Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

A comparison of DNA extraction procedures for the detection of *Mycobacterium ulcerans*, the causative agent of Buruli ulcer, in clinical and environmental specimens

Lies Durnez [a,b,*], Pieter Stragier [b], Karen Roebben [b], Anthony Ablordey [b,c], Herwig Leirs [a,d], Françoise Portaels [b]

[a] *Evolutionary Ecology Group, Department of Biology, University of Antwerp, Antwerp, Belgium*
[b] *Mycobacteriology Unit, Department of Microbiology, Institute of Tropical Medicine, Antwerp, Belgium*
[c] *Bacteriology Department, Noguchi Memorial Institute for Medical Research, Accra, Ghana*
[d] *Danish Pest Infestation Laboratory, University of Aarhus, Faculty of Agricultural Sciences, Department of Integrated Pest Management, Kongens Lyngby, Denmark*

ARTICLE INFO

Article history:
Received 23 June 2008
Received in revised form 22 September 2008
Accepted 2 October 2008
Available online 17 October 2008

Keywords:
Buruli ulcer
Clinical specimens
DNA extraction
Environmental specimens
Modified Boom
Mycobacterium ulcerans

ABSTRACT

Mycobacterium ulcerans is the causative agent of Buruli ulcer, the third most common mycobacterial disease in humans after tuberculosis and leprosy. Although the disease is associated with aquatic ecosystems, cultivation of the bacillus from the environment is difficult to achieve. Therefore, at the moment, research is based on the detection by PCR of the insertion sequence IS*2404* present in *M. ulcerans* and some closely related mycobacteria. In the present study, we compared four DNA extraction methods for detection of *M. ulcerans* DNA, namely the one tube cell lysis and DNA extraction procedure (OT), the FastPrep procedure (FP), the modified Boom procedure (MB), and the Maxwell® 16 Procedure (M16).

The methods were performed on serial dilutions of *M. ulcerans*, followed by PCR analysis with different PCR targets in *M. ulcerans* to determine the detection limit (DL) of each method. The purity of the extracted DNA and the time and effort needed were compared as well. All methods were performed on environmental specimens and the two best methods (MB and M16) were tested on clinical specimens for detection of *M. ulcerans* DNA.

When comparing the DLs of the DNA extraction methods, the MB and M16 had a significantly lower DL than the OT and FP. For the different PCR targets, IS*2404* showed a significantly lower DL than mls*A*, MIRU1, MIRU5 and VNTR6. The FP and M16 were considerably faster than the MB and OT, while the purity of the DNA extracted with the MB was significantly higher than the DNA extracted with the other methods. The MB performed best on the environmental and clinical specimens.

This comparative study shows that the modified Boom procedure, although lengthy, provides a better method of DNA extraction than the other methods tested for detection and identification of *M. ulcerans* in both clinical and environmental specimens.

© 2008 Elsevier B.V. All rights reserved.

1. Introduction

Mycobacterium ulcerans is the causative agent of Buruli ulcer (BU), the third most common mycobacterial disease in humans after tuberculosis and leprosy (Portaels, 1995; Meyers and Portaels, 2003). This disease is mainly endemic in Central and West Africa, where it affects mostly poor communities (Debacker et al., 2004; Portaels, 1995). Epidemiological evidence strongly associates BU with aquatic ecosystems and *M. ulcerans* is considered an environmental pathogen (Portaels, 1995). Cultivation of the bacillus from the environment is however difficult to achieve: presently only one fully characterized *M. ulcerans* isolate has been recovered from an aquatic insect (Portaels et al., 2008). Therefore, at the moment, detection is based on demonstrating the presence by PCR of the insertion sequence IS*2404* in *M. ulcerans* and some closely related *Mycobacterium* species, namely, *M. liflandii*, *M. pseudoshottsi* and the mycolactone-producing *M. marinum* strains (Stinear et al., 1999; Stragier et al., 2007). This insertion sequence element has been identified in water, fish, aquatic insects, detritus, leeches, crustaceans, mollusks and mosquitoes, suggesting the presence of IS*2404* positive mycobacteria in these specimens (Eddyani et al., 2004; Kotlowski et al., 2004; Johnson et al., 2007).

Confirmation of the IS*2404* positive environmental specimens remains difficult because of the low *M. ulcerans* DNA concentration present in environmental specimens (Portaels et al., 2008). Only few researchers have used confirmation PCR targeting other regions in the

* Corresponding author. Mycobacteriology Unit, Department of Microbiology, Institute of Tropical Medicine, Nationalestraat 155, B-2000 Antwerp, Belgium. Tel.: +32 3 247 63 36; fax: +32 3 247 63 33.
E-mail address: ldurnez@itg.be (L. Durnez).

0167-7012/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.mimet.2008.10.002

Table 1
Environmental specimens tested and results for all DNA-extraction methods

Collection site		Number of specimens	Number of IS*2404*-positive specimens			
			MB	M16	OT	FP
Arra Nile	Algae	4	0	0	2	0
	Plant	2	0	0	1	0
Ndirindiri swamp	Insect	10	1	0	0	0
	Plant	4	0	0	0	0
Nile Fish Ferry	Fish organ	4	1	0	0	0
Obongi H/C	Goat faeces	1	0	0	0	0
Obongi bathing place	Plant	9	0	0	3	0
	Insect	16	0	0	4	0
	Goat faeces	1	0	0	0	0
Merea Etele seasonal stream	Insect	3	0	0	1	0
Gbalala Moyo river/swamp	Insect	7	0	0	1	0
	Soil	1	0	0	0	0
Borehole dirty water Obongi H/C	Water	1	0	0	0	0

M. ulcerans genome or plasmid such as the insertion sequence IS*2606*, KR or ER (present in the *mlsA* and *mlsB* gene) (Johnson et al., 2007; Williamson et al., 2008). Since these targets have lower copy numbers than IS*2404*, a positive IS*2404* PCR cannot always be confirmed (Johnson et al., 2007). Moreover, these targets are not specific for *M. ulcerans* (Johnson et al., 2007; Williamson et al., 2008). In recent years, VNTR and MIRU–VNTR methods have been developed for genotyping *M. ulcerans* isolates from clinical specimens (Ablordey et al., 2005; Stragier et al., 2005, 2006), also allowing differentiation of *M. ulcerans* from related IS*2404* positive species (Stragier et al., 2007). These fingerprinting methods are very successful when performed on isolates and on clinical specimens (Ablordey and Stragier, unpublished results), but results from environmental specimens remain poor (Johnson et al., 2007; Williamson et al., 2008; Ablordey and Stragier, unpublished results).

PCR is usually preceded by a DNA extraction to remove inhibitory substances and to concentrate the DNA. For the detection of *M. ulcerans* DNA, several DNA extractions have been developed and are currently used (Boom et al., 1990; Roberts and Hirst, 1997; Stinear et al., 2000; Kotlowski et al., 2004; Johnson et al., 2007). Since the *M. ulcerans* DNA concentration is very low in environmental specimens, the DNA extraction is an important step for detecting *M. ulcerans* in the environment. Moreover, when screening the environment for the presence of the bacillus, a simple and easy to use DNA extraction method is preferred, without loosing the small amount of *M. ulcerans* DNA present in the specimens.

In the present study, three commonly used DNA extraction methods were compared for detection of *M. ulcerans* DNA, namely the one-tube cell lysis and DNA extraction procedure (OT), the FastPrep procedure (FP) and the modified Boom procedure (MB), and one automated method that has not been used before for the detection of *M. ulcerans*-DNA, namely, the Maxwell® 16 Procedure (M16). For each of these methods we compared the sensitivity (using PCR with different targets in *M. ulcerans*), the purity of the extracted DNA, the time and effort needed, and their ability to detect *M. ulcerans* in environmental specimens to determine the best method in further studies of the environmental reservoir of *M. ulcerans*. The MB and M16 were also tested on clinical specimens.

2. Materials and methods

2.1. Serial dilutions and preparation of spiked specimens

Serial dilutions were developed starting with a suspension of *M. ulcerans* (ITM971116) containing 1.3×10^7 Acid Fast Bacilli (AFB)/ml. The number of AFB was counted using a counting chamber (Sheppard and McRae, 1965). Ten fold dilutions were made from 10^{-1} of the original suspension up to 10^{-8} of the original suspension. Of these dilutions 10 µl was used in the experiments, containing 1.3×10^4 up to 1.3×10^{-3} AFB, and 190 µl of ddH_2O or insect homogenate was added. When a bigger volume was required in the DNA extraction protocol, this was achieved by adding the appropriate amount of ddH2O in the first step of the protocol.

Every DNA extraction procedure was carried out in triplicate with the dilution series in ddH_2O and in triplicate with the dilution series in insect homogenate.

2.2. Preparation of clinical and environmental specimens

The DNA extraction procedures were applied on 63 environmental specimens collected in Uganda in September—October 2004. These specimens contained algae and plant material, insects, fish organs, faeces of goats, soil, and water (Table 1). Specimens were homogenized in 2 ml ddH_2O, using sterile mortars and pestles. Of this homogenate 200 µl was used for each DNA extraction procedure. When a bigger volume was required in the DNA extraction protocol, this was achieved by adding the appropriate amount of ddH2O in the first step of the protocol.

A total of 186 tissue specimens from clinically suspected cases of BU from Benin (Allada and Zagnanado) and the Democratic Republic of Congo (Kimpese) were homogenized in 2 ml phosphate buffered saline (Oxoid, Hampshire, England; pH 7.3 ± 0.2), using sterile mortars and pestles. The DNA extraction was performed on the homogenate after decontamination with 3 ml HCl (1 N) and neutralization with an equal amount of NaOH (1 N). Direct Smear Examination (DSE) using the Ziehl—Neelsen staining was performed on the homogenate after decontamination and bacilli were enumerated as previously described (Rieder et al., 1998).

2.3. DNA extraction procedures

2.3.1. FastPrep® SPINKit DNA extraction procedure

The FP was carried out according to manufacturer's FastDNA kit (MP Biomedicals, Brussels, Belgium) protocol for “animal tissue, cultured cells, insects, bacteria, bone, etc”, with minor adjustments: 200 µl of the specimen was added to a Lysing Matrix A tube containing 600 µl of lysis buffer (CLS-TC) and homogenized in the FastPrep® Instrument for 40 s at speed 6.0. Subsequently the specimens were centrifuged for 10 min at 14000 *g*. A volume of 650 µl of the supernatants was added to 650 µl of Binding Matrix and incubated with gentle agitation at room temperature on a rotator for 5 min. The specimens were centrifuged at 14000 *g* for 10 s to pellet Binding Matrix and the supernatants were discarded. The pellet was resuspended in 500 µl SEWS-M (salt/ethanol wash solution, DNase-

Table 2
Primers used for detection and identification of *M. ulcerans*

Primer name	Forward primer sequence	Reverse primer sequence	Annealing temperature	Reference
IS*2404* (1st run)	agggcagcgcggtgatacgg	cagtggattggtgccgatcgag	64 °C	Guimaraes-Peres et al. (1999)
IS*2404* (2nd run)	ggcgcagatcaacttcgcggt	ctgcgtggtgctttacgcgc	64 °C	Guimaraes-Peres et al. (1999)
mls*A*	gagatcggtcccgacgtctac	ggcttgactcatgtcacgtaag	58 °C	Mve-Obiang et al., 2005
MIRU1	gtgcccaatcccatcaagc	gacattccgatgccgaggt	58 °C	This publication
MIRU5	ccctgtccatccctaccagtt	ggcaaggtgatcgcgtca	58 °C	Stragier et al. (2005)
VNTR6	gaccgtcatgtcgttcgatcctagt	gacatcgaagaggtgtgccgtct	58 °C	Ablordey et al. (2005)

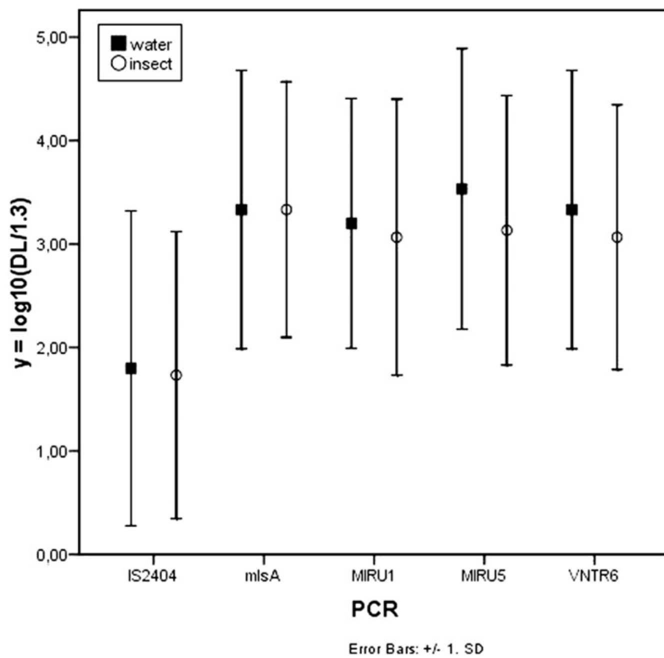

Fig. 1. Comparison of detection limits (DLs) with controls dissolved in water and in insect homogenate. The *y*-axis is expressed as $\log_{10}$ (DL/1.3).

free) and transferred to a spin module. The spin modules were centrifuged twice at 14 000 *g* for 1 min and the content of the catch tube was discarded. DNA was eluted by resuspending the Binding Matrix above the spin filter with 100 µl of DNA elution solution and incubating at 55 °C for 5 min. The spin modules were centrifuged at 14 000 *g* for 1 min to elute the DNA. The recovery tube with the eluted DNA was kept at −20 °C until further analysis.

2.3.2. Modified Boom DNA extraction procedure

The MB was carried out as previously described (Portaels et al., 2008): 250 µl of specimen was added to 250 µl of lysis buffer (1.6 M GuHCl, 60 mM Tris pH 7.4, 1% Triton X-100, 60 mM EDTA, Tween-20 10%), 50 µl Proteinase K (20 mg/ml) and '500 µl' glass beads. The specimens were vortexed and incubated overnight at 60 °C in a shaking water bath. To capture DNA 40 µl diatomaceous earth stock solution (10 g diatomaceous earth obtained from Sigma Aldrich Chemi GmbH in 50 ml of H_2O containing 500 µl of 37% (wt/vol) HCl) was added to each specimen. The specimens were vortexed and incubated at 37 °C in a shaker incubator for 90 min. The specimens were centrifuged at 14 000 *g* for 10 s and the supernatants were removed. The resulting pellets were washed twice with 70% ethanol and once with acetone. The washed pellets were dried at 55 °C for 30 min. The dried pellets were resuspended in 100 µl TE and incubated at 55–60 °C for 20 min. The specimens were centrifuged at 14 000 *g* for 10 s and the resulting supernatants were kept at −20 °C until further analysis.

Table 4
Significance values for comparison of the different DNA extraction methods

		p-values				
		IS*2404*	mls*A*	MIRU1	MIRU5	VNTR6
K–W test: difference between all methods		<0.001	<0.001	<0.001	<0.001	<0.001
M–W test	MB vs M16	0.700	0.056	0.092	0.465	0.336
	MB vs OT	0.003	0.002	0.001	0.003	0.005
	MB vs FP	0.002	0.001	0.001	0.002	0.002
	M16 vs OT	0.003	0.003	0.002	0.003	0.004
	M16 vs FP	0.002	0.002	0.002	0.002	0.002
	OT vs FP	0.002	0.180	0.002	0.180	0.065

2.3.3. One-Tube cell lysis and DNA extraction procedure

The OT was carried out as described by Kotlowski et al. (2004) with minor adjustments: 250 µl of specimen was added to 150 µl lysis buffer (10 mM 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}), 300 µl chloroform and 300 µl Tris-saturated phenol (pH 6.9). The specimens were placed at −20 °C for 1 h. Subsequently, specimens were centrifuged in tubes at 10 000 *g* for 20 min. Supernatants were transferred to fresh tubes. Isopropanol to 1/4 volume of the supernatants was added and the mixtures were loaded onto silica-cellulose membranes in columns. Specimens were allowed to filter through the membrane by centrifuging at 4000 *g* for 1 min. The membranes were washed twice with 300 µl absolute ethanol (by centrifuging at 4000 *g* for 1 min). DNA was eluted with 400 µl hot (about 75 °C) TE buffer (by centrifuging at 4000 *g* for 1 min) and precipitated with 3 M sodium acetate (1/10 of the volume was added) and two portions of absolute ethanol. The DNA was precipitated overnight at −20 °C and the supernatants were removed after centrifugation at 10 000 *g* for 20 min. The pellets were washed with 70% ethanol and the resulting pellets were suspended in 100 µl 0.5× TE buffer (pH 8.0) and stored at −20 °C until further analysis.

2.3.4. Maxwell® 16 DNA extraction procedure

The Maxwell® 16 DNA extraction procedure was carried out with the Maxwell® 16 DNA Purification Kit and the Maxwell® 16 Instrument (Promega, Leiden, The Netherlands), according to manufacturer's instructions: 200 µl of specimen was added to 200 µl of lysis buffer (10 mM Tris–HCl pH 7.5, 10 mM NaCl, 10 mM EDTA, 50 ml 10% SDS solution l^{-1}) and 10 µl Proteinase K (20 mg/ml) and incubated overnight at 60 °C in a shaker incubator. Subsequently 400 µl of the specimen was added to the first well of the Maxwell® 16 Cartridges, containing lysis buffer. The cartridges as well as elution tubes with 300 µl elution buffer were loaded into the Maxwell® 16 Instrument and the program of DNA purification of Tissue was used. The Maxwell® 16 Instrument purifies the DNA using MagneSil Paramagnetic Particles, which provide a mobile solid phase that optimizes capture, washing and elution of the DNA. After the machine has run, about 150 µl of eluted DNA are kept at −20 °C until further analysis.

Table 3
Exponents of the detection limits (DLs) of the different PCR assays for the different DNA extraction methods

	MB			M16			OT			FP		
	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	max
IS*2404*	0.5	0	1	0.7	0	1	2.7	2	3	4	4	4
mls*A*	2	2	2	2.5	2	3	4.5	4	>4	>4	>4	>4
MIRU1	1.8	1	2	2.3	2	3	4	4	>4	>4	>4	>4
MIRU5	2.7	2	3	2.3	1	3	4.5	4	>4	>4	>4	>4
VNTR6	2.3	2	3	2	1	3	4.2	3	>4	>4	>4	>4

The minimal and maximal exponents of the DLs that could be observed in this study are also given. To calculate the DL, a reverse transformation has to be carried out: DL = 1.3 × 10E(*y*) in which *y* equals the value mentioned in the table.

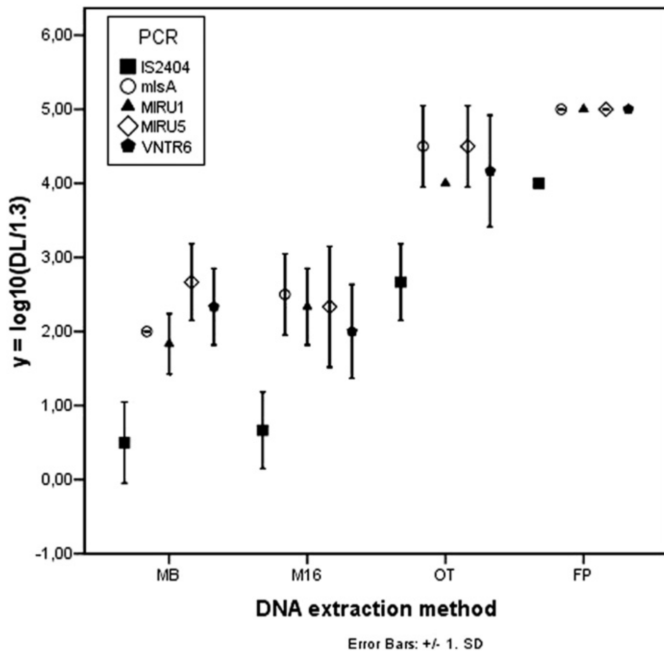

Fig. 2. Comparison of detection limits (DLs) of different PCR assays for the different DNA extraction methods. The *y*-axis is expressed as $\log_{10}$ (DL/1.3).

2.4. Comparison of time and effort of the DNA extraction procedure

In order to compare the time and effort needed for the analysis of 10 specimens with each DNA extraction procedure, several criteria were enumerated: manipulation time needed before the overnight step (if any), waiting time before overnight step (if any), manipulation time needed after overnight step, waiting time after overnight step, and the number of pipeting steps. An estimation of the maximum number of specimens possible to process in one working day is also given, since this is a measure for the through-put of a procedure, which is important when a lot of specimens need to be analyzed.

2.5. PCR conditions

Primers used for this study with their annealing temperature are listed in Table 2. For PCR with IS*2404* primers (1st run) and mls*A* primers, a 50 µl reaction mixture was used containing 25 µl 2× PCR buffer (3.3 mM $MgCl_2$, 100 mM KCl, 20 mM Tris, 0.20% Triton X), 0.5 µl each of forward and reverse primer (25 pmol/µl), 4 µl 2 mM PCR nucleotide mix (Pharmacia Amersham biotech, Uppsala, Sweden), 10 µl ddH_2O, 1 unit of Go Taq polymerase enzyme (Promega, Leiden, The Netherlands), and 10 µl DNA template. For IS*2404* PCR, amplification was carried out in two runs, the second run with a 25 µl reaction mixture containing 12.5 µl 2× PCR buffer (3.3 mM $MgCl_2$, 100 mM KCl, 20 mM Tris, 0.20% Triton X), 1 µl each of forward and reverse primer (25 pmol/µl), 2 µl 2 mM PCR nucleotide mix (Pharmacia Amersham biotech), 8.5 µl ddH_2O, 0.5 units of Go Taq polymerase enzyme (Promega), and 1 µl of the PCR product of the first run (Portaels et al., 2008). PCR amplifications with MIRU1, MIRU5, and VNTR6 primers were performed in 50 µl mixtures containing 1.0 U of HotStarTaq polymerase (QIAGEN, Hilden, Germany), 5 µl of 10× PCR buffer (QIAGEN, Hilden, Germany), 10 µl of Qsolution (QIAGEN, Hilden, Germany), 1.5 mM MgCl2 (QIAGEN, Hilden, Germany), 4 µl 2 mM PCR nucleotide mix (Pharmacia Amersham biotech), 1 µl each of forward and reverse primer (25 pmol/µl), 20.8 µl ddH_2O, and 10 µl of sample DNA (Stragier et al., 2005; Ablordey et al., 2005).

Cycling conditions began with an initial denaturation at 94 °C for 15 min, 40 cycles (25 for the second run with IS*2404* primers) of 94 °C for 45 s, annealing temperature (Table 2) for 45 s, 72 °C for 45 s and a final extension of 72 °C for 10 min.

The amplified DNA was subjected to gel electrophoresis using a 2% agarose gel and detected by ethidium bromide staining and UV transillumination.

All amplicons were confirmed by sequencing.

2.6. Purity of the DNA extract

The ratio of absorbance at 260 nm and 280 nm was used to assess the purity of DNA. A ratio of ~1.8 is generally accepted as “pure” for DNA. If the ratio is appreciably lower, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm. This ratio was measured with the NanoDrop® ND-1000 (Thermo Scientific) according to manufacturer's instructions.

2.7. Statistical methods

All statistical analyses were performed in SPSS 16.0.

To compare the detection limits (DLs) of the methods applied on dilution series in water and in insect homogenate, a Wilcoxon matched pairs signed rank (W-M-P) test was used.

To compare the DLs and the purity of the different methods, non parametric tests were used. The Kruskall–Wallis (K–W) test was used when comparing all methods. When a significant difference was found with the K–W test, the Mann–Whitney U (M–W) test was used to compare the methods side by side to detect the difference.

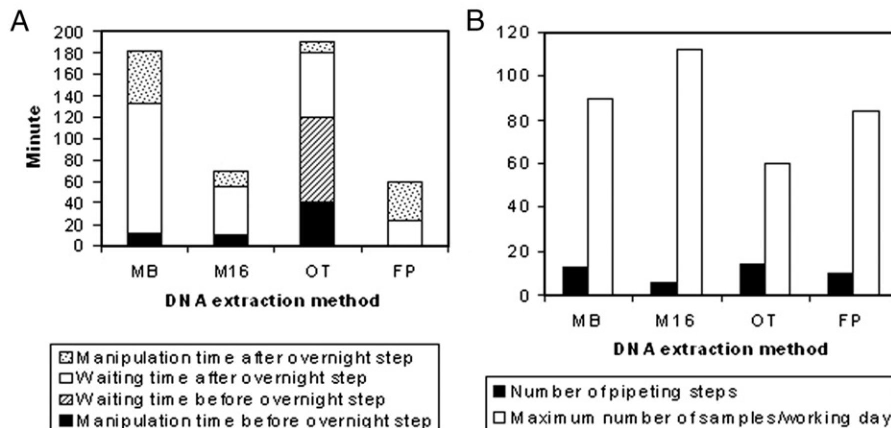

Fig. 3. Comparison of time and effort of the different DNA extraction methods. A. Comparison of time needed for the DNA extraction of ten specimens. B. Comparison of pipeting steps and number of specimens possible to handle in one working day.

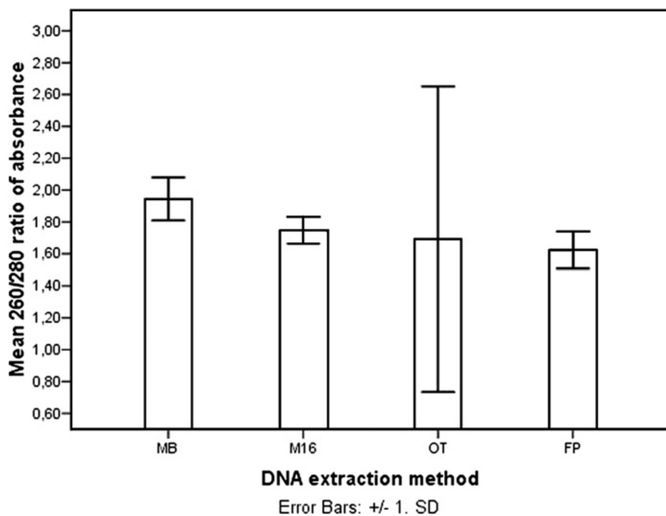

Fig. 4. Comparison of DNA purity obtained by the different DNA extraction methods.

The Friedman test (K related samples) was used to compare the sensitivity of the different PCR assays. When a significant difference was found with this test, the W–M–P test was used to detect the difference.

The Kappa test was used to estimate the concordance between the methods applied on environmental specimens and on clinical specimens. The Chi square test was used to compare the number of positive clinical specimens detected by MB and M16.

3. Results

3.1. Comparison of detection limits of the DNA extraction procedures for the different PCR assays

The DL, which is the lowest amount of AFB detected by the different PCR assays following the DNA extraction methods, was used as an indirect measure for the sensitivity of the DNA extraction methods. These DLs vary from 1.3×10^0 AFB up to more than 1.3×10^4 AFB, with an exponential increase. In order to easily compare the DLs statistically and graphically, the exponent of the DL was used in the comparisons instead of the DL itself. The values that are compared in the statistical tests are therefore transformed into $\log_{10}$ (DL/1.3) for all analyses.

It was tested whether the insect homogenate has an effect on the sensitivity of the DNA extraction methods and PCR assays. No such effect was observed when comparing the results of the controls dissolved in water and in insect homogenates as paired samples for the IS*2404*-, mls*A*-, MIRU1-, MIRU5- or VNTR6-assays (W-M-P Test, $p=0.705$; 1.000; 0.083; 0.055; and 0.096 respectively). The results are shown in Fig. 1.

The mean, minimum and maximum of $\log_{10}$ (DLs/1.3) for the different PCR assays and different methods are shown in Table 3. To calculate the mean DL, these values can be retransformed to: 1.3×10E (*y*). For the IS*2404* PCR, this results in mean DLs of 4 AFB for the MB, 6.5 AFB for the M16, 652 AFB for the OT and 13000 AFB for the FP. With the non-parametric K–W test on the $\log_{10}$ (DLs/1.3), a significant difference was shown between the different methods for all PCR assays ($p<0.001$ in all cases), with the MB and M16 performing better (having a lower DL) than the OT and FP, and the OT performing better than the FP for some of the PCR targets (M–W test, significance values are shown in Table 4). A significant difference was also shown between the different PCR targets (Friedman test: $p<0.001$), with the IS*2404* PCR assay having a significantly lower DL than the mls*A*-, MIRU1-, MIRU5- and VNTR6-assay (W-M-P test, $p<0.001$ in all cases). No significant difference was found between the different PCR assays targeting mls*A*, MIRU1, MIRU5 and VNTR6 (W-M-P test, $p>0.05$ in all cases). The results are shown in Fig. 2.

3.2. Comparison of time and effort

A comparison of time and effort needed for DNA extraction of about 10 specimens using the four different procedures was estimated. These results are shown in Fig. 3. The FP is the fastest (60 min), followed by the M16 (70 min), the MB (182 min) and the OT (190 min). The number of manipulation steps is the lowest for the M16, followed by the FP, the MB and the OT. The total waiting time is the longest for the OT (140 min), followed by the MB (120 min), the M16 (45 min), and the FP (24 min). With the M16 the largest number of specimens can be processed in one working day, followed by the MB, the FP and the OT.

3.3. DNA purity

The results of the 260/280 ratio of absorbance for each DNA extraction method are shown in Fig. 4. A significant difference in DNA purity obtained in the different DNA extraction methods was demonstrated (K-W test: $p<0.001$), the MB resulting in a higher DNA purity as compared to the M16, the OT and the FP (M-W test: $p<0.001$ (MB vs FP and MB vs M16) and $p=0.006$ (MB vs OT)).

3.4. Environmental specimens

The MB resulted in two IS*2404* positive specimens, namely an insect (cicada) and the organs of a fish (Nile perch). With the OT, 12 specimens tested IS*2404*-positive (six specimens of plant material and six insects), but with this method we observed contamination of the negative controls during the experiments. The M16 and FP did not demonstrate any IS*2404* positive specimens (Table 1). None of the IS*2404* positive specimens could be confirmed by the mls*A*-, MIRU1-, MIRU5- or VNTR6-assay. When comparing the concordance of the MB and the OT, the kappa-value was −0.058, which indicates that the concordance is not better than what has been found by chance. For the M16 and FP, no kappa-values could be calculated because of the fact that no IS*2404* positive specimens were found with these methods.

3.5. Clinical specimens

A total of 186 specimens from clinically suspected cases of BU were tested with the MB and the M16, since, as mentioned in the above paragraph, these methods performed significantly better than the OT and FP. Although no significant difference was found when comparing the DLs of these two methods with serial dilutions, the MB detected significantly more IS*2404* positive clinical specimens than the M16: in 89 out of 186 (47.8%) clinical specimens IS*2404* was detected with the MB while only 40 out of 186 (21.5%) clinical specimens were IS*2404* positive with the M16 ($X^2=36.282$, $df=1$, $p<0.001$) (Table 5). This difference was significant for both specimens with a low DSE score (zero up to 4 AFB) and specimens with a high DSE score (data not

Table 5
Number of IS*2404* negative and positive clinical specimens analyzed with the modified Boom procedure (MB) and the Maxwell® 16 Procedure (M16)

		M16		Total number of specimens
		IS*2404* negative specimens	IS*2404* positive specimens	
MB	IS*2404* negative specimens	93	4	97
	IS*2404* positive specimens	53	36	89 (47.8%)
Total number of specimens		146	40 (21.5%)	186

shown). The kappa-value, which is a measure for concordance of the methods, was 0.372.

3.6. Discussion

In view of the difficulty in cultivating *M. ulcerans* from environmental specimens, PCR based detection of IS*2404* seems to be the preferred way of detecting *M. ulcerans* in this kind of specimens. In spite of the sensitivity of nested IS*2404* PCR, the low cell population in environmental specimens (Portaels et al., 2008) and difficulties in achieving effective cell lysis (Kotlowski et al., 2004) influence the sensitivity of the detection assay when working with environmental specimens. In patient samples, this seems much less of an issue because of the higher *M. ulcerans* load. However, clinical specimens can also have a low bacillary load. The DNA extraction method employed needs therefore important consideration when detecting *M. ulcerans* DNA in both environmental and clinical specimens. In the present study we compared three commonly used DNA extraction methods (MB, OT and FP) and one automated method (M16).

The MB method showed a comparable DL as obtained in other studies (5 AFB in Phillips et al., 2005). The FP however, showed in our study a much higher DL than in a study carried out by Fyfe et al. (2007). In the present study, the FP followed by a conventional gel-based PCR assay for IS*2404* resulted in a DL of 13,000 AFB, as compared to a DL of 1 to 10 AFB demonstrated by Fyfe et al. (2007) when using the FP followed by a real-time PCR assay for IS*2404*. This difference can be explained by the fact that a real-time PCR assay is 100 to 1000 times more sensitive than the conventional gel-based PCR assay (Fyfe et al., 2007). Another possible explanation for part of the difference is the fact that Fyfe et al. (2007) manually purified the DNA after lysis with the FP instrument while in our study the DNA was purified by using a spin kit. The manual purification and the spin kit for purifying the DNA have however not been compared and may require further study.

With the OT there were some problems of contamination, both when working with dilution series and with environmental specimens. This is probably due to the fact that this DNA extraction makes use of spin column technology. The spin columns cannot be placed tightly enough into the collection tubes during the various centrifugation steps. Therefore cross-contamination because of an aerosol may occur. The same has been observed in other studies on DNA extraction methods (Queipo-Ortuño et al., 2007). Contamination with the FP was not observed, although with that method also a spin column is used. Probably in this study the DL of that method was not low enough to detect any contamination if occurred.

The present study clearly shows a difference in sensitivity of the PCR assays with different PCR targets that could be used for detection and identification of *M. ulcerans*. This difference is expected based on the copy numbers of the different targets in the *M. ulcerans* genome. IS*2404* is present in the chromosome and on the pMUM001 plasmid and has more than 200 copies (Stinear et al., 2007) while KR or ER, which are only present on the plasmid, have 4 to 8 copies (Williamson et al., 2008). MIRU1, MIRU5 and VNTR6 only occur once in the chromosome (Stragier et al., 2005; Ablordey et al., 2005). The fact that in our study the IS*2404* assay is more sensitive than the mls*A* assay, contradicts the results published by Williamson et al. (2008), who found a similar DL for both assays.

The IS*2404* assay has been used by many researchers to detect *M. ulcerans* in both clinical and environmental specimens. However, given the low bacillary load of *M. ulcerans* in environmental specimens and the higher sensitivity of the IS*2404* PCR assay as compared to other PCR assays that are specific for *M. ulcerans*, it is difficult to confirm the IS*2404* positive specimens. In the present study, we were not able to confirm any of the IS*2404* positive environmental specimens. In a study carried out by Johnson et al. (2007), in which a real-time PCR assay was used following the FP, only 13 out of 48 IS*2404* positive mosquito pools were confirmed with KR and IS*2606* PCR and from only 2 IS*2404* positive pools VNTR locus 9 could be amplified. Probably by using a more sensitive DNA extraction procedure, combined with a real-time PCR assay, the confirmation rate of IS*2404* positive specimens could be improved. Additional studies are needed to confirm the superiority of the MB combined with real-time PCR.

Our original rationale was that DNA extraction procedures need particular attention when working on environmental specimens, while this is of less importance for clinical specimens. However, when comparing the two DNA extraction methods that performed best in the dilution series experiments on a number of clinical specimens, it was clear that also for clinical specimens the DNA extraction procedure is an important step. The subtle difference that was observed with the dilution series and that was not statistically significant, shows to be significant when working with both clinical and environmental specimens. Therefore it is recommended that every DNA extraction method, both manual and automated, that is introduced in a laboratory, should be extensively tested on dilution series, but also on specimens that will be processed with these DNA extraction methods.

This comparative study shows that the modified Boom procedure, although lengthy, provides a better method of DNA extraction than the other methods tested for detection and identification of *M. ulcerans* in both clinical and environmental specimens.

Acknowledgements

This research was supported by a PhD-grant of the Flemish Interuniversity Council, by the Directorate-General for Development Cooperation (Brussels, Belgium), the Damien Foundation and the European Commission (project INCOCT-2005-051476-BURULICO). We thank Martine Debacker for the advice in statistics, and Anita Van Aerde and Pim de Rijk for their excellent technical assistance.

References

Ablordey, A., Swings, J., Hubans, C., Chemlal, K., Locht, C., Portaels, F., Supply, P., 2005. Multilocus variable-number tandem repeat typing of *M. ulcerans*. J. Clin. Microbiol. 43, 1546–1551.

Boom, R., Sol, C.J., Salimans, M.M., Jansen, C.L., Wertheim-van Dillen, P.M., van der Noordaa, J., 1990. Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. 28, 495–503.

Debacker, M., Aguiar, J., Steunou, C., Zinsou, C., Meyers, W.M., Guédénon, A., Scott, J.T., Dramaix, M., Portaels, F., 2004. *Mycobacterium ulcerans* disease (Buruli ulcer) in a rural hospital, southern Benin, 1997–2001. Emerg. Infect. Dis. 10, 1391–1398.

Eddyani, M., Ofori-Adjei, D., Teugels, G., De Weirdt, D., Boakye, D., Meyers, W.M., Portaels, F., 2004. Potential role for fish in transmission of *Mycobacterium ulcerans* disease (Buruli ulcer): an environmental study. Appl. Environ. Microbiol. 70, 5679–5681.

Fyfe, J.A.M., Lavender, C.J., Johnson, P.D.R., Globan, M., Sievers, A., Azuolas, J., Stinear, T.P., 2007. Development and application of two multiplex real-time PCR assays for the detection of *Mycobacterium ulcerans* in clinical and environmental specimens. Appl. Environ. Microbiol. 73, 4733–4740.

Guimaraes-Peres, A., Portaels, F., de Rijk, P., Fissette, K., Pattyn, S.R., van Vooren, J., Fonteyne, P., 1999. Comparison of two PCRs for detection of *Mycobacterium ulcerans*. J. Clin. Microbiol. 37, 206–208.

Johnson, P.D.R., Azuolas, J., Lavender, C.J., Wishart, E., Stinear, T.P., Hayman, J.A., Brown, L., Jenkin, G.A., Fyfe, J.A.M., 2007. *Mycobacterium ulcerans* in mosquitoes captured during outbreak of Buruli ulcer, Southeastern Australia. Emerg. Infect. Dis. 13, 1653–1660.

Kotlowski, R., Martin, A., Ablordey, A., Chemlal, K., Fonteyne, P.A., Portaels, F., 2004. One-tube cell lysis and DNA extraction procedure for PCR-based detection of *Mycobacterium ulcerans* in aquatic insects, mollusks and fish. J. Med. Microbiol. 53, 927–933.

Meyers, W.M., Portaels, F., 2003. Buruli ulcer: *Mycobacterium ulcerans* infection, In: Warrell, D.A., Cox, T.M., Firth, J.D., BenzJr. Jr., E.J. (Eds.), Oxford Textbook of Medicine, 4th ed. Oxford University Press, Oxford, England, pp. 583–585.

Mve-Obiang, A., Lee, R.E., Umstot, E.S., Trott, K.A., Grammer, T.C., Parker, J.M., Ranger, B.S., Grainger, R., Mahrous, E.A., Small, P.L.C., 2005. A newly discovered mycobacterial pathogen isolated from laboratory colonies of *Xenopus* species with lethal infections produces a novel form of mycolactone, the *Mycobacterium ulcerans* macrolide toxin. Infect. Immun. 73, 3307–3312.

Phillips, R., Horsfield, C., Kuijper, S., Lartey, A., Tetteh, I., Etuaful, S., Nyamekye, B., Awuah, P., Nyarko, K.M., Osei-Sarpong, F., Lucas, S., Kolk, A.H.J., Wansbrough-

Jones, M., 2005. Sensitivity of PCR targeting the IS*2404* insertion sequence of *Mycobacterium ulcerans* in an assay using punch biopsy specimens for diagnosis of Buruli ulcer. J. Clin. Microbiol. 43, 3650–3656.

Portaels, F., 1995. Epidemiology of mycobacterial diseases. In: Schuster, M. (Ed.), Mycobacterial Diseases of the Skin. Elsevier Science Inc., New York, N.Y, pp. 207–222.

Portaels, F., Meyers, W.M., Ablordey, A., Castro, A.G., Chemlal, K., de Rijk, P., Elsen, P., Fissette, K., Fraga, A.G., Lee, R., Mahrous, E., Small, P.L.C., Stragier, P., Torrado, E., Van Aerde, A., Silva, M.T., Pedrosa, J., 2008. First cultivation and characterization of *Mycobacterium ulcerans* from the environment. PLOS Negl. Trop. Dis. 2, e178.

Queipo-Ortuño, M.I., Tena, F., Colmenero, J.D., Morata, P., 2007. Comparison of seven commercial DNA extraction kits for the recovery of *Brucella* DNA from spiked human serum samples using real-time PCR. Eur. J. Clin. Microbiol. Infect. Dis. 27, 109–114.

Rieder, H.L., Chonde, T.M., Myking, H., Urbanczik, R., Laszlo, A., Kim, S.J., Van Deun, A., Trébucq, A., 1998. The Public Health Service National Tuberculosis Reference Laboratory and the National Laboratory Network. International Union Against Tuberculosis and Lung Disease, Paris.

Roberts, B., Hirst, R., 1997. Immunomagnetic separation and PCR for detection of *Mycobacterium ulcerans*. J. Clin. Microbiol. 35, 2709–2711.

Sheppard, C.C., McRae, D.H., 1965. A method for counting acid-fast bacteria. Int. J. Lepr. 36, 78–82.

Stinear, T., Ross, B.C., Davies, J.K., Marino, L., Robins-Browne, R.M., Oppedisano, F., Sievers, A., Johnson, P.D., 1999. Identification and characterization of IS*2404* and IS*2606*: two distinct repeated sequences for detection of *Mycobacterium ulcerans* by PCR. J. Clin. Microbiol. 73, 1018–1023.

Stinear, T., Davies, J.K., Jenkin, G.A., Hayman, J.A., Oppedisano, F., Johnson, P.D.R., 2000. Identification of *Mycobacterium ulcerans* in the environment from regions in Southeast Australia in which it is endemic with sequence capture-PCR. Appl. Environ. Microbiol. 66, 3206–3213.

Stinear, T.P., Seemann, T., Pidot, S., Frigui, W., Reysset, G., Garnier, T., Meurice, G., Simon, D., Bouchier, C., Ma, L., Tichit, M., Porter, J.L., Ryan, J., Johnson, P.D.R., Davies, J.K., Jenkin, G.A., Small, P.L.C., Jones, L.M., Tekaia, F., Laval, F., Daffé, M., Parkhill, J., Cole, S.T., 2007. Reductive evolution and niche adaptation inferred from the genome of *Mycobacterium ulcerans*, the causative agent of Buruli ulcer. Genome Res. 17, 192–200.

Stragier, P., Ablordey, A., Meyers, W.M., Portaels, F., 2005. Genotyping *Mycobacterium ulcerans* and *Mycobacterium marinum* by using mycobacterial interspersed repetitive units. J. Bacteriol. 187, 1639–1647.

Stragier, P., Ablordey, A., Bayonne, L.M., Lugor, Y.L., Sindani, I.S., Suykerbuyk, P., Wabinga, H., Meyers, W.M., Portaels, F., 2006. Heterogeneity among *Mycobacterium ulcerans* isolates from Africa. Emerg. Infect. Dis. 12, 844–847.

Stragier, P., Ablordey, A., Durnez, L., Portaels, F., 2007. VNTR analysis differentiates *Mycobacterium ulcerans* and IS*2404* positive mycobacteria. Syst. Appl. Microbiol. 30, 525–530.

Williamson, H.R., Benbow, M.E., Nguyen, K.D., Beachboard, D.C., Kimbirauskas, R.K., McIntosh, M.D., Quaye, C., Ampadu, E.O., Boakye, D., Merritt, R.W., Small, P.L.C., et al., 2008. Distribution of *Mycobacterium ulcerans* in Buruli ulcer endemic and non-endemic aquatic sites in Ghana. PLOS Negl. Trop. Dis. 2, e205.