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Immunomagnetic Separation and PCR for Detection of *Mycobacterium ulcerans*

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We have developed a technique based on the use of monodisperse magnetic beads to isolate *Mycobacterium ulcerans* from heterogenous mixtures, prior to PCR amplification. Using this method, we were able to detect *M. ulcerans* in water samples taken from Phillip Island, Australia, the site of several outbreaks of *M. ulcerans* disease in recent times.

Infection with *Mycobacterium ulcerans* produces progressive, necrotizing skin ulcerations in humans. Treatment of the lesions is usually by surgical excision, as chemotherapy is ineffective in halting the progression of the infection. First reported in Australia in 1948 (6), the disease is known to occur in many tropical regions throughout the world. Despite several epidemiological studies of this organism, the identity of the natural reservoir, the route of infection, and the mechanism of transmission are yet to be established. This may be due to the fact that conventional isolation techniques are based on culture methods, which are prone to contamination by less fastidious organisms. The detection of organisms in clinical and environmental samples using PCR overcomes this type of limitation. However, false-negative results may occur if polymerase inhibitors, commonly present in such samples are not removed prior to amplification.

Immunomagnetic separation (IMS) has recently been shown to be an effective method for isolating both eukaryotic cells (8) and prokaryotic organisms (2) from complex substances such as food (5), sewage effluent, and blood (7). This technique uses monodisperse magnetic beads coated with antibodies against surface epitopes of the bacteria of interest, to isolate the organisms from heterogenous samples. In this way, the bacteria are concentrated, and substances which may be inhibitory to subsequent analyses are effectively removed. Combined with PCR, this method promises to provide a sensitive detection assay for organisms which may otherwise go undetected. This report describes a rapid method for the isolation and identification of *M. ulcerans*, using an integrated immunomagnetic PCR technique.

Bacterial strains and growth conditions. *M. ulcerans* strains were cultured on Lowenstein-Jensen medium at 30°C until growth was confluent and then subcultured in Dubos broth supplemented with 5% sheep serum for 8 weeks at 30°C with constant agitation. Other mycobacterial species were cultured on nutrient agar.

Production and purification of IgG. Antibodies were raised in mice against a variety of *M. ulcerans* isolates. Serum was harvested, and high-titer fractions were pooled. Immunoglobulins (Ig) were precipitated with 50% ammonium sulfate and purified by using a DEAE Affi-Gel Blue column (Bio-Rad) according to the manufacturer's instructions.

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Direct coating of the immunomagnetic particles. The amount of antibody required for optimal coating of the immunomagnetic particles was determined to be 15 μg of IgG in a final volume of 100 μl. This was incubated with 15 μl of 2.8-μm-diameter immunomagnetic particles (6×10^8 to 7×10^8 beads per ml) precoated with sheep anti-mouse IgG (Dynabeads M-280; Dynal), with bidirectional mixing for 24 h at 4°C. Beads were washed three times for 5 min each with phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) (PBS-BSA), resuspended in 100 μl of PBS-BSA, and stored at 4°C (Fig. 1a).

Indirect coating of the immunomagnetic particles. Indirect coating differs from the approach described above in that the organisms are first incubated with antibody and added to the beads secondarily (Fig. 1b). Samples were incubated with an excess of antibody at 4°C for 24 h, centrifuged, and washed, and the pellets were resuspended in PBS-BSA. The antibody-coated cells were then incubated with 15 μl of the coated beads for 24 h at 4°C with bidirectional mixing and washed as described above.

IMS. A final volume of 15 μl of coated beads was used per ml of test sample. The samples were incubated with the beads for 1 h at 4°C with bidirectional mixing, washed three times in PBS-BSA, and resuspended in a final volume of 200 μl of the same buffer. The bead-bacterium aggregates were beaten with 100 μl of 0.1-μm-diameter glass beads (Sigma) for 3 min to disrupt the cells and then heated to 95°C for 10 min to heat kill any remaining viable bacteria. The samples were stored at 4°C until PCR testing.

Detection of *M. ulcerans* in spiked samples. A series of pilot experiments was used to assess the efficiency of the IMS procedure. One trial used dilutions of *M. ulcerans* isolate 186510 ranging from 10^6 to 10^1 organisms incubated with the coated beads. Ten microliters of the suspension was placed on a glass slide with 0.3 mg of acridine orange per ml and examined under a fluorescent microscope. Aggregates were often seen, due to the bacteria binding to several particles simultaneously. Attached bacteria could be visualized at concentrations down to 10^4 organisms per ml. A second experiment used a suspension of one of five mycobacterial species (*M. chelonei*, *M. fortuitum*, *M. marinum*, *M. phlei*, and *M. smegmatis*) at a concentration of 10^6/ml, spiked with serial dilutions of *M. ulcerans* ranging from 10^6 to 10^2/ml. This mixed suspension was incubated with the coated beads, and aliquots of the supernatant were subsequently plated onto Middlebrook agar and incubated at 37°C for 3 days. As *M. ulcerans* will not grow in this period, the number of other mycobacterial cells not bound to

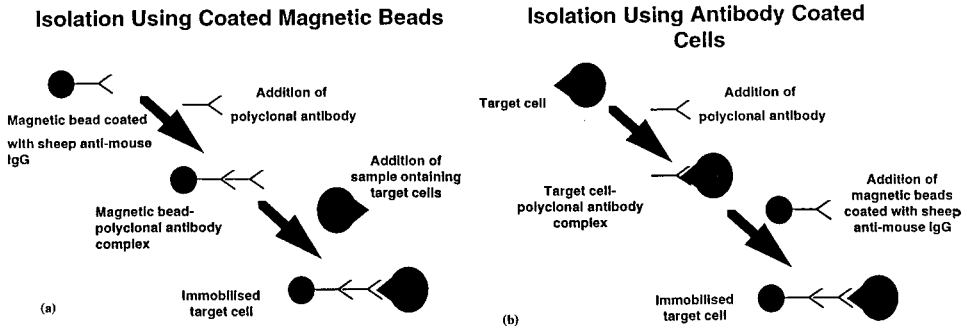

FIG. 1. Schematic representation of the direct (a) and indirect (b) techniques used for IMS. The direct method uses beads coated with antibody, which are then added to the cell suspension. The indirect method uses cells incubated with an excess of antibody which are secondarily added to the bead suspension.

the beads could be determined. It was found that between 70 and 85% of the initial mycobacterial cells added to the tubes had bound to the beads. This figure remained relatively constant, regardless of the number of *M. ulcerans* cells added to the samples.

Nested PCR. The reaction mixture contained 1× PCR buffer, 2 mM $MgCl_2$, 100 μM each deoxynucleoside triphosphate, 20 pM each primer (Table 1) (4), 1 U of *Taq* polymerase per 50 μl, and 1 μl of DNA. The profile consists of 30 cycles of 1 min at 94°C, 1 min at 64°C, and 1 min at 72°C. The second PCR uses 1 μl of the first PCR as template and uses 20 cycles each consisting of 30 s at 95°C, 60 s at 68°C, and 90 s at 72°C. DNA from a clinical sample and water were used as positive and negative controls, respectively. A successful PCR yielded amplification products of 384 and 185 bp. Amplification of IMS-treated spiked river water samples could detect approximately 10 organisms; for samples that were not pretreated, 10^3 organisms were necessary for detection. Samples containing mixed mycobacterial cultures allowed identification of *M. ulcerans* following the second round of amplification, to 100 organisms. All samples were positive for the external 384-bp product, which does not differentiate between mycobacterial species. The indirect IMS treatment produced consistently better results than direct IMS treatment, producing an increase in sensitivity of up to 10-fold (Fig. 2). There was no difference in sensitivity, however, following the second amplification cycle (Fig. 3).

Analysis of environmental samples. Water samples were collected from several sites on Phillip Island in Victoria, Australia, the site of recent outbreaks of *M. ulcerans* infection. Following IMS-PCR, approximately 10% of samples returned positive results for the 185-bp amplification product, out of a total of 150 tested.

Conventional cultural methods for the isolation and identification of *M. ulcerans* are time-consuming and prone to contamination with less fastidious organisms. PCR assays have provided rapid diagnoses for a number of diseases and are particularly useful for slow-growing or fastidious organisms, such as *M. ulcerans*. However, PCR performed directly on clinical and environmental material has been reported to show lower sensitivity than when performed on purified DNA (9), due to polymerase inhibitors often being present in these samples. To remove such inhibitors, several workers have performed DNA purification techniques prior to PCR amplification (1).

To assess how well the technique could detect low numbers of target organisms present in highly contaminated samples, we mixed 10-fold dilutions of *M. ulcerans* with nontarget bacteria. When these samples were amplified, we were able to show that against a population of 10^6 nontarget organisms, the beads were able to capture approximately 100 organisms. This will prove particularly useful for attempts to isolate *M. ulcerans* from the environment.

The majority of isolations of *M. ulcerans* from clinical specimens in Australia have come from two geographically isolated regions. Mossman, in far-north Queensland has been the site of a number of cases among the aboriginal population, and Phillip Island is the site of several more recent outbreaks (3). Despite several epidemiological studies in both areas, the organism has never been isolated from the environment. This is primarily due to the need, in the past, to culture the organism and make a definitive identification based on biochemical profiles. Water, soil, and vegetation samples were collected from these areas and subjected to the IMS technique followed by PCR. Water samples produced positive results in many cases for *Mycobacterium* species, several of which were determined to be *M. ulcerans*.

TABLE 1. Sequences and locations of primers used in the *M. ulcerans* PCR

Primer	Sequence (5′-3′)	Position in gene[a]	Direction of amplification
My-1	gAgATCgAgCTggAggATCC	197–216	Forward
My-2	AgCTgCAgCCCAAAggTgTT	535–554	Reverse
Mu-65[b]	CCgAgACCCTgCTCAAATCg	382–402	Forward

[a] Plus strand in the 5′-to-3′ direction.
[b] Mu-65 is biotinylated.

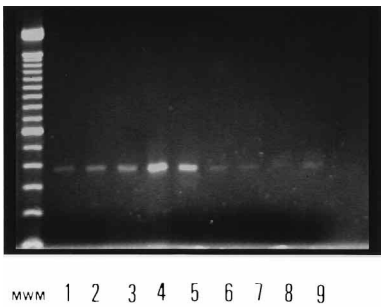

FIG. 2. External PCR of river water samples spiked with known quantities of *M. ulcerans*. Lanes 1 to 3, direct IMS treatment; lanes 4 to 6, indirect IMS treatment; lanes 7 to 9, no treatment prior to PCR. Lanes 1, 4, and 7, 10^6 organisms; lanes 2, 5, and 8, 10^5 organisms; lanes 3, 6, and 9, 10^4 organisms. Lane MWM, molecular weight standards (100-bp ladder [Promega]).

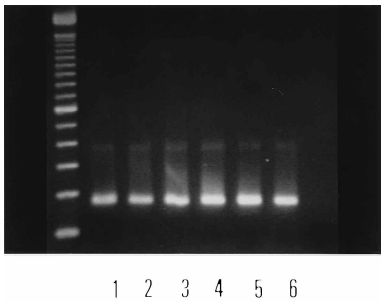

FIG. 3. Internal PCR (185 bp) of *M. ulcerans* using an aliquot of 1 μl from the external PCR as template. Lanes 1 to 3, direct IMS treatment; lanes 4 to 6, indirect IMS treatment. Lanes 1 and 4, 10^6 organisms; lanes 2 and 5, 10^5 organisms; lanes 3 and 6, 10^4 organisms. Lane MWM, molecular weight standards (100-bp ladder [Promega]).

We thank Zeta Blacklock for supplying Queensland isolates of *M. ulcerans* and the reference strain of *M. marinum* and Kathy Jackson and David Leslie for supplying the Victorian isolates of *M. ulcerans*. Thanks are also due to Paul Johnson for the tour of Phillip Island and helping with the collection of samples there and to May Smith for providing the environmental samples from north Queensland.

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