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One-tube cell lysis and DNA extraction procedure for PCR-based detection of *Mycobacterium ulcerans* in aquatic insects, molluscs and fish

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The purpose of this study was to develop a simple procedure for cell lysis and DNA extraction for direct detection of *Mycobacterium ulcerans* in aquatic insects, gills and intestinal contents of fish, molluscs and human tissue samples using a nested PCR method specific for the insertion sequence IS*2404*. The simultaneous action of sodium *N*-lauroyl sarcosine, guanidinium isothiocyanate, chloroform and Tris-saturated phenol on mycobacteria, followed by a DNA purification method using mini-columns fitted with silica-cellulose membranes was successfully employed to extract DNA from cultured bacteria, environmental and human tissue samples. All specimens were collected from Buruli ulcer endemic regions. *M. ulcerans* DNA was detected in 11 of 57 aquatic insects, one of six molluscs and three of 15 fish, supporting the hypothesis that the fauna of major Buruli ulcer endemic foci in swampy terrain of tropical and subtropical regions can be a source of *M. ulcerans* infection.

INTRODUCTION

Mycobacterium ulcerans is a human pathogen causing chronic necrotic skin diseases (MacCallum *et al.*, 1948; Meyers *et al.*, 1974; Hayman & McQueen, 1985; van der Werf *et al.*, 1999). The environmental sources and the mode of transmission of *M. ulcerans* are not definitely known. Molecular techniques are, however, making important inroads in unravelling the identification of likely sources of *M. ulcerans*. The discovery of *M. ulcerans*-specific insertion sequence IS*2404* (Ross *et al.*, 1997b) permits the identification of the bacterium by PCR directly from environmental samples. Molecular detection of *M. ulcerans* from environmental sources presents numerous problems: the concentration of bacilli in the natural environment is low [estimated at 0·5 cells per 100 ml of water and 40 cells per 100 g of detritus (Stinear *et al.*, 2000)], effective lysis of mycobacteria is difficult, and PCR inhibitors (e.g. humic acids and phenolic substances) are not completely removed by standard DNA extraction protocols. Three methods have been employed to overcome some of these difficulties. The first study of environmental sources of *M. ulcerans* employed gel filtration chromatography using a column containing polyvinylpyrrolidone (PVP) for removal of PCR inhibitors such as humic acids and phenolic substances from soil (Ross *et al.*, 1997a). A second method used antibodies to *M. ulcerans* to coat magnetic beads which attracted and concentrated the bacteria (Roberts & Hirst, 1997). The third method was a sequence capture-PCR procedure in which *M. ulcerans* DNA sequences were concentrated, and irrelevant DNA along with other potential inhibitors of the amplification reaction were removed (Stinear *et al.*, 2000).

The above methods for *M. ulcerans* detection in environmental samples involve elaborate and laborious sample preparation procedures that are prone to contamination. Development of DNA extraction procedures that are less sophisticated and prone to contamination would be most useful. Here, we propose a one-tube cell lysis and DNA extraction procedure for PCR-based detection of *M. ulcerans* directly from insects, molluscs and gills and intestinal contents of fish.

METHODS

Bacterial strains and growth conditions. Bacterial strains investigated in this study are listed in Table 1. *Mycobacterium avium* and *M. ulcerans* strains were cultured on Löwenstein-Jensen (L-J) medium at 37 °C for 1 month and at 33 °C for 3 months, respectively, while the *Mycobacterium tuberculosis* strain was cultivated at 37 °C for 2 months. Cultured *M. avium* strains were used as a model for optimization of the DNA extraction procedure because colonies of this organism are easily homogenized in TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8·0). The yield of extraction and purity of DNA were determined in ten *M. ulcerans* isolates.

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Abbreviations: BU, Buruli ulcer; GITC, guanidinium isothiocyanate; LCTP, lysis buffer, chloroform, Tris-saturated phenol.

Collection of specimens. A total of 78 environmental specimens collected from Buruli-ulcer (BU)-endemic regions in Benin were analysed: aquatic insects ($n = 57$) belonging to the families Belostomatidae (*Appasus* sp.), Dytiscidae, Hydrophilidae and Naucoridae (*Naucoris* sp., *Macrocoris* sp.), six small molluscs (*Bulinus senegalensis*), five *Tilapia* and ten small fish (not classified). In addition, five samples of human tissue from patients with BU were investigated and three samples of transport medium previously inoculated with human tissue (modified Dubos medium) (Table 2). Environmental and human tissue samples weighing up to 200 mg were homogenized in 4 ml TE buffer. Aliquots of 2 ml were used for culture on L-J medium at 30 °C and microscopic examination. The remaining two 1 ml suspensions of each sample were subjected to nested-PCR assay.

Extraction of chromosomal DNA

Optimization of DNA extraction from *M. avium*. Cultures of five *M. avium* strains were harvested and suspended in 5 ml TE buffer. Eighty Eppendorf tubes with equal volumes of the five-strain-cocktail of *M. avium* suspensions ($\sim 10^9$ c.f.u.) were prepared and centrifuged at 4000 ***g*** for 5 min. The resulting pellets were subjected to simultaneous lysis and DNA extraction varying the following to determine the optimal conditions for maximum yield of extracted DNA: different concentrations of sodium *N*-lauroyl sarcosine (Sarcosyl) in lysis buffer (Fig. 1), different proportions of lysis buffer, chloroform and Tris-saturated phenol (Fig. 2) and different incubation temperatures (Fig. 3). After cell lysis, samples were centrifuged at 10 000 ***g*** for 20 min at 4 °C. The supernatants were transferred to new tubes, precipitated with absolute ethanol and resuspended in 50 µl 0·5× TE buffer. Subsequently, 10 µl of chromosomal DNA was subjected to electrophoresis using 1 % agarose gels. The gels were stained with ethidium bromide and visualized under UV light. Densitometric measurement of chromosomal DNA from gels (TotalLab 1; Nonlinear Dynamics) was used for determination of the relative quantity of DNA.

Extraction of chromosomal DNA from environmental specimens and bacterial cultures. Two 1 ml suspensions of each sample were centrifuged in screw-cap tubes at 4 °C, 4000 ***g*** for 15 min. Supernatants were removed and the pellets suspended in 150 µl lysis buffer [10 mM

Table 2. Detection rates of positive samples in nested PCR

Specimens	IS*2404*-PCR positive (%)	Total no.
Aquatic insects (whole)	11 (19)	57
Belostomatidae		
Appasus sp.	6	43
Macrocoris sp.	1	2
Dytiscidae	0	1
Hydrophilidae	1	1
Naucoridae		
Naucoris sp.	3	10
Molluscs (whole)	1	6
Bulinus senegalensis	1	6
Fish (gills and content of intestines)	3 (20)	15
Tilapia sp.	1	5
Small fish	2	10
Human (ulcerative lesions)	8 (100)	8
Skin, muscle and adipose tissues	5	5
Transport medium previously inoculated with human tissue	3	3

Table 1. Bacterial strains tested in the present study and amount of DNA extracted from bacteria

ATCC, American Type Culture Collection; ITM, Institute of Tropical Medicine in Antwerp.

Species and strain no.	Origin	DNA [mg (g cells)$^{-1}$]	Purity (OD 260/280)
M. avium			
ATCC 15769	Chicken		
ATCC 25291	Chicken		
ITM 96-348	Cat, Belgium	3 ± 0·4	1·85
ITM 96-799	Bovine, Belgium		
ITM 2672	Goat, Norway		
M. ulcerans			
ATCC 19432	Australia	4·00	1·74
ITM 97-104	Benin	5·28	2·00
ITM 92-9146	Benin	5·26	1·65
ITM 98-912	China	1·99	1·65
ITM 94-5151	Congo	2·26	1·81
ITM 97-483	Ghana	1·97	1·63
ITM 94-511	Ivory Coast	4·02	2·16
ATCC 33728	Japan	3·58	2·03
ITM 94-1328	Malaysia	3·75	1·68
ITM 5150	Zaire	5·73	1·93
M. tuberculosis			
ATCC 35827	H37Rv-Km-Res	5·15	1·74

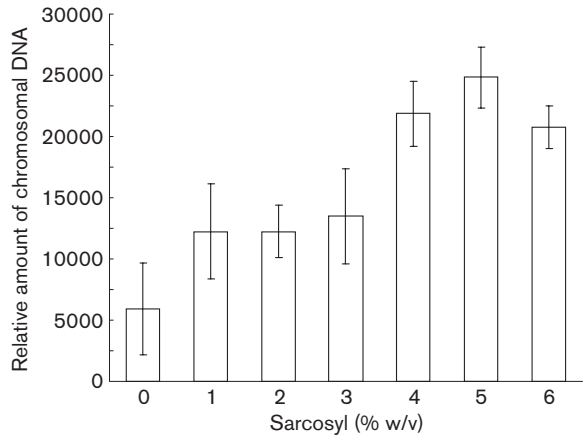

Fig. 1. Different concentrations of Sarcosyl in lysis buffer used for extraction of five-strain-cocktail of *M. avium* chromosomal DNA. Intensity of chromosomal DNA was measured using TotalLab 1. Results are given as mean values from three different experiments ± SD.

Tris/HCl, pH 8·0; 5 mM EDTA, pH 8·0; 4 M guanidinium isothiocyanate (GITC), pH 7·5; 50 g Sarcosyl l^{-1}, 2·5 g SDS l^{-1}, 5 g sodium citrate l^{-1} and 5 g Triton X-100 l^{-1}]. Volumes of 300 µl chloroform and 300 µl Tris-saturated phenol (pH 6·9) were added to each tube. For DNA extraction from mycobacterial cultures, two or three loops of cells were scraped from L-J slants and suspended in lysis buffer without homogenization, and subsequently mixed with chloroform and Tris-saturated phenol. The samples were placed at −20 °C for 1 h. Subsequently, samples were centrifuged in tubes at 4 °C for 20 min at 10 000 ***g***. Supernatants were transferred to fresh tubes. 2-Propanol to 1/4 volume of the supernatants was added and the mixtures loaded onto silica-cellulose membranes in columns (A & A Biotechnology, Gdynia, Poland). Samples were allowed to filter through the membrane by gravity. The membranes were washed twice with 300 µl absolute ethanol (by gravity). DNA was eluted with 400 µl hot (about 75 °C) TE buffer (by gravity) and precipitated with two portions of absolute ethanol. The resulting pellets were suspended in 25 µl 0·5× TE buffer (pH 8·0) and stored at −20 °C until further analysis. The DNA concentration was measured using a Genesys spectrophotometer (Spectronic Unicam, Rochester, NY, USA).

To assess the influence of components of the lysis buffer, Tris-saturated phenol and chloroform on the viability of mycobacteria, about 10^9 *M. ulcerans* ATCC 19432 and *M. avium* ATCC 15769 cells, harvested from L-J slants were suspended in 150 µl lysis buffer mixed with 300 µl Tris-

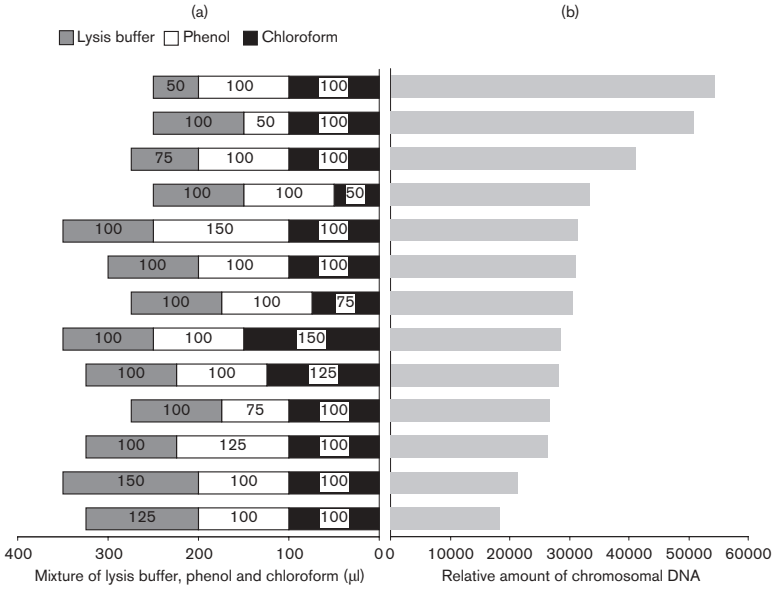

Fig. 2. Extraction of chromosomal DNA from five-strain-cocktail of *M. avium*. (a) Different volumes of phenol, chloroform and lysis buffer used; (b) relative amount of chromosomal DNA measured by TotalLab 1 software. Results are given as the sum of relative amounts of genomic DNA measured from the three series.

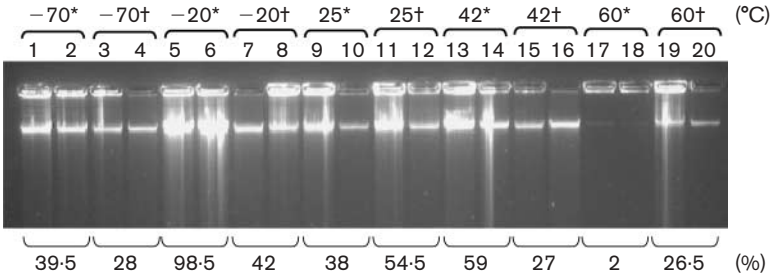

Fig. 3. Extraction of chromosomal DNA from five-strain-cocktail of *M. avium*. Relative amount of chromosomal DNA measured by TotalLab 1 software. *Extraction with phenol/chloroform, †extraction without phenol/chloroform.

saturated phenol (pH 6·9) and 300 µl chloroform. After a freezing stage at −20 °C for 1 h and centrifugation for 20 min at 10 000 ***g***, the intermediate phases, which are likely to contain viable bacilli, were suspended in 1 ml PBS (8 g NaCl, 0·2 g KCl, 1·44 g Na_2PO_4, and 0·24 g KH_2PO_4 in 1 l distilled water, pH 7·4). Suspensions of 100 µl were inoculated onto each of five L-J and five Middlebrook 7H12 media plates and incubated at 30 °C. The L-J slants were checked for growth after 1 and 3 months, while cultures in the BACTEC 460 TB instrument (Becton Dickinson) were read every second day for 1 month.

PCR conditions, DNA electrophoresis, restriction analysis and sequencing. The IS*2404* fragment was amplified in a nested format using the following combinations of primers PGP1, 5′-AGGG-CAGCGCGGTGATACGG; PGP2, 5′-CAGTGGATTGGTGCCGATC-GAG (this study) and PGP3, 5′-GGCGCAGATCAACTTCGCGGT; PGP4, 5′-CTGCGTGGTGCTTTACGCGC (Guimaraes-Peres *et al.*, 1999). The expected sizes of PCR products in the first and second PCR runs are 332 and 218 bp, respectively. For the first round of nested PCR, 5 µl of the DNA extract was added to 45 µl PCR mixture containing 20 pM PGP1 and PGP2, 1 U AmpliTaq DNA polymerase (Roche), 0·2 mM each dNTP, 10 mM Tris/HCl (pH 8·4), 1·5 mM $MgCl_2$, 50 mM KCl and 0·1 % Triton X-100, and overlaid with mineral oil. Cycling conditions were as follows: denaturation at 94 °C for 5 min; amplification for 40 cycles of 94 °C for 45 s, 64 °C for 45 s and 72 °C for 45 s and a final extension at 72 °C for 10 min. For the second PCR, 1 µl of the first-run product was amplified in a 25 µl reaction mixture with 10 pM PGP3 and PGP4 primers at an annealing temperature of 64 °C for 35 cycles. Seven microlitres of amplified DNA was subjected to electrophoresis using 2 % agarose gels. The gels were stained with ethidium bromide and visualized under UV light. The nested PCR-assays were performed in duplicates on the same collection of samples. The nested PCR assay was repeated three times when we had discrepant results. In these cases, the final result was scored 'positive' when we had two positive results and negative when we had two 'negative' results. In the assessment test for the estimation of a suitable volume of template DNA for the first round run in the nested PCR assay 2, 5, 7 and 10 µl genomic DNA were used for detection of *M. ulcerans* in a set of 11 aquatic insects. The specificity of the nested PCR reaction was determined by digesting the second round PCR products with the endonucleases *Rsa*I and *Hpa*II. The PCR products were cloned using the TOPO TA cloning kit (Invitrogen).

RESULTS AND DISCUSSION

Optimization of cell lysis and DNA extraction

Mycobacteria have a mycolate-rich cell wall that is difficult to lyse using lysozyme, proteinase K and other enzymes. Mechanical protocols for disruption of mycobacteria using Mini-BeadBeaters are very efficient for pure cultures (Via & Falkinham, 1996; Zhang & Ishaque, 1997, Kim *et al.*, 2001) but need to be modified, depending on the chemical composition and rheological properties of samples.

In the present study, we have developed a one-step procedure of cell lysis and DNA extraction from aquatic insects, gills and intestinal contents of fish, molluscs and human tissue samples. Due to the tendency of *M. ulcerans* cells to form clumps in suspension (Stinear *et al.*, 2000), *M. avium* strains were used to optimize the DNA extraction procedure. The inclusion of Sarcosyl in this study as a component of lysis buffer yielded the maximum amount of genomic DNA at 5 % concentration (Fig. 1). In addition, SDS at 0·25 % (w/v) concentration was found to be soluble in 4 M GITC and incorporated in the lysis buffer to enhance cell lysis. Application of lysis buffer, chloroform and Tris-saturated phenol (LCTP) in the ratio of 1 : 2 : 2 (Fig. 2) for simultaneous cell lysis and DNA extraction made it possible to obtain the highest relative amount of DNA (Fig. 3). A concentrated solution of GITC is among the most effective protein denaturants and nuclease inhibitors (Gordon, 1972; Chomczynski & Sacchi, 1987; Nelson & Krawetz, 1992), which is useful in DNA extraction protocols for mycobacteria (Noordhoek *et al.*, 1995; Chakravorty & Tyagi, 2001). Hippel & Wong (1964) found that the higher destabilizing effect of the thiocyanate anion combined with the guanidinium cation on the native form of collagen, ribonuclease and DNA makes GITC a much more effective denaturing agent than guanidinium chloride.

The novel use of a phenol/chloroform mixture, together with GITC and Sarcosyl at −20 °C in our DNA extraction procedure significantly improved the yield of extracted DNA from *M. avium* (Fig. 3, lanes 5 and 6) in comparison to the yield from samples treated only with lysis buffer without phenol and chloroform (Fig. 3, lanes 7 and 8). Phenol dissolves proteins (Cohn & Conant, 1926) and lipids leaving water-soluble matter (carbohydrates, nucleic acids, etc.) in the aqueous layer (Kirby, 1956), while the small hydrophobic molecules of chloroform diffuse through the cellular membrane into the cytoplasm leading to cell lysis and inactivation of DNases (Reyes & Latorre, 1979; Da Silva Tatley *et al.*, 1992). The anticoagulant agent sodium citrate and non-ionic detergent Triton X-100 were included in lysis buffer to prevent the aggregation of mycobacteria in homogenized samples, hence facilitating the interaction between the mycobacterial cell wall and components of the LCTP mixture.

Ziehl-Neelsen staining of *M. avium* smears showed remarkable differences in the morphological appearances before (Fig. 4a) and after cell lysis (Fig. 4b). All the cell lysates of *M. ulcerans* ATCC 19432 and *M. avium* ATCC 15769 resulting from the chemical treatments, showed no growth on L-J and Middelbrook 7H12 (BACTEC 12B) media after 3 and 1 months incubation, respectively.

Binding of DNA in the presence of a chaotropic agent to silica or glass is well known (Vogelstein & Gillespie, 1979; Yang *et al.*, 1979; Marko *et al.*, 1982). This concept has been used in the purification of nucleic acids directly from human serum and urine by Boom *et al.* (1990). In the present study, mini-columns fitted with silica-cellulose membranes were used for DNA purification from clinical and environmental samples, except soil, for PCR-based detection of *M. ulcerans*. A limitation of the application of this method to soil samples is that DNA in the presence of 4 M GITC, is adsorbed to silica in soil and cannot be recovered easily in the aqueous phase after phenol/chloroform extraction. The purity of extracted DNA was in the range 1·63–2·16. The use of mini-columns in purifying DNA from environmental and human tissue samples clearly improved the sensitivity of the assay (Fig. 5b) compared to the samples that were not subjected to

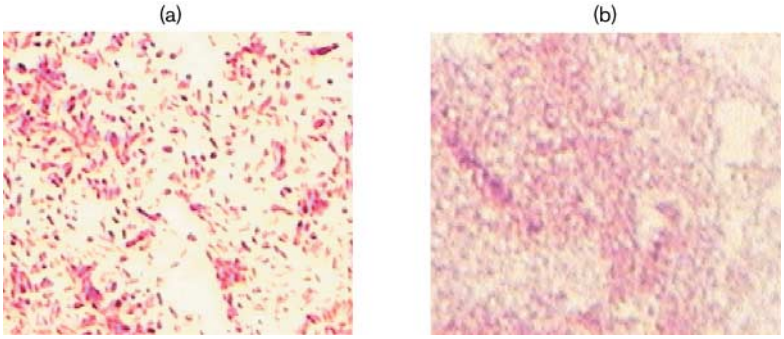

Fig. 4. Five-strain-cocktail of *M. avium* after Ziehl-Neelsen staining, original magnification, ×100. (a) *M. avium* cells before lysis; (b) *M. avium* cells after cell lysis using DNA extraction procedure after optimization.

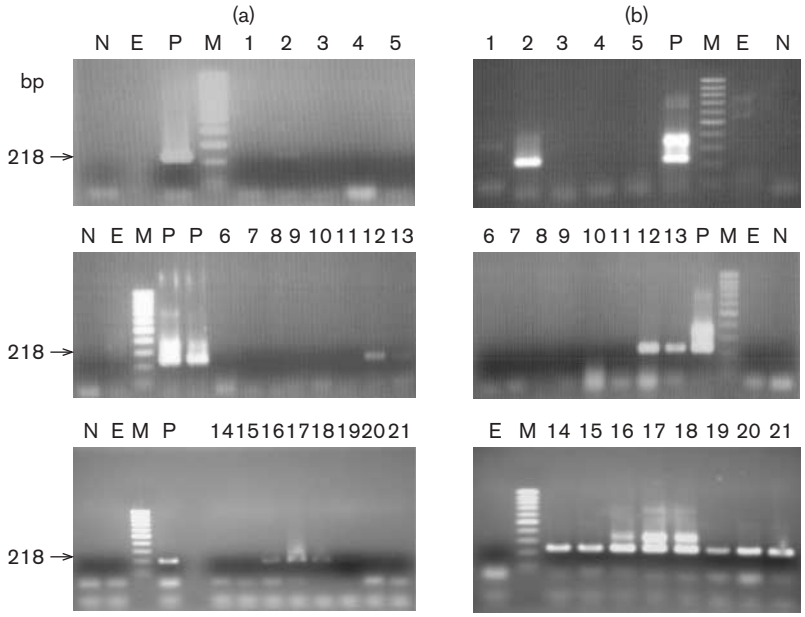

Fig. 5. Specific detection of *M. ulcerans* IS*2404* from *B. senegalensis*, *Tilapia* gills and intestines and human tissue samples from BU patients by nested PCR. (a) Samples after chemical lysis and DNA extraction followed by ethanol precipitation; (b) samples after chemical lysis and DNA extraction followed by column purification and ethanol precipitation. Lanes: 1–5, *B. senegalensis*; 6, 8, 10, 12, *Tilapia* gills; 7, 9, 11, 13, *Tilapia* intestines; 14–16, human skin, muscle and adipose tissues; 17, 18, human skin and adipose tissues; 19–21, transport medium; P, positive control (*M. ulcerans* ATCC 19432); N, negative control (PCR mastermix); E, negative control (blank test of DNA extraction); M, DNA size marker (100 bp ladder; MBI Fermentas).

column purification (Fig. 5a). Boddinghaus *et al.* (2001) also demonstrated the effectiveness of the use of silica membranes in the removal of PCR inhibitors.

The medium yield of 3 ± 0·4 mg DNA g^{-1} from *M. avium* and 3·8 ± 1·4 mg g^{-1} from *M. ulcerans* cells harvested from L-J slants is comparable to other DNA extraction methods for slow-growing mycobacteria (Belisle & Sonnenberg, 1998; Santos *et al.*, 1992; Via & Falkinham, 1996; Zhang & Ishaque, 1997). The mean yield of genomic DNA for ten different *M. ulcerans* strains was 3·8 ± 1·4 mg DNA (g cells)$^{-1}$ of (Table 1). In the assessment test for the estimation of a suitable volume of template DNA for the first run of the nested PCR, one of 11 insects tested positive (Fig. 6a, lane 2) with 10 µl genomic DNA compared to three positives (Fig. 6b, lanes 2, 4 and 9) using 5 µl genomic DNA. Using both 2 and 7 µl of template DNA did not increase the number of positive results in comparison to 5 µl genomic DNA (data not shown). The weaker extra upper band that appeared in Fig. 6(a), (lane 2) and Fig. 6(b), (lane 4) may have resulted from non-specific amplification. Since human and other eukaryotic genomes are larger than those of prokaryotes, the probability of non-specific amplification in the PCR method increases in the presence of genomic DNA isolated from eukaryotic cells.

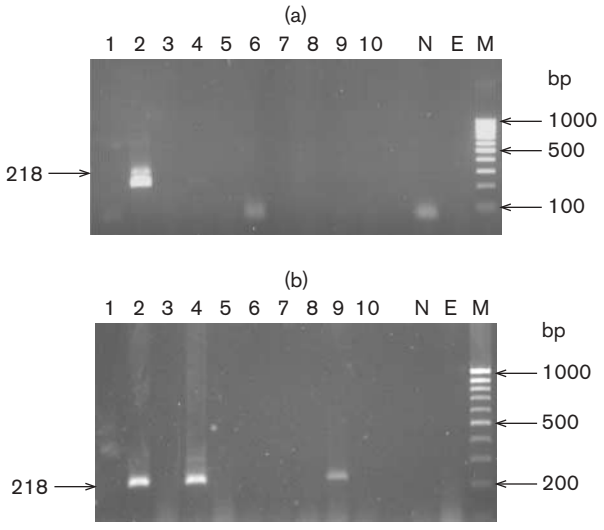

Fig. 6. PCR-based detection of *M. ulcerans*. Ten (a) and five (b) microlitres of purified genomic DNA were used in 50 µl of PCR mixture in the first run. Lanes: 1, fish; 2–10, aquatic insects; N, negative control (PCR mastermix); E, negative control (blank test of DNA extraction); M, DNA size marker (100 bp ladder).

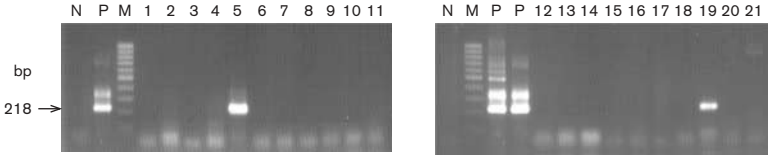

Fig. 7. Direct detection of *M. ulcerans* IS*2404* in aquatic insects from Benin after DNA extraction and column purification followed by ethanol precipitation. Lanes: 1–21, aquatic insects; M, DNA size marker (100 bp ladder); P, positive control (*M. ulcerans* ATCC 19423); N, negative control (PCR mastermix).

Non-specific bands can be removed and sensitivity increased (Alsallami & Kotlowski, 2001) by performing amplification reactions at higher annealing temperatures.

Detection of *M. ulcerans* in aquatic insects, molluscs and fish

On the basis of IS*2404* PCR detection of *M. ulcerans* in water and mud (Ross *et al.*, 1997a; Roberts & Hirst, 1997; Stinear *et al.*, 2000) and in aquatic insects (Portaels *et al.*, 1999), Portaels *et al.* (2001) hypothesized that mycobacteria (*M. ulcerans*) present in water and mud are mechanically concentrated by small water-filtering organisms like microphagous fish, mosquito larvae, small crustacea or molluscs or even some protozoa such as amoeba (Drancourt *et al.*, 2002). These water-filtering organisms can be ingested subsequently by water bugs, which become reservoirs of the bacteria. Some of these aquatic bugs have been demonstrated to infect animals (Marsollier *et al.*, 2002) and may infect humans through biting. Application of the DNA extraction procedure developed in this study followed by nested PCR specific to IS*2404* enabled us to detect *M. ulcerans* in 11 of 57 aquatic insects (~19 %), one of six molluscs, and three of 15 fish collected from BU-endemic regions (Table 2). In addition, five human tissue samples and three samples of transport medium used for storage of clinical samples gave positive results in the nested PCR (Table 2). Fig. 7 shows the results of PCR-based detection of *M. ulcerans* in aquatic insects. Characteristic banding patterns obtained from restriction analysis of PCR products using *Rsa*I and *Hpa*II endonucleases, and sequencing of the PCR product from one positive sample of *Tilapia* sp. confirmed specific amplification of the IS*2404* fragment in the PCR assay. Attempts to culture *M. ulcerans* from the environmental samples investigated failed. We succeeded in culturing *M. ulcerans* only once out of more than 1000 samples from aquatic insects from Benin; after several passages in mice (Chemlal *et al.*, 2002; F. Portaels, K. Chemlal, P. Elsen, P. L. C. Small, A. Van Aerde, W. M. Meyers & M. T. Silva, manuscript in preparation). Suggested explanations for the inability to culture *M. ulcerans* from environmental specimens were discussed elsewhere (Portaels *et al.*, 2001).

In conclusion, the DNA extraction method developed in this study is much simpler to perform than methods described previously and is desirable when dealing with highly infectious microbes (category 3 organisms). In addition, the simultaneous lysis and purification makes extraction of DNA possible directly from aquatic insects, molluscs and fish in one tube, reducing the risk of contamination in the nested PCR due to transfer of DNA from one tube to another. Detection of *M. ulcerans*-specific DNA sequence in the following families of aquatic insects: Belastomatidae (*Appasus* sp. and *Macrocoris* sp.), Hydrophilidae and Naucoridae (*Naucoris* sp.) confirms earlier suggestions implicating aquatic insects in the transmission of BU disease in swampy terrains of tropical and subtropical countries.

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