any of the PCR-positive samples, which is similar to previous studies. These studies have examined a large number of epidemiologically incriminated water samples collected in areas of endemicity or during epidemics (5, 9, 12, 13). These data suggest that the organisms in the environment may be in a noncultivable state or in low numbers or may be overgrown by other mycobacteria in culture. Thus, PCR may be the only means of environmental detection of this organism.

Whether one or both of these two sites identified as containing *M. ulcerans* were responsible for the outbreak is difficult to determine. However, the occurrence of a relatively large number of cases in a sparsely populated area suggested that an irrigation system with the capacity to aerosolize bacteria efficiently over a wide area is more likely responsible for the majority of cases. This theory is consistent with the location of the golf course irrigation system at the center of the outbreak. The possibility that aerosolized bacteria from the swamp and the golf course were not the cause of the infections but were passively contaminated from an unknown source seems unlikely. They were the only significant bodies of water within the outbreak area, and other environmental specimens from the area tested negative.

Previous studies of infection with *M. ulcerans* have indicated an association with human-made bodies of water, but the bacterium has not previously been linked to artificial habitats such as reticulated water systems (14). Although this is the first time that *M. ulcerans* has been shown to be associated with and possibly transmitted by a water reticulation system, the colonization of artificial systems is a feature of several other mycobacteria, including *M. marinum*, *M. kansasii*, *M. xenopi*, *M. chelonae*, *M. gordonae*, and *M. avium* (2, 14).

The origin of the *M. ulcerans* strain responsible for the outbreak and the manner of its introduction to the Cowes

environment are open to conjecture. The environmental disturbance associated with the building of a road across the swamp 1 year before the outbreak offers a possible explanation for the initiation of the outbreak. This swamp may have provided a suitable environment for growth of *M. ulcerans*, which led to the contamination of the irrigation system. It is interesting to note that the construction company used to supply sand for the road construction has a quarry within an area where cases of *M. ulcerans* were reported prior to the Cowes outbreak (7). However, testing of this quarry site by PCR failed to reveal the presence of *M. ulcerans* DNA.

In summary, we have used PCR to detect *M. ulcerans* DNA for the first time in water samples, thereby supporting the postulated epidemiology of disease due to this organism. Our finding suggests that, like many other mycobacteria, *M. ulcerans* is able to colonize artificial aqueous environments, from which it may have an enhanced capacity to cause human disease. The PCR assay provides a method of detecting this organism during investigations of outbreaks and of monitoring water sources for the control and prevention of *M. ulcerans* infections.

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