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(54) Title: METHOD FOR DETECTING AND/OR ISOLATING MYCOBACTERIA

(57) Abstract: The present invention relates to the field of mycobacteria, and more specifically to a method for efficient and selective isolation of mycobacteria, which allows for the application of tools for the detection and identification of mycobacteria. The method of the invention may be applied to non-tuberculosis mycobacteria, also called environmental mycobacteria. Detection and identification of one of more strains of non tuberculosis mycobacteria can subsequently be performed.

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METHOD FOR DETECTING AND/OR ISOLATING MYCOBACTERIA

FIELD OF THE INVENTION

The present invention relates to the field of mycobacteria and more specifically
5 to a method for efficient and selective isolation of mycobacteria strains. Subsequent to the isolation of the mycobacteria strains, detection, quantification and identification of these strains can be performed. The method of the invention may be applied to non-tuberculosis mycobacteria (NTM).

10 BACKGROUND OF THE INVENTION

Mycobacteria are gram positive bacteria with a very slow-growth rate. On solid culture, the growth rate goes up to 30 days. Besides the tuberculosis causing bacteria from the *M. tuberculosis*-complex, or the paratuberculosis causing *M. avium*-complex and *M. leprae*, the so-called environmental bacteria (EM)
15 are saprophytic, aquatic bacteria with a human pathogenicity. Recent examinations found them to be present in a wide variety of water-samples such as in drinking water and in biofilms of water distribution systems (LeDantec C et al., 2002; Vaerewijck MJ et al., 2005). Their presence bears a certain danger for older or immunosuppressed persons and, in particular, tap-
20 water was shown to be the source of Mycobacterial infections in aged and immune-compromised persons (Primm TP et al., 2004). Most applications for a direct detection of Mycobacteria in a sample are limited by the low number of Mycobacteria initially present in the sample. In fact, due to the slow growth-rate of EM, the diagnosis of their presence with conventional culture-based
25 methods is time-consuming, laborious and inefficient. Fast molecular methods for the detection and identification of Mycobacteria are usually applied after the culture of the bacteria.

The concentration of bacteria with para-magnetic beads is used regularly in

microbiological applications, but most of the studies published thus far are not driven by the interest to capture very low amounts of bacteria, since, in most of the cases, a pre-amplification phase is included or the initial number of bacteria in the sample is already very high (> 1000 cfu/ml). Examples of a few
5 of these published studies include the immunomagnetic isolation method described in Fu et al. (2005) which exhibits an efficiency of 50% on *E.coli* and therefore allows for the recovery of 50% of the bacteria present in the sample. Similarly, the same efficiency is observed for the immunomagnetic isolation method on *Campylobacter jejuni* (Yu et al., 2005). Grant et al. (2000) deal with
10 the isolation of *M. avium* from milk. The process disclosed in Grant et al. (IMS-PCR) only has a sensitivity of 20 cfu/ml or 20,000 cfu/L.

Therefore, it is important that new, fast, efficient and sensitive methods to detect the presence of mycobacteria, such as NTM strains, in samples such as
15 water-samples be developed. It is also important to provide methods to selectively isolate the mycobacteria strains to be analysed from a sample.

SUMMARY OF THE INVENTION

An object of the present invention is to provide a method for isolating
20 Mycobacteria strains.

More particularly, and in accordance with the present invention, this object is achieved with a method for isolating mycobacteria from a sample, the method comprising the steps of:

- 25 a) contacting a sample suspected of containing mycobacteria with magnetic beads coated with an antibody immunologically specific to mycobacteria under three-dimensional motion conditions to form bead-mycobacteria complexes, and
- b) magnetically recovering the bead-mycobacteria complexes to obtain a sample containing isolated mycobacteria strains.

Another object of the present invention is achieved with a method for detecting mycobacteria from a sample, the method comprising the steps of:

5 a) contacting a sample suspected of containing mycobacteria with magnetic beads coated with an antibody immunologically specific to mycobacteria under three-dimensional motion conditions to form bead-mycobacteria complexes;

b) magnetically recovering the bead-mycobacteria complexes to obtain a sample containing isolated mycobacteria strains; and

10 c) determining the presence or absence of the defined mycobacterium strain by the detection of said defined mycobacterium strain.

A further object of the present invention is achieved with a method for detecting viable mycobacteria from a sample, the method comprising the steps
15 of:

a) contacting a sample suspected of containing mycobacteria with magnetic beads coated with an antibody immunologically specific to mycobacteria under three-dimensional motion conditions to form bead-mycobacteria complexes;

20 b) magnetically recovering the bead-mycobacteria complexes to obtain a sample containing isolated mycobacteria;

c) adding a viability determining agent to the sample containing isolated mycobacteria;

25 d) extracting genomic DNA from the sample containing isolated mycobacteria of step c); and

e) detecting the genomic DNA of a defined mycobacterium strain; wherein the detection of a predetermined number of mycobacteria genomes of the defined mycobacterium strain is indicative of a viable mycobacterium strain.

The methods of the present invention advantageously provide a way for isolating and concentrating small quantities of mycobacteria from a sample of any given size. It also avoids the culture of the sample in order to obtain a sufficient concentration of bacteria in order to proceed with the detection thereof. Another advantage is that the method of the present invention yields a good quality DNA for further analysis and quantification.

The objects and other advantages of the present invention will become more apparent upon reading of the following non-restrictive description of preferred embodiments thereof, given for the purpose of exemplification only, with reference to the accompanying figures.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Estimation of the capture efficiency (%) of different types of immuno-magnetic capture beads.

100µl of Dynabeads™ of different sizes and coatings (Dyna, Invitrogen) were added to 1.10^7 cfu ($1E07$ cfu) *M. abscessus* in 500µl 1xPBS supplemented with 0.1% Tween20™. After two hours of incubation at room-temperature on a horizontal rotary shaker (10 rpm), the beads were captured through magnetic force (Dyna, MPC bead retriever) and washed 2 times for 5 minutes in 1ml 1xPBS supplemented with 0.1% Tween20. The capture efficiency was estimated through culture of the supernatant before and after bead capture. The beads used in the assays were treated as follows: **2.8µm BSA coated:** 100µL tosylactivated magnetic beads 2.8µm (Dyna) were incubated with 100 µl BSA in 1xPBS (1mg/ml) over night at 37°C on a horizontal rotary shaker and washed with 0.2M Tris-buffer pH 8.0. **4.5µm-IgG-35111 and 2.8µm-IgG-35111:** The polyclonal antiserum 35111 was purified through a ProteinA-column. 1ml of tosylactivated beads in the two respective sizes (Dyna) were incubated overnight at 37°C with the derived IgG-fraction from 35111 (1mg/ml

in 0.1M phosphate buffer) and washed in 0.2M Tris pH 8.0 to block remaining active sites on the bead-surface. **2.8µM-ProtA-IgG-35111:** 2.8µm tosylactivated beads were incubated with recombinant ProteinA (1mg/ml in 0.1M phosphate buffer, Sigma) at 37°C and washed with 0.2M Tris pH 8.0.

5 The ProteinA coated beads were incubated for 1 h at room-temperature with the IgG-fraction of 35111 (1mg/ml in 1xPBS). **4.5µm-IgG-affinity:** 4.5µm tosylactivated beads (Dynal) were incubated with an affinity-purified fraction of 35111-IgG purified for their affinity against Mycobacterial surface-proteins.

Figure 2: Capture efficiency (%) as a function of bacterial concentration using 2.8µM-ProtA-IgG-35111-beads.

10 100µl of 2.8µM-ProtA-IgG-35111-beads were added to 500 µl 1xPBS supplemented with 0.1% Tween20 spiked with *M. abscessus* in different quantities as indicated in the figure. The incubation was performed on a horizontal rocking-platform at room-temperature for 2h. Capture efficiency was

15 estimated through culture of supernatant before and after bead capture. The capture efficiency decreased with bacterial concentration using two-dimensional incubation. This figure shows the capture efficiencies in dependency of the number of bacteria present in the assay (x-axis cfu *M. abscessus* in assay) the given capture efficiency for a "low" number of bacteria

20 ($5 \cdot 10^3$ (5E03)) is below 25% while a high number of bacteria present results in a capture efficiency of above 75%. It is shown that the use of a two-dimensional incubation on a rocking platform in combination with a small sample volumen (500µl) as used in other approaches for the immunomagnetic bead concentration is not satisfying to ensure an efficient capture of low

25 bacterial concentrations.

Figure 3: Capture-efficiency as dependent on sample volume.

Three different volumes of sample (0.5ml, 5ml and 25ml) were spiked with a fixed number of *M. abscessus* bacteria ($1 \cdot 10^5$ cfu (1E05 cfu)). *M. abscessus*

bacteria were captured with 100µl (2×10^8 beads) of 2.8µM-ProtA-IgG-35111 beads. Capture efficiency was estimated through plate culture. The incubation during 30 minutes at room-temperature was performed using a 360° rotary shaker with the tubes fixed orthogonally as indicated in **Figure 11**. Note the difference of capture efficiency in the 0.5ml sample as shown in **Figure 2** through the change of incubation: the capture efficiency for $5 \cdot 10^5$ cfu ($5E05$ cfu) with a two-dimensional incubation on a rocking platform is 40%, the capture efficiency for $1 \cdot 10^5$ cfu ($1E05$ cfu) with a three-dimensional incubation is over 70%. This figure combines in fact two changes compared to the result presented in Figure 2: The assay was performed with a three-dimensional incubation (Figure 11) and the volume in which the assay was performed was changed. Compared to Figure 2, Figure 3 shows that through the use of three-dimensional incubation from a volume of 500µl, the capture efficiency of a comparable number of CFU in the test ($5 \cdot 10^5$ ($5E05$) (Figure 2) and $1 \cdot 10^5$ ($1E05$) (Figure 3) is already 2.5-fold higher (48% for the 2-dimensional incubation and 65% for the three-dimensional incubation). In this assay, it is shown that the recommended ratio of bead number ($2 \cdot 10^8$ beads ($2XE08$ beads)) and assay volume (500µl) as recommended by Dynal) is not efficient. Using a 10-fold larger assay volume (5000µl) together with the same number of beads ($2 \cdot 10^8$ ($2XE08$)) the capture efficiency was over 75%.

Figure 4: Capture efficiency is independent of *M. abscessus* concentrations with three-dimensional incubation.

5ml 1xPBS supplemented with 0.1% Tween20 were spiked with different amounts of *M. abscessus*. The assay was performed as indicated in Figure 1 with a three-dimensional incubation as indicated in **Figure 11**. The capture efficiency was estimated through culture of the supernatant, before and after bead-capture.

Figures 5A and 5B: Photograph of the agarose gel for gene-specific PCR for Mycobacteria that have been concentrated (Fig 5A) and immuno-captured (Fig. 5B) showing that bead capture increases the detection sensitivity in water-samples.

5 200ml of water were spiked with the indicated numbers of *M. abscessus*. After a non-specific concentration of the 200ml sample through filtration and detachment in 5ml of 1xPBS supplemented with 0.1% Tween20, the bacteria were either concentrated through centrifugation at 3000g for 15 minutes (Figure 5A) or immuno-captured through the addition of 2.8µm-ProtA-IgG-
10 35111 beads and subsequent three-dimensional incubation during 30 minutes at room-temperature (Figure 5B). The presence of the captured Mycobacteria was shown through genomic DNA-preparation and a genus-specific PCR for the hsp65-gene. Note that the sensitivity of the assay is enhanced 500-fold through immuno-magnetic capture. smcIII is a tap-water sample with a
15 bacterial load of 1.10^3 cfu (1E03 cfu) Mycobacteria.

Figure 6: Schema of test-procedure for Mycobacteria in water-samples.

Water-samples are concentrated non-specifically through filtration on a 0.45µm polycarbonate membrane (Step 1). The total bacteria present on the filter are
20 detached in 5ml capture-buffer through intensive vortexing for 15 minutes (Step 2). The solubilised bacteria are specifically coated through antibody-coated paramagnetic beads (Step 3). The genomic DNA from the bead-captured Mycobacteria is prepared through a developed protocol (Step 4). The prepared DNA serves as template for a Mycobacteria genus-specific PCR
25 inside the hsp65-gene (Step 5).

Figure 7: Course of immunisations of rabbits with Mycobacterial cell wall preparations and the estimation of the serum titers against their antigen.

Rabbits were immunised through injections with native cell wall-preparations of
30 the following Mycobacterial species: *M. kansasii*, *M. xenopi*, *M. avium*, *M.*

abscessus and *M. gordonae*. 500 µl lysate (1mg/ml) was mixed with 500µl of incomplete Freund's Adjuvant and injected subcutaneously at five different locations on the rabbit. Each antigen was injected into two different rabbits. Ten days after the immunisation, 5 ml of blood were collected. An ELISA test
5 was used to estimate the serum titer. An immunisation was regarded as successful when a titer above 1.10^4 (1E04) was reached.

Figures 8A to 8D: Estimation of the sera cross-reactivity against the different Mycobacterial antigens.

10 ELISA-plates were coated with membrane preparations from six different Mycobacterial species as indicated. For each serum the titer of response to the different antigens was estimated. Note the broad cross-reactivity of serum 35111 (Figure 8B) and the strong reactivity of 36147 against its designated antigen *M. xenopi* (Figure 8C). Serum 36040 shows a five-fold higher reactivity
15 against its antigen *M. avium* (Figure 8D). *M. gordonae* (Figure 8A) is specifically recognised by the developed antiserum 37174.

Figures 9A to 9E: Quantification of Mycobacterial charge through real-time PCR.

20 Genomic DNA prepared from *M. abscessus* was diluted in consecutive steps down to a concentration of 1pg/µl. The template DNA was added to a Sybergreen-based MasterMix. Real-time PCR with the primer-combination tb11-12 (SEQ ID NO:1 and SEQ ID NO:2) amplified a sequence from the hsp65 gene. **Figure 9A** shows the development of the fluorescence signals in
25 dependence of the cycle number, **Figure 9B** shows the linearity of the amplification reaction over a range of 1.10^3 pg (1E03 pg) down to 1 pg genomic DNA, the equivalent of 200 mycobacterial genomes. **Figures 9C and 9D** shows the analysis of a quantitative assay of water-samples spiked with different dilutions of *M. abscessus*. The assay was performed as indicated in
30 **Figure 6**. The assay detected bacterial concentrations down to $1.5.10^3$ cfu

(1,5E03 cfu) (**Figure 9C**). Note the linearity of the assay is not given for low amounts of bacteria (**Figure 9D**). **Figure 9E** shows the agarose gel of the quantitative PCR showing the formation of primer-dimers, when amplifying low amounts of template DNA.

5

Figures 10A and 10B: Molecular Live/Dead assay for Mycobacteria using Propidium-Monoazide (PMA)

Dead and live Mycobacteria (standardised on McF1) were mixed in 6 different ratios (0%, 10%, 25%, 50%, 80% and 100% living bacteria) in 1ml samples.

10 The samples were divided into two fractions: 1) incubation at room temperature for 20 min (-PMA); 2) incubation with PMA 50µM end concentration for 20 min at room temperature in the dark (+PMA). After the incubation, both samples were illuminated for 2 minutes with 650W on ice. The genomic DNA of both fractions (-PMA and +PMA) was isolated. **Figure 10A**
15 shows the quantitative analysis of the DNA amount through real-time PCR. Note the enhancement of the Cp-value (Crossing point value) or Ct-value for the +PMA sample on 7 units with 0% living bacteria, which is the equivalent of a 1.10^4 (1E04) diminution of the isolated DNA. **Figure 10B** shows the percentage of DNA isolated after PMA treatment vs percentage of living
20 bacteria in sample; DNA is measured by spectroscopic analysis at 260 nm of the isolated DNA from both samples (Nanotrop apparatus).

Figure 11: Schema showing the position of the sample tube during incubation for immunomagnetic bead capture.

25 The pre-concentrated sample is transferred into 5ml of 1xPBS supplemented with 0.1% Tween20. The 15 ml polystyrene tube is fixed in 45° angle from the rotor using a 2 cm distance from the axe of the rotor. The optimal turning speed to prevent sedimentation of the beads and not to disturb the fragile bead-bacteria connection was shown to be 2 rpm. The incubation was
30 performed at room-temperature during a time spin of 30 minutes using an

ELMI-Intelli Mixer RM2™. After the capture the bacteria-charged beads were captured with a magnetic bead retriever (Dyna), the supernatant was aspirated. The beads were washed through a 2-fold incubation with 1xPBS supplemented with 0.1% Tween20 for 5 minutes. After the final magnetic
5 capture, the bead-bacteria complexes were transferred in a buffer adapted in the down-stream applications.

Figure 12: Photograph of the agarose gel of the sodA PCR-reaction following the sodA qPCR assay.

10 Based on a list of Mycobacterial housekeeping-genes (Department of Biochemistry and molecular biology, Oswaldo Cruz, Rio de Janeiro, Brasil) (<http://www.dbbm.fiocruz.br/genome/mycobac/tubhousekeep.html>), the inventors searched for available sequence information from non tuberculosis Mycobacteria (NTM) with emphasis on the NTM found in water (*M .kansasii*,
15 *M. gordonae*, *M. xenopi*, *M. abscessus*, *M. avium*) using the BLAST-program (NCBI, USA). When a sufficient number of sequences from different mycobacterial species were identified from a potential housekeeping gene, the online program "ClustalW" (EMBL-tools, Germany) was used to produce significant alignments of the chosen sequences. Highly conserved stretches
20 were chosen to design primers with the online program "Primer 3" (Rozen S and Skaletsky HJ, 2000) with the goal to design a PCR-reaction resulting in short amplicons (100 to 200bp) to enhance the reproducibility of a potential quantitative assay. Chosen primer-pairs were analysed with the online-tool referred to as "Net-primer" (PREMIER Biosoft International 3786 Corina Way,
25 Palo Alto, CA 94303-4504, USA, for potential secondary structures and primer-dimer formation. A final "Blast search" of the developed primers against the Blast nucleotide-database was performed to ensure the specificity of the chosen sequences for the genus Mycobacteria as well as the non-identity with closely related species like *Nocardia* and *Rhodococcus*. A primer combination
30 was chosen, when at last the entire sequence of one primer was 100% unique

- for *Mycobacteria*. The chosen assay amplifies a 150bp variable stretch surrounded by two highly conserved regions (Position 484-510 and 670-700 on the *M. avium* superoxide dismutase A-gene (*sodA*; EMBL AccNr. AF180816; Liu,X.; Feng,Z.; Harris,N.B.; Cirillo,J.D.; Bercovier,H.; Barletta,R.G.;
- 5 Identification of a secreted superoxide dismutase in *Mycobacterium avium* ssp. paratuberculosis FEMS Microbiol. Lett. 202(2):233-238 (2001)).

The analysis of the *sodA* PCR-reaction on agarose gel.

- The chosen primer pair *sod-f* (SEQ ID NO:11) and *sod-r* (SEQ ID NO:12) was
- 10 used in a standard PCR reaction (50µl-assay volume primer end concentration 10pM) using the following thermal cycle scheme:

5 minutes at 95°C initial denaturation phase, 40 cycles denaturation for 1minute at 95°C, 1 minute annealing at 56°C and 1 minute extension at 72°C with a final extension step at 72°C for 10 minutes.

- 15 The photo shows the results of the PCR-reaction, *sod-f-sod-r*, on a series of consecutive genomic DNA dilutions 10 ng/µl to 100pg/µl from five different *Mycobacteria* resolved on a 1.5% agarose-gel. Note the absence of visible primer-dimers on the gel also at low DNA-concentrations.

20 **Figure 13: Evaluation of the specificity of the *sodA*-qPCR assay.**

- Serial dilutions of prepared genomic DNA from the mycobacteria mostly found in environmental water samples were used in a quantitative PCR assay using the designed primer pair SEQ ID NO:11 and SEQ ID NO:12 (Figure 12). The quantitative PCR-reactions were performed on a Roche Lightcycler™
- 25 Instrument using a 384-well plate. The reactions were performed in a 10µl volume. The PCR-mix consisted of 2µl DNA, 0.05µl of each primer (100µM concentration), 5µl Roche Sybergreen PCR Master Mix™ 2x concentrated and 2.9µl water.

- 30 The PCR reaction contained a 5 minute activation phase at 95°C, the

amplification was performed as follows: 15sec at 95°C, 15sec at 56°C and polymerisation for 30 sec at 72°C. The specificity of the amplicon was estimated through a melting curve analysis. The assay included a negative contamination control consisting of PCR-grade water treated in the same manner as the samples. Each sample was performed in a duplicate qPCR-reaction. The analysis of the data was performed through the Roche Lightcycler software. Ct-values, slope and PCR efficiency were estimated using the second derivative maximum method. Note the efficiency of the qPCR reaction (slope of the calculated lines) is equivalent for all the species tested.

The estimated PCR-efficiency (slope of the calculated lines) of the *sodA* qPCR with genomic DNA templates is the same for all tested mycobacterial species.

Figure 14: Reliability of the *sodA* qPCR assay

Prepared genomic DNA from *M. abscessus* in a concentration of 1ng/μl was diluted in 12 independent assays consecutively down to 0.1pg/μl. 2μl of each dilution was used as template in the qPCR assay targeting the *sodA* gene as described in Figure 13. The estimated ct-values were transformed into DNA-concentrations using the absolute quantification module of the Lightcycler software. Linear fitting, calculation of the r^2 and P-value were performed using the InStatTM-software (Graph-Pad, USA).

Figure 15: Immunocapture qPCR with spiked water samples

1 liter water samples spiked with a *M. abscessus* culture dilution were concentrated as indicated in the immuno-capture protocol as in Example 2. After genomic DNA preparation the total number of genomes present in the sample (Δ) was estimated with the *sodA* qPCR-assay. The obtained ct values were transformed into the concentration of Mycobacterial genomic DNA in pg/μl using the standard curve from Figure 14. These obtained DNA concentrations were transformed in Mycobacterial genomes using as

estimation an average weight for a mycobacterial genome as 5.5 femtogram. The calculated genomes values in Figure 15 are drawn to the totality of the sample.

- 5 To calculate the total loss of DNA during the immuno-capture procedure, the assay was also performed on a genomic DNA-dilution also transformed into genomes per assay ($\diamond$). Note that the loss of genomes through immunocapture is related to the initial concentration of bacteria in the spike: high concentrations resulted in 90% of the spiked bacterial DNA recovered, while
10 the recovery for low concentrations of initial bacteria (>100 bacteria) drops to an average of 40%.

Figure 16: Calculation of the mycobacterial charge in a spiked water sample with immunocapture qPCR

- 15 Figure 16 shows the calculated 95% confidence interval for the genome number calculation from spiked 1 liter water samples with the immunocapture PCR. The correlation is linear over a range from 100 to $1 \cdot 10^7$ ($1E02$ to $1E07$) genomes/ liter water. The absolute limit of quantification is 100 genomes per liter water, while the analytical sensitivity is below 10 genomes. Due to the
20 losses of bacteria during sample treatment, the real bacterial charge expressed in genomes per liter is underestimated up to 50% depending on the initial bacterial concentration.

- The correlation in a 95% confidence interval for the genome number
25 calculation is linear over a range from 100 to 1×10^7 genomes per liter water, while the analytical sensitivity is below 10 mycobacterial genomes.

Figure 17: Test of the immunocapture qPCR on tap water samples from Ile de France

The mycobacterial content of 1 liter water samples was preconcentrated and immunocaptured as indicated in the protocol of **Example 2**. After genomic DNA preparation the mycobacterial charge was quantified with the sodA qPCR assay. For a further characterisation of the pathogenic potential, mycobacterial species could be directly identified from samples with an estimated mycobacterial genome number above 1000 (Par5, Iss, Par14, Drin bef) (**Figure 18**).

Estimation of mycobacterial genome numbers. 1000ml tap water samples were preconcentrated and the mycobacteria present specifically immunocaptured as indicated in **Example 2**. After genomic DNA preparation the mycobacterial charge expressed as mycobacterial genome numbers per liter sample was quantified with the sodA qPCR assay.

Figure 18: Direct mycobacterial species identification from the sample

The mycobacterial genomic DNA from samples with an estimated mycobacterial charge over 1000 genomes as estimated in Figure 17 was used as a template in a hsp65 PCR reaction (Telenti, AF et al., 1993). Mycobacterial species could be directly identified from samples with estimated mycobacterial genome numbers above 1000 (Par5, Iss, Par14, Drin bef) (Figure 17). The obtained PCR product was digested in two separate reactions with the restriction enzymes HaeIII and BestEI. Figures 18A and 18B show the obtained restriction patterns resolved on a 5% agarose gel. The fragment lengths were estimated with the program Quantity One™ (BioRad, USA). Identified fragments lengths were used to identify the mycobacterial species with the online data base "PraSite" (Centre Hospitalier Université Vaudois, Lausanne, <http://app.chuv.ch/prasite/index.html>). The table presents the

identified Mycobacterial species in the samples together with the corresponding restriction patterns.

Figure 19: staining and microscopy displaying of Mycobacteria after bead-capture

Dilutions of *Mycobacterium abscessus* cultures were incubated with anti-Mycobacteria ssp-coated magnetic beads. After washing, the Mycobacteria fixed on the magnetic beads were stained with Ziehl-Nielsen staining or with fluorescent auramine O stain. The pictures were taken with a fluorescence microscope using a 100x objective.

While the invention will be described in conjunction with example embodiments, it will be understood that it is not intended to limit the scope of the invention to such embodiments. On the contrary, it is intended to cover all alternatives, modifications and equivalents as may be included as defined by the appended claims.

DETAILED DESCRIPTION OF THE INVENTION

1) Definitions

As used herein, the terms or expressions listed below will have the following meaning:

As used herein, the term "sample" may be a liquid sample. Such a "liquid sample" may be, but is not limited to, a water, milk or sputum sample. A water sample may include an environmental water sample as tap water or untreated water sample. For the purposes of the present invention, the liquid sample may also include a biofilm sample, wherein the biofilm is understood by a person skilled in the art as being a complex aggregation of microorganisms

marked by the excretion of a protective and adhesive matrix often found on the surface of water in water treatment systems. As one skilled in the art may appreciate, the sample may contain one or a plurality of mycobacteria strains amongst other types of bacteria or contaminants found in tap water or in untreated water.

As used herein, a "mycobacterium strain" may be any type of mycobacteria such as a non-tuberculosis mycobacterium (NTM). A non-exhaustive list of NTM strains detected by the method of the invention may be : *M. abscessus*, *M. kansasii*, *M. xenopi*, *M. avium* and *M. gordonae*. Of course, a person skilled in the art will understand that other NTM strains, such as those listed as part of Table 3, could also be detected by the method of the present invention, but that the above mentioned strains are the ones most commonly found in tap water or untreated water.

As used herein, the term "detecting" refers to the identification of a component of a microorganism, such as an epitope or a particular nucleotide sequence, which thereby determines the presence of the microorganism. It will be further understood that any one strain from the plurality of mycobacteria strains in the sample may be detected independently of any other strain. Also, it will be understood that more than one strain from the plurality of mycobacteria strains can be advantageously detected at the same time, and such, independently of any other strains. The term "detecting" can also refer to staining and microscopy visualisation of mycobacteria. Classical fluorescent stains of mycobacteria are for example Ziehl-Nielsen stain or fluorescent Auramine O dye (Kommareddi S, Abramowsky C, Swinehart G, Hrabak L (1984). "Nontuberculous mycobacterial infections: comparison of the fluorescent auramine-O and Ziehl-Neelsen techniques in tissue diagnosis". *Hum Pathol* **15** (11): 1085-9). Auramine O can be used together with Rhodamine B as the Truant auramine-rhodamine stain for *Mycobacterium tuberculosis*

As used herein, the expression "defined mycobacterium strain" denotes the mycobacterium strain that the method is tailored to detect, insofar as the method may be tailored to detect as many mycobacterium strains as possible
5 from the sample, either specifically or non-specifically, independently of one another, or may be tailored to detect a single mycobacterium strain.

Used in the present context, "low concentration of mycobacteria" is understood to be measured as a Mycobacterial charge under 1000 mycobacterial
10 genomes in 1 liter of water as estimated via qPCR.

As used herein, the expression "a viability determining agent" refers to an agent that selectively allows the determination of whether a cell is dead or alive (viable). More particularly, such an agent may penetrate cells which can
15 be considered dead and therefore may inhibit its nucleic acid amplification for determining its viability. A contemplated viability determining agent by the present invention may be a membrane-impermeant dye (e.g. propidium mono-azide (PMA)) that selectively penetrates cells with compromised membranes, such cells being considered dead.

20

As used herein, the expression "a predetermined number of mycobacteria genomes is indicative of a viable mycobacterium strain" refers to a defined number of genomes associated with a specific mycobacterium strain. In general, about 1000 or more mycobacteria genomes will be an indication that
25 a sample contains viable mycobacteria strains. For instance, in the experiments performed with locally obtained water (Region Ile de France) (see Example section) no Mycobacteria were grown on solid culture from samples with an estimated mycobacterial charge under 1000 genomes per liter.

30 As used herein, the term "specific" or "immunologically specific" or "specifically

directed" means that it is characteristic of the antibody to possess substantially greater affinity for mycobacteria than for other microorganisms contemplated by the present invention.

5 **2) Method for isolating mycobacteria**

The inventors have developed and optimised a method for selectively isolating Mycobacteria strains from a sample. The method is advantageously based on the non-specific and specific isolation of Mycobacteria present in a sample.

10

More specifically, the invention provides methods for the selective isolation and/or detection of mycobacteria from a sample.

15

Accordingly, an embodiment of the present invention is to provide a method for isolating mycobacteria from a sample which comprises a step a) of contacting a sample suspected of containing mycobacteria with magnetic beads coated with an antibody immunologically specific to mycobacteria under three-dimensional motion conditions to form bead-mycobacteria complexes.

20

A preferred embodiment of the present invention is to provide a method for isolating a NTM strain from a sample which comprises a step a) of contacting a sample suspected of containing a NTM strain with magnetic beads coated with an antibody immunologically specific to the NTM strain under three-dimensional motion conditions to form bead-NTM complexes.

25

Prior to step a), one may wish to add an optional non-specific filtering step so as to provide a concentrate of the sample. Such optional step may be achieved by a filter that allows the retention of mycobacteria (e.g. NTM strains), such as a 0.45µm filter.

30

This optional filtration step may be followed by a resuspension step of the bacteria stuck on the filter in a small volume (e.g. 5 ml) of buffer. Such a small volume may thereafter be used as a starting sample for the isolating method of the present invention. The buffer in which the bacteria or concentrate are
5 resuspended may be any buffer suitable to be used in accordance with the isolating and/or detecting methods of the invention

Preferably the volume of the starting sample is comprised between 0.5 and 25 ml, preferably comprised between > 0.5 and 25 ml and more preferably
10 5 ml. Indeed, a volume of 0.5 ml may be used with the three dimensional motion conditions.

If the sample is sputum, prior to step a) homogenization and decontamination of sputum sample could be performed. For example, the method of Kubica
15 (Kent, P. T., and G. P. Kubica. 1985. Public health mycobacteriology: a guide for the level III laboratory, p. 57–68. U.S. Department of Health and Human Services publication no. (CDC) 86-21654 6. Centers for Disease Control and Prevention, Atlanta, Ga.) could be used for this purpose.

20 As used herein, the term "coated" refers to having a coating material, such as an antibody specific for Mycobacteria, on the beads. The coating material may totally or partially cover the bead.

As used herein, the expression "three-dimensional motion conditions" refers to
25 the conditions that will allow the sample to be mixed or shaken in about all possible directions at slow tilt rotation, preferably 1-20 rpm, more preferably 2 rpm. Such conditions may be achieved, for instance, by placing the sample into a tube fixed orthogonally on a 360° rotary shaker, as shown in Figure 11. The sample is mixed or shaken for a defined period of time so as to permit the
30 bead-mycobacteria complexes to form. As used herein, the expression "for a

defined period of time", refers to the time necessary to let the mycobacteria cells to adhere to the beads. It must be at least 2 min and preferably about 30 min; however, it may also be a few hours or even overnight, if convenient.

- 5 The isolating method of the invention also comprises a step b) of magnetically recovering the bead-mycobacteria complexes to obtain a sample containing isolated mycobacterium strains.

10 As used herein, the expression "magnetically recovering" consists of capturing or retrieving the bead-mycobacteria complexes so as to obtain a sample containing isolated mycobacteria strains. The recovery of the complexes may be accomplished with a magnetic bead retriever, or with a magnetic stand as described in Example 2 and Figure 6.

- 15 Optionally, one skilled in the art may wish to subsequently wash the beads to eliminate components which could be non-specifically fixed to beads. In this connection, gentle washings may be performed by using, for instance, a solution of 1XPBS supplemented with 0.1% Tween20.

- 20 Another optional step, further to the step of recovering the bead-mycobacteria complexes, consists of separating mycobacteria from the beads. Such a step may be achieved by strong shaking (e.g. vortexing) of the sample.

25 Further to the isolating method of the invention, a step of detecting the presence or absence of at least one mycobacterium strain (e.g. NTM strain) in the sample containing isolated mycobacterium strains may be performed. In this connection, the method according to this embodiment of the present invention can further comprise, after step b), a step of detecting the presence or absence of a mycobacterium strain from the sample containing isolated

mycobacterium strains.

3) Method for detecting mycobacteria

5 Another embodiment of the present invention relates to a method for detecting mycobacteria comprising the steps of:

- 10 a) contacting a sample suspected of containing mycobacteria with magnetic beads coated with an antibody immunologically specific to mycobacteria under three-dimensional motion conditions to form bead- mycobacteria complexes;
- b) magnetically recovering the bead-mycobacteria complexes to obtain a sample containing isolated mycobacteria strains; and
- c) determining the presence or absence of a defined mycobacterium strain by the detection of said defined mycobacterium strain.

15

Another preferred embodiment of the present invention relates to a method for detecting NTM comprising the steps of:

- 20 a) contacting a sample suspected of containing a NTM strain with magnetic beads coated with an antibody immunologically specific to NTM under three-dimensional motion conditions to form bead-NTM complexes;
 - b) magnetically recovering the bead-NTM complexes to obtain a sample containing isolated NTM strains; and
 - c) determining the presence or absence of a defined NTM strain
- 25 by the detection of said defined NTM strain.

Following the selective isolation of the mycobacteria or NTM strains according to the present invention, a downstream analysis can be performed to estimate the proportion of dead bacteria and live bacteria in the concentrated bacteria sample. Therefore, in a related aspect of the invention, also provided is a method for detecting viable mycobacteria (e.g. NTM strain) from a sample. Such a particular mycobacteria detecting method comprises the steps of:

a) contacting a sample suspected of containing mycobacteria with magnetic beads coated with an antibody immunologically specific to mycobacteria under three-dimensional motion conditions to form bead-mycobacteria complexes;

b) magnetically recovering the bead-mycobacteria complexes to obtain a sample containing isolated mycobacteria;

c) adding a viability determining agent to the sample containing isolated mycobacteria;

d) extracting genomic DNA from the sample containing isolated mycobacteria of step c); and

e) detecting the genomic DNA of a defined mycobacterium; wherein the detection of a predetermined number of mycobacteria genomes is indicative of a viable mycobacterium.

One in the art will understand that the comparison of a sample of mycobacteria to which the viability determining agent was added with a non-treated sample will allow the estimation of the proportion of dead bacteria and live bacteria in the sample of concentrated bacteria.

One in the art will also understand that the presence or absence of mycobacteria strains may also be determined by the culture of the mycobacteria concentrated sample, for instance, onto Petri dish.

One in the art will also understand that the presence or absence of mycobacteria may also be determined by fluorescent staining and/or microscopy observation of the mycobacteria captured on the beads.

- 5 As already mentioned above, one may wish prior to step a), to perform an optional step of filtering the sample non-specifically in order to concentrate the sample.

This optional filtration step may also be followed by a resuspension step of the
10 bacteria stuck on the filter in a small volume (e.g. 5 ml) of buffer. Such a small volume may thereafter be used as a starting sample for the isolating and detecting methods of the present invention. The buffer in which the concentrate or bacteria are resuspended may be any buffer suitable to be used in accordance with the isolating and/or detecting methods of the
15 invention. For instance, such a buffer may be 1XPBS supplemented with 0.1% Tween20.

It will be understood by one skilled in the art that the step of detecting mycobacteria and particularly NTM strains in a sample can be achieved by immuno detection. For example, the immuno detection can be by an enzyme-
20 linked immunosorbent assay (ELISA) or by any other immuno detection method known to one skilled in the art.

Also, according to the present invention, the step of detecting may be achieved by molecular DNA detection which may consist of DNA amplification (e.g.
25 PCR), of nucleic acid hybridization (e.g. Southern blot) or of any other method known by a person skilled in the art.

Advantageously, one may contemplate performing a step for non selective DNA amplification (e.g. PCR) of all mycobacteria or all NTM followed by
30 hybridization (e.g. Southern Blot) of the products from the amplification with a

probe which is specific to one or more species of mycobacteria. This coupling will allow the evaluation of the total quantity of mycobacteria in the analyzed sample and the determination of the presence of one or more specific mycobacteria or NTM strains, depending on the probes used.

5

In the case where one may wish to utilize a hybridization-based detection system for molecular DNA detection, a nucleic acid probe is chosen that is complementary to a target nucleic acid sequence, (e.g. a NTM) and then by selection of appropriate conditions the probe and the target sequence "selectively hybridize," or bind, to each other to form a hybrid molecule. A nucleic acid probe that is capable of hybridizing selectively to a target sequence under "moderately stringent" conditions typically hybridizes under conditions that allow detection of a target nucleic acid sequence of at least about 15-25 nucleotides in length having at least approximately 70% sequence identity with the sequence of the selected nucleic acid probe. Stringent hybridization conditions typically allow detection of target nucleic acid sequences of at least about 15-25 nucleotides in length having a sequence identity of greater than about 90-95% with the sequence of the selected nucleic acid probe. Hybridization conditions useful for probe/target hybridization where the probe and target have a specific degree of sequence identity, can be determined as is known in the art (see, for example, *Nucleic Acid Hybridization: A Practical Approach*, editors B. D. Hames and S. J. Higgins, (1985) Oxford; Washington, D.C.; IRL Press).

25 Primers or probes used in accordance with the molecular DNA detection contemplated by the present invention are those that are specific to all mycobacteria strains. In a preferred embodiment, the contemplated primers or probes are those that are specific to all NTM strains. For instance, the primers or probes can be selected in a nucleotide sequence of NTM genes encoding

heat shock proteins (e.g. HSP65). One may wish to use pairs of primers already known in the art such as those disclosed in Talenti *et al.*:

Tb11f : 5'-ACCAACGATGGTGTGT-3' (SEQ ID NO :1),

5 melting temperature (TM) : 60°C 190,9 µM

Tb12r : 5'-CTTGTCGAACCGCATA-3' (SEQ ID NO:2),

TM 62°C 201.5 µM

(length of amplicon : 439 bp)

10 The following primer pairs respectively disclosed in Kim *et al.* and Yadav *et al.* can also be used:

HSPF3: 5'-ATCGCCAAGGAGATCGAGCT-3' (SEQ ID NO: 3), TM 60°C

HSPR4: 5'-AAGGTGCCGCGGATCTTGTT-3' (SEQ ID NO: 4), TM 60°C

and

15 hspSHf: 5'-CTGGTCAAGGAAGGTCTGCG-3' (SEQ ID NO: 5)

hspSHr: 5'-GATGACACCCTCGTTGCCAAC-3' (SEQ ID NO: 6);

(length of amplicon : 228 bp)

Other primers or probes may be produced in order to be used in accordance
20 with the present invention. For instance, the present inventors have designed and produced primers which can also be used as primers in accordance with the present invention. Those primers or probes have been selected in a nucleotide sequence of NTM genes encoding housekeeping genes. The housekeeping genes targeted may be selected from the group of genes
25 including, for example, the DNA gyrase B gene (*gyrB*); the super oxide dismutase gene (*sodA*), and the NADH-dependent enoyl-acyl-carrier-protein reductase gene (*inhA*).

In this connection, the pairs of primers designed from these genes by the inventors include the following:

Primers based on the *gyrB* gene:

gyrB-f 5-TTCGCCAACACCATCAACAC-3 (SEQ ID NO: 7)

gyrB-r 5-GTGTTGCCCAACTTGGTCTT-3 (SEQ ID NO: 8);

5 Primers based on the *inhA* gene:

inhA-f 5-GCATCAACCCGTTCTTCGAC-3 (SEQ ID NO: 9)

inhA-r 5-ACCGTCATCCAGTTGTACGC-3 (SEQ ID NO: 10)

Primers based on the *sodA* gene:

10 *sodA*-f 5-CCACTCGATCTGGTGGAA-3 (SEQ ID NO: 11)

sodA-r 5-TGGTCGTACAGCTGGAAGGT-3 (SEQ ID NO: 12)

One skilled in the art will appreciate that the genome number of at least one mycobacterium strain (e.g. NTM strain) in the sample containing isolated
15 mycobacteria can be estimated. One skilled in the art will understand that the genome number can be estimated by DNA amplification, e.g. by quantitative PCR (qPCR).

20 **4) Beads and antibodies used in accordance with the present invention**

The beads used in accordance with the method of the present invention can have a diameter of about 2 to about 5 microns, and advantageously, of about 2.8 microns. Such beads may be hydrophobic beads with p-toluenesulphonyl (tosyl) reactive groups on their surface, such as those described in the
25 Example section hereinbelow.

Furthermore, the antibody used may be linked to the bead via a linker. As used herein, the term "linker" refers to a molecule that acts as an intermediary

molecule in the sequestering of the anti-mycobacteria antibody to the bead's surface. The linker is attached (e.g. covalently) to the bead's surface and interacts (e.g. non-covalently) with the anti-mycobacteria antibody to maintain such antibody at the surface of the bead. A linker which may be used in accordance with the present invention consists of Protein A or of an antibody that can bind the anti-mycobacteria antibody contemplated by the present invention. Other commonly used linkers for the coating of beads with antibodies, such as a rabbit anti-IgG antibody or Protein G for example, can be used in the method of invention.

10

The choice of the linker depends on the animal from which the anti-IgG antibody is obtained.

The contemplated antibody used in accordance with the present invention may be a polyclonal antiserum or a fraction thereof which has an affinity profile to specifically bind to the majority or all the mycobacteria strains in a sample. Such a fraction may be, for instance the IgG fraction from a polyclonal antiserum raised against a mycobacterium strain.

15

A preferred antibody used in accordance with the present invention is a polyclonal antibody which is able to recognize all mycobacteria strains (of *M. tuberculosis* complex and NTM) with an equivalent specificity. For instance, a contemplated antibody used in accordance with the present invention may be the 35 111 polyclonal antibody obtained by immunisation of rabbits with lyophilised *M.abscessus* cell wall preparations and able to recognize all mycobacteria strains with the same affinity as shown in Figure 8B.

20

25

Furthermore, it will also be understood by one skilled in the art that a monoclonal antibody that specifically binds to a mycobacterium strain (e.g. a NTM strain) can also be used to coat magnetic beads in accordance with the

30

present invention. The term "monoclonal antibody" means an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts.

5 The "monoclonal antibodies" also include clones of antigen-recognition and binding-site containing antibody fragments (Fv clones) isolated from phage antibody libraries using the techniques described in Clackson et al., Nature, 352:624-628 (1991) and Marks et al., J. Mol. Biol., 222:581-597 (1991), for example.

10

In light of the above, one skilled in the art will understand that it is within the scope of the present invention to use an antiserum with a higher affinity for a specific strain. In other words, one may wish, in a case where the isolation and/or detection of a target mycobacterium strain (e.g. NTM strain) are of particular interest, to use an antibody specific to such a mycobacterium strain (e.g. a NTM strain). Alternatively, one may wish to solely determine whether a given sample contains mycobacteria strains (e.g. NTM strains) in general. In this connection, an antibody which has the capacity of detecting all mycobacterium strains (e.g. all NTM strains) may be used in accordance with the methods of the present invention.

20

The person skilled in the art will surely appreciate that the present invention consists in an evaluation of different parameters for a magnetic bead capture of mycobacteria. After an optimisation of various parameters, the inventors have shown and presented in the Examples herein below that it is possible to capture even very low amounts of bacteria with a high efficiency (over 75%). This result has not been reached in prior art assays.

25

5) Further advantages of the present invention

The person skilled in the art will further appreciate that the mycobacteria (and especially NTM) isolation method of the present invention solves the need:

5

a) to concentrate a small number of mycobacteria present in an original sample;

b) to separate mycobacteria from other substances like other bacteria or inhibitors present in the sample;

10

c) to culture mycobacteria in order to obtain a concentration high enough so as to allow their detection;

d) to obtain a good quality DNA for analysis; and

e) to decrease the time required to perform a mycobacteria identification procedure in a sample from 6 weeks to about 2 days.

15

Especially the isolation method of the invention permits to remove inhibitors of PCR that are in mycobacteria preparations obtained by traditional isolation process consisting in centrifuging sample and recovering a mycobacteria pellet. Then, the isolation method of the invention permits a reliable and reproducible PCR detection of mycobacteria.

20

The Examples provided herein below will further contribute to the description of the present invention.

EXAMPLE 1:

25

The invention describes for the first time the important influence of sample size on capture efficiency. The inventors have shown that a volume of 5 ml was optimal to capture Mycobacteria with the highest efficiency. The inventors showed that the manner of incubation influences in strong manner the capture efficiency. To ensure an optimal fixation and orientation of the purified IgG on

the magnetic beads a non-covalent fixation via covalent attached protein A was optimal. The example presented herein below describes a new method with optimised steps resulting in superior efficiency of Mycobacterial capture, thus enhancing the sensitivity of downstream detection procedures.

5

Materials

Beads

Dynabeads® M-450 Tosylactivated: Hydrophobic naked beads with p-toluenesulphonyl (tosyl) reactive groups. For coupling of antibodies for cell applications. Proteins are absorbed hydrophobically on initial coupling with covalent binding of primary amine groups (NH₂ and sulphhydryl groups (SH)) occurring overnight. Coupling reactions can be performed at neutral pH although high pH and incubation at 37°C will promote covalent binding.

Dynabeads® M-280 Tosylactivated : Hydrophobic, monodisperse magnetic particles (2.8 µm in diameter) with p-toluene-sulfonyl (tosyl) groups, further surface activation not required. Allows easy coupling of antibodies with optimal orientation for affinity purification of proteins. Covalent coupling to primary amino- or sulphhydryl groups in proteins/peptides. Coupling overnight at temperatures up to 37°C and pH 8-9.

20

Polyclonal antibody

Protocol for the immunisation of rabbits with lyophilised *M.abscessus* cell wall preparations

25

Preparation of antigen:

- 1) 100ml of Sauton medium supplemented with 0.1 Tween 20 are spiked with a colony of *M. abscessus*

- 2) *M. abscessus* is grown to a density of McF1 ($2 \cdot 10^8$ mycobacteria/ml) (Manual of Clinical Microbiology, Balows et al., 5^e ed., 1991) (3 to 4 days) at 37°C on rotary shaker
- 3) *M. abscessus* culture is centrifuged at 5000g 10 min
- 5 4) Pellet is two times washed with 50 ml sterile 1xPBS buffer
- 5) Washed Pellet is suspended in a 15ml Greiner Tube with 5 ml 1xPBS supplemented with 0.1% CHAPS and 0.1% Triton X-100
- 6) Addition of 7gr sterile glass-beads ($\varnothing$ 4mm)
- 7) Slight horizontal shaking of the tube per hand 1min
- 10 8) Recuperation of supernatant
- 9) Freeze drying of supernatant through lyophilisation
- 10) Solubilisation of freeze dried material in 2ml 1xPBS
- 11) Estimation of protein content

Immunisation of rabbits

- 15 1) Rabbits variety New Zealand 2 kg weight
- 2) Collection of 0 serum 5ml estimation of serum titer against antigen
- 3) 1st immunisation with 0,5 ml *M. abscessus* lyophilisat (1mg/ml) in 1xPBS in emulsion with 0,5 ml Freud's adjuvant incomplete (1 ml per
- 20 rabbit)
- 4) Intradermic injection 5 different emplacements per rabbit
- 5) Collection of 5ml serum after 1st immunisation: 3 weeks later, estimation of serum titer
- 6) 2nd and 3rd immunisation same procedure as points 3 to 5
- 25 7) If antibody-titer(50%) after the third immunisation has reached a value over $1 \cdot 10^4$ ($1E04$) the immunisation is successful otherwise a 4th immunisation will be performed
- 8) Final Bleeding of animal
- 9) Preparation of serum

10) Estimation of cross-reactivity against other Mycobacterial species and other bacteria (Nocardia)

11) Preparation of IgG fraction from obtained serum. Optical measurement at 280nm

5

Primer or probes:

hsp 65 gene

Telenti et al., 1993

- 10 Tb11 : 5'-ACCAACGATGGTGTGT-3' TM : 60°C 190,9 µM (SEQ ID NO :1)
Tb12 : 5'-CTTGTCGAACCGCATA-3' TM 62°C 201.5 µM (SEQ ID NO :2)
(439 bp)

Kim et al., 2005

- 15 HSPF3: 5'-ATCGCCAAGGAGATCGAGCT-3' TM 60°C (SEQ ID NO :3)
HSPR4: 5'-AAggTgCCg Cgg ATC TTg TT-3' TM 60°C (SEQ ID NO :4)

Yadav et al., 2003

- hspSHf: 5'-CTGGTCAAGGAAGGTCTGCG-3' (SEQ ID NO :5)
20 hspSHr: 5'-GATGACACCCTCGTTGCCAAC-3' (SEQ ID NO:6)
(228 bp)

gyrB-gene

- 25 gyrB-f 5-TTCGCCAACACCATCAACAC-3 (SEQ ID NO:7)
gyrB-r 5-GTGTTGCCCAACTTGGTCTT-3 (SEQ ID NO: 8)

inhA-gene

- 30 inhA-f 5-GCATCAACCCGTTCTTCGAC-3 (SEQ ID NO:9)
inhA-r 5-ACCGTCATCCAGTTGTACGC-3 (SEQ ID NO:10)

sodA-gene

sodA-f 5-CCACTCGATCTGGTGGAA-3 (SEQ ID NO:11)

sodA-r 5-TGGTCGTACAGCTGGAAGGT-3 (SEQ ID NO:12)

5 Method

For a rapid and precise analysis on their presence in samples, it is preferable to separate the Mycobacteria from the sample matrix and to concentrate the bacteria present in a very small volume. This enables a downstream processing of the sample for an analysis with molecular or immunological methods. The inventors have determined a procedure to efficiently isolate the Mycobacterial strains in environmental water-samples like tap-water or untreated water-samples.

The concentration of bacteria with magnetic beads is used regularly in microbiological applications, like in the detection of *Salmonella* or *E.coli*, but most of the studies published so far have not driven by the interest to capture very low amounts of bacteria, since, in most of the cases, a pre-amplification phase is included or the initial number of bacteria is already very high (>1000 cfu/ml). When considering the studies published so far, capture-efficiencies below 50% of the total bacteria present in the sample have been reported (Fu *et al.*, 2005; Yu *et al.*, 2001).

Experimental procedure

To ensure a high efficiency and specificity of the bead-capture, immunizations of rabbits with the prepared surfaces of the outer Mycobacterial layer were performed to obtain polyclonal antisera (**Figure 7**). The antiserum chosen for the experiments described herein (35111) showed a high sensitivity against all the Mycobacteria used in the present study (*M. abscessus*, *M. kansasii*, *M. xenopi*, *M. avium* and *M. gordonae*) (**Figure 8**).

In an initial experiment (**Figure 1**), different sizes of magnetic beads were tested (2.8µm and 4.5µm), different attachments of the polyclonal antibody via Protein A and different preparations of the antigenic activity (IgG-fraction, affinity purified) were also tested. Here the inventors show that the most efficient capture of the bacteria could be reached through a Protein A based non-covalent fixation of the purified IgG-fraction of 35111 on 2.8µm magnetic beads from Dynal.

With this combination, the present method of the invention was able to capture over 75% of 1.10^7 cfu ($1E07$ cfu) *M. abscessus* in a test volume of 0.5ml. The incubation was performed at room-temperature on a rocking platform in slow-motion to prevent the settling of the beads during the two hour incubation period. In the following experiment, the inventors showed that this process is only efficient for high concentration of bacteria in the sample. **Figure 2** indicates the efficacy of capture for low numbers of bacteria dropped to around 20% of the initial bacteria present in the spiked sample. This result indicated that the conventionally used incubation method is insufficient to ensure an efficient capture.

To evaluate the influence of sample-size and incubation on the efficiency of the bacterial capture, the procedure was performed with a constant number of bacteria (1.10^5 cfu ($1E05$ cfu) *M. abscessus*) present in 3 different sample sizes (0.5 ml, 5ml and 25ml) with a constant number of antibody coated beads (1.10^8 ($1E08$)). The highest efficiency was obtained through the incubation in a 5ml sample volume in a 15ml Greiner tube (**Figure 3**).

The inventors showed that the capture of different concentrations of Mycobacteria in sample volume of 5ml can be performed with a consistent efficiency over 75% when using a 360° rotor in slow tilt rotation (2 rpm) and the 15ml tubes fixed in a 45° angle (see **Figures 4 and 11**).

Through the developed protocol (see **Table 1**), the detection method of the present invention was able to capture and concentrate Mycobacteria in different concentrations in a very efficient manner. The application of this detection method or capture procedure showed a 100-fold increase in sensitivity through bead-capture in comparison to a concentration of the bacteria through centrifugation (**Figure 5**).

Results:

The inventors report the development and optimisation of different parts of a fast test-procedure to detect the presence of Mycobacteria in water-samples. The test is based on the non-specific and specific concentration of Mycobacteria in combination with a direct PCR-reaction.

In detail, the inventors worked on three different components of the test: 1) the non-specific concentration of bacteria through filtration of 500 to 1000 ml water samples and their detachment, 2) The immunomagnetic concentration of Mycobacteria and 3) genomic DNA preparation and genus-specific PCR to detect the presence of captured Mycobacteria (**Figure 6**).

To ensure a specific and efficient immunomagnetic bead-capture, the inventors raised polyclonal antisera against outer cell-wall preparations of environmental Mycobacteria (**Figure 7**). For the optimisation of the capture procedure, an antiserum (35111) was used which showed a high sensitivity against all five Mycobacteria employed in this study (*M. kansasii*, *M. abscessus*, *M. xenopi*, *M. avium* and *M. goodnae*) (**Figure 8**). Through an optimised fixation of the IgG-fraction from 35111 on Dynabeads (Invitrogen), the inventors were able to capture up to 90% of the Mycobacteria present in a spiked 5 ml sample even at very low concentrations of Mycobacteria (**Figure 4**).

The detection of the bead-captured Mycobacteria is performed through a direct

PCR-procedure. After a genomic DNA preparation adapted on low numbers of gram-positive bacteria, a genus-specific PCR detects the presence of Mycobacteria in the sample. In comparison to a non-specific concentration of the sample through centrifugation, the specific immunomagnetic capture was
5 shown to be 500-fold more sensitive (**Figure 5**). The developed test-procedure was validated against a set of different water-samples (**Table 2**). The inventors showed that all samples with a Mycobacterial count over than 50 cfu were correctly identified through the developed procedure of the present invention.

To quantify the number of Mycobacteria present in water samples the
10 inventors worked on a genus-specific real-time PCR-procedure. An assay on diluted genomic mycobacterial DNA showed linearity over a range of ten concentrations down to DNA-concentration of 1pg/ μ l, the equivalent of 200 mycobacterial genomes (**Figure 9A and B**). The analysis of spiked water samples with real-time PCR showed that $2 \cdot 10^3$ cfu ($2E03$ cfu) of Mycobacteria
15 can be detected (**Figure 9C and D**).

To estimate the ratio of dead/live Mycobacteria in the sample the inventors worked on the use of propidium-mono-azide (PMA) for a covalent marking of dead bacteria (Nocker et al., 2006). PMA penetrates into dead bacteria and sticks to them after illumination to their double-stranded DNA in a covalent
20 fashion, thus preventing their isolation. The analysis of *M. abscessus* samples with a different fraction of living bacteria (0%, 10%, 25%, 50%, 80% and 100%) through quantitative real-time PCR showed a 5000-fold reduction of genomic DNA when adding PMA to dead-bacteria compared to the non-treated sample (**Figure 10A**). The amount of genomic DNA isolated from the
25 PMA-treated samples increased with the fraction of living bacteria in the sample, while the amount of genomic DNA isolated from non-PMA treated samples stayed nearly constant (**Figure 10A**). This is confirmed through the spectroscopic analysis of the isolated DNA (**Figure 10B**).

EXAMPLE 2:**Protocol for the qPCR immunocapture**

Reference is made to Figure 6.

5 Steps 1 and 2: Sample pre-concentration through filtration

The spiked samples were filtrated through a 0.45µm Isopore filter membrane (Millipore) on a Sartorius filtration-ramp using sterile plastic funnels. The filter with the bacteria was transferred sterile into a 50ml Greiner tube with 5ml of sterile detachment buffer (1xPBS supplemented with 0.01% Triton X-100). To
10 mechanically detach the bacteria on the filter, the fixed tube was vortexed at highest speed for 5 minutes. The supernatant with the detached bacteria was transferred into a 15ml Greiner tube.

Step 3a: Preparation of antibody-coated micro-beads

15 The beads for the immunocapture were prepared as follows: 1ml of 2.8µm tosylactivated micro beads (2×10^9 ; Dynal, Invitrogen) were washed in sterile 1xPBS. After the addition of 500 µl 1xPBS supplemented with recombinant ProteinA (Sigma P7837) to a final concentration of 1mg/ml, the tube was incubated over night at 37°C under slow rotation to prevent the settlement of
20 beads to allow a covalent attachment of the ProteinA-molecule on the tosylactivated surface. The incubation was followed by a wash with 1xPBS. The ProteinA-coated beads were incubated with a purified IgG fraction of the antiserum 35111 dissolved in 500µl of 1xPBS at a concentration of 1mg/ml. After 1 hour slow-tilt rotation at room-temperature the beads were used for the
25 immunocapture procedure.

Step 3b: The immunocapture of Mycobacteria

25µl (5×10^7) of the antibody-coated magnetic beads were added to 15ml

Greiner tubes with the detached bacteria in buffer. The tubes were rotated slowly for 45 minutes at room-temperature. After the incubation, the beads with the attached bacteria were recuperated with a magnetic stand (Dyna, Invitrogen). After removal of the supernatant the beads were solubilised in
5 950µl of phosphate buffer (FastDNA Spin for soil kit, MP Biomedicals) supplemented with Blue-Dextran (Sigma D4772) in a final concentration of 1pg/µl.

Step 4: The preparation of genomic DNA

10 The bead-captured bacteria in phosphate buffer were transferred into screw-cap tubes supplemented with a mixture of beads in different sizes optimised for the mechanical disruption of bacteria in earth and water samples (Fast DNA spin Kit™). After the addition of lysis buffer from the kit, the tubes were beaten for 90 seconds in a "bead beater"™ (Biospec Products, USA) at
15 highest frequency. The preparation of genomic DNA from the disrupted bacteria was performed according to the protocol. The isolated genomic DNA was eluted in a final volume of 50µl water molecular biology grad (Gibco).

Step 5: The quantitative PCR

20 To detect and quantify the total mycobacterial charge of the sample, the inventors performed a PCR-assay targeting the superoxide dismutase A-gene (*sodA*) in the genomic DNA from Mycobacteria. *sodA* is a highly conserved housekeeping gene of Mycobacteria. Based on public available sequence information, the inventors identified a short variable stretch surrounded by two
25 highly conserved regions (Position 484-510 and 670-700 on the *M. avium* *sodA*-gene (EMBL AccNr. AF180816).

In this region, the inventors designed a primer pair, SEQ ID NO: 11 and SEQ ID NO: 12, showing 100% identity with the Mycobacteria most frequently found

in environmental water samples (*M. avium*, *M. gordonae*, *M. kansasii*, *M. xenopi* and *M. abscessus*).

The quantitative PCR-reactions were performed on a Roche Lightcycler Instrument using a 384-well plate. The reactions were performed in a 10µl
5 volume. The PCR-mix consisted of 2µl DNA, 0.05µl of each primer (100µM concentration), 5µl Roche Sybergreen PCR Master Mix 2x and 2.9µl water.

The PCR reaction was divided into three phases: 1) a 5 minute activation at 95°C, 2) the amplification: denaturation for 15 seconds at 95°C, annealing for 15 seconds at 56°C and polymerisation for 30 sec at 72°C for 45 cycles and 3)
10 the melting-temperature analysis of the obtained products to analyse the specificity of the amplicons. Each assay included a negative contamination control consisting of PCR-grade water treated in the same manner as the samples. The qPCR-reactions of the samples were performed in duplicate. To ensure a standardised analysis of the data, the inventors used the automated
15 algorithms of the Roche Lightcycler analysis-software using the second derivative maximum method. Ct-values, slope and PCR efficiency were estimated.

For a comparison of the different assays, the values for the sensitivity were expressed as numbers of genomes per reaction. As the assay targets the
20 whole genus Mycobacteria, in which the different species possess slightly different genomes sizes, the inventors calculated the average weight for a standard mycobacterial genome as 5.5 femtogram from available mycobacterial complete genome-sequences (*Mycobacterium tuberculosis* (strains H37Rv and CDC1551), *M. bovis* AF2122/97, *M. avium* subsp.
25 paratuberculosis K10, *M. leprae* TN, and *M. smegmatis* MC2 155) and the inventors estimated the copy number of the *sodA* gene as 1 for all Mycobacteria.

EXAMPLE 3: qPCR Immunocapture of Mycobacteria in water-samples: a rapid and sensitive method for routine testing

1) Introduction

Mycobacteria

5 The genus Mycobacteria consists of over 150 species with different pathogenicity for humans. Besides the obligate pathogens *M. leprae* and the *Mycobacterium tuberculosis* group, potentially pathogenic Mycobacteria for humans and animals are the *M. avium*-complex and the environmental or atypical Mycobacteria.

10 Infections with EM

EM are responsible for non-tuberculosis infections in patients with AIDS and elderly persons. EM are also the source of infections in medical facilities due to inattentive treatment of sterile instruments during treatments. The exposure to EM through tap-water could be the source of EM-infections in humans as their
15 presence in drinking water and in bio-films of water distribution systems was demonstrated.

Test procedures

Routine testing in water microbiology laboratories through conventional
20 culture-based methods are rarely performed as the slow growth-rates of EM make these procedures a time-consuming and laborious task.

The rapid test

The inventors developed a fast (6 hours) and sensitive (<100 genomes per
25 liter) test-procedure to detect the presence of non-tuberculosis Mycobacteria in water-samples.

The test is based on a non-specific and a specific immunomagnetic concentration of the Mycobacteria present in the sample followed by a direct

quantitative PCR-analysis (qPCR).

In detail, the inventors worked on three different components of the test: (1) the non-specific concentration of bacteria through filtration of 500 to 1000 ml water samples and their detachment; (2) the immunomagnetic concentration of mycobacteria and (3) the genomic DNA preparation and genus-specific PCR to detect and quantify the mycobacterial content in the sample.

2) Materials and Methods

Antisera against mycobacteria

To ensure a specific and efficient immunomagnetic bead-capture, the inventors raised polyclonal antisera against outer cell-wall preparations of environmental mycobacteria (EM). For the capture procedure, the inventors used an antiserum (35111) which showed a high sensitivity against all five Mycobacteria employed in this study (*M. kansasii*, *M. abscessus*, *M. xenopi*, *M. avium* and *M. goodnae*)

Optimisation of bead capture

In experiments with mycobacteria spiked samples the inventors showed that the size of the paramagnetic beads and the fixation of the antiserum had an important impact on the capture efficiency (**Example 1**).

Besides the antibody coated beads, the volume used for the capture procedure is important. Using constant concentration of beads and bacteria with different assay volumes, the inventors showed that 5 ml is optimal (**Example 1**).

After the application of an adapted incubation protocol to prevent the sedimentation of the optimised magnetic beads through slow-tilt rotation, the capture efficiency of the Mycobacteria was always between 75 to 90% even at

very low concentrations of mycobacteria (**Example 1**).

qPCR assay

The detection and quantification of the bead-captured Mycobacteria is performed through a quantitative PCR-assay (qPCR). After a genomic DNA isolation adapted on the low numbers of gram-positive bacteria present in a sample, a genus-specific PCR detects the presence of mycobacteria in the sample.

The inventors developed a mycobacteria genus-specific PCR assay using the mycobacterial household gene *sodA* (**Figure 12**). The inventors showed that the qPCR assay has the same PCR-efficiency for the set of the most important EM in water (*M. kansasii*, *M. xenopi*, *M. avium*, *M. gordonae* and *M. abscessus*) (**Figure 13**). Further experiments with the whole mycobacterial collection of the Institut Pasteur showed that nearly all mycobacterial DNA-templates (95 of 102) showed a positive result in the assay. The closest genus *Rhodococcus* was negative (**Table 3**).

With a dilution of genomic DNA the inventors demonstrated the high reproducibility of the PCR-assay (**Figure 14**). The assay has an absolute sensitivity of 10 femtogram/reaction (the equivalent of two mycobacterial genomes) and is linear over a range of 5 log.

The combination of immunocapture and qPCR

In experiments with 1000 ml mycobacteria spiked water-samples, the inventors performed the complete immuno-capture qPCR procedure as in **Example 2**. To estimate the loss of DNA during the whole procedure, the inventors compared the results of the qPCR with the results of genomic DNA dilutions as a template. For low mycobacterial concentrations in the spike, the loss was estimated as 40% of the Mycobacteria present (**Figure 15**). The estimation of

the assay sensitivity revealed a 5 log linear relation between the amount of spiked mycobacteria and the calculated number of mycobacterial genomes (**Figure 16**). The quantitative sensitivity of the assay is below 100 mycobacteria per 1000ml sample while the analytical sensitivity is below 10 mycobacteria.

3) Results

The estimation of mycobacterial genome numbers

The inventors used the developed immunocapture qPCR to estimate the number of genomes in tap water samples from the Ile de France region. (**Figure 17**). In this ongoing examination, the number of mycobacterial genomes in positive samples was between 100 and 10000 genomes per liter.

Mycobacteria species identification and comparison with culture

For a further characterisation of the pathogenic potential in the samples, the inventors could use the genomic DNA directly isolated from samples with over 1000 genomes/liter to identify the mycobacterial species with PCR-restriction analysis (PRA), only applicable on cultured colonies until now (**Figure 18**). The inventors identified typical EM in these samples (**Table 4**). A parallel examination of these samples via culture-based identification confirmed the results obtained. This whole test-procedure, including species identification, can be finalised in only two work days, compared to the culture-based approach which was usually finalised in a time span of 6 weeks.

For the first set of analysed tap-water samples (30 samples) the inventors observed that samples with mycobacterial genome numbers over 1000 always showed the presence of colonies, when analysed in parallel with culture (**Table 4**).

Table 1 : Procedure for immunomagnetic capture of Mycobacteria**• Bead-coating**

- Incubation of 1 ml Dynabeads Tosylactivated (2.8µm, Dynal Invitrogen) in 500 µl Protein A-solution (1mg/ml in 1xPBS pH7.5), at 37°C;
- Blocking of non-reacted Tosyl-groups with 200 mM Tris pH 7.5, 2h 37°C;
- 2x wash 1xPBS RT;
- Storage of beads in 1xPBS 0.1% Tween20 supplemented with BSA 1mg/ml 0.01% sodium-azide; and
- All incubations are performed on the Elmi Intelli –Mixer RM-2 at 2 rpm.

• Capture procedure

- 5 ml of 1xPBS supplemented with 0.1% Tween20 -buffered sample in 15 ml Greiner Tube (Polystyrol);
- Addition of 100 µl antibody-coated beads ($2 \cdot 10^8$ (2E08)) washed before use in 1ml 1xPBS supplemented with 0.1% Tween20;
- Incubation at room temperature 30 minutes at 2 rpm, fixation of tubes in 45° angle on the mixer (**Figure 11**);
- Magnetic capture of Mycobacteria-coated beads; and
- 2x wash with 5 ml 1xPBS, gentle movement of the tubes to resolubilise the beads, no vortex.

Table 2: Validation of the immuno-capture procedure (Figure 6) for Mycobacteria.

Sample	Date	Sample volume	Direct PCR	Solid culture	PCR	Species identification
Name/ type of sample		Bead- capture/ culture	hep65- gene (tb11- tb12)	Number of slow- growing colonies on 5 weeks cultured filter (37°C) Decontamination 100ml sample with cpc	Hep65 (tb11-tb12)/ Intragenic 16srRNA on cultured colonies	PRA analysis of hep65 gene on PCR positive colonies
Domestic and environmental samples						
Clmt-1 (Tap- water)	5.12.07	400ml/ 100ml	-	1	Negative	
Clmt-2 (Tap- water)	15.12.07	400ml/ 100ml	-	-		
Smc-1 (Tap- water)	5.12.07	400ml/ 100ml	+	20	+/+	<i>M.chelonae</i> / <i>M.abscessus</i>
Smc-2 (Tap- water)	15.12.07	400ml/ 100ml	+	10	+/+	<i>M.chelonae</i> / <i>M.abscessus</i>
Smc-3 (Tap- water)	21.01.07	400ml/ 100ml	+	12	+/+	<i>M.chelonae</i> / <i>M.abscessus</i>
Plt-1 (Tap- water)	5.12.07	400ml/ 100ml	-	2	+/+	<i>M.gordonae</i> / <i>M.abscessus</i>
Plt-2 (Tap- water)	15.12.07	400ml/ 100ml	-	2	+/+	<i>M.gordonae</i>
Her-1 (Tap- water)	28.11.06	400ml/ 100ml	+	14	+/+	<i>M.scrofulaceum</i> or <i>M.chelonae</i>
Cnl-1 River	5.12.07	400ml/ 100ml	- (inh)	2	Negative	
Cnl-2 River	15.12.06	400ml/ 100ml	- (inh)	1	Negative	

Samples water-works						
Wpre-1 (untreated water)	23.11.06	400ml/ 100ml	-	3	Negative	
Wpre-2 (Untreated water)	28.02.07	400ml/ 100ml	-	-		
Wprn-1 (Untreated water)	23.11.06	400ml/ 100ml	-	-		
Wprn-2 (Untreated water)	28.02.07	400ml/ 100ml	-	2	+/+	<i>M.chelonae</i>
Wprn-1 (untreated water)	28.02.07	400ml/ 100ml	-	1	+/+	<i>M.scrofulaceum</i>
Wppc-1 (Treated water)	23.11.06	400ml/ 100ml	+	-		
Wppc-2 (Treated water)	28.02.07	400ml/ 100ml	+	-		
Wppn-1 (Treated water)	23.11.06	400ml/ 100ml	-	-		
Wppn-2 (Treated water)	28.02.07	400ml/ 100ml	+	5	+/+	<i>M.chelonae</i> / <i>M.abscessus</i>
Wppm-1 (Treated water)	28.02.07	400ml/ 100ml	-	-		

Water-filters						
Fdw - 1 (Water-filter prof)	5.09.06	Content in 400ml stirred 300/100	+	No culture		Direct Identification InnoLipa: <i>M.kansasii</i>
Fdw - 2 (Water-filter prof)	7.01.07	Content in 400ml stirred 300/100	-	-		
FveoA (Water-filter prof)	1.12.06	Content in 400ml stirred 300/100	-	-		
FveoB (Water-filter prof)	1.12.07	Content in 400ml stirred 300/100	-	-		
FBr-1106 (Water-filter household)	7.01.07	Content in 400ml stirred 300/100	+	5	+/+	<i>M.chelonae/M.abscessus</i>
FBr-1206 (Water-filter household)	1.12.07	Content in 400ml stirred 300/100	+	8	+/+	<i>M.chelonae/M.abscessus</i>

In Table 2, the inventors analysed four different types of water-samples: 1) domestic and environmental water samples (10 samples), 2) water from the water works before and after treatment (10 samples in total), 3) the bacterial content of water-filters after the statutory time of utilisation (6 samples) and 4) samples from the formation of biofilms in an experimental set-up (4 samples). Each of the samples was divided into two fractions and independently analysed 1) via immunomagnetic capture and direct PCR for Mycobacteria and 2) through disinfection of the sample with 0,001% cpc (v/v) to eliminate background bacterial flora, filtration through a 0,45µM polycarbonate membrane and its culture on 7H11 ODAC-agar for 5 weeks. Slow growing colonies were sub-cultured and the mycobacteria were identified at the species-level through PCR of a 441bp fragment from the hsp65-gene. Note the correct identification of all samples with Mycobacterial counts over 50 cfu through the immunomagnetic capture method.

Table 3 : Species reactivity of the sodA qPCR assay.

No.	AccNo. Pasteur	Mycobacteria species	Positive result in QPCR	Melting temperature of the product I	Melting temperature of the product II
1	140420019	<i>M.abscessus</i>	+	91,15	88,42
2	144160001	<i>M.hodleri</i>	+	91,08	
3	141550001	<i>M.immunogenum</i>	+	90,57	
4	144100001	<i>M.lentiflavum</i>	+	91,2	
5	144290001	<i>M.elephantis</i>	+	91,84	
6	141430001	<i>M.komosense</i>	+	89,74	
7	144400001	<i>M.neworfeanse</i>	+	91,1	
8	144030001	<i>M.hibernae</i>	+	91,63	
9	141270001	<i>M.aichiense</i>	+	90,03	
10	140410020	<i>M.peregrinum</i>	+	91,56	
11	141350001	<i>M.senegalense</i>	+	91,39	
12	141300001	<i>M.phlei</i>	+	90,53	
13	141360001	<i>M.rabinowitshi</i>	+	91,22	
14	140310002	<i>M.avium</i>	+	91,67	
15	141020001	<i>M.simiae</i>	+	91,11	
16	141330001	<i>M.smegmatis</i>	+	91,13	
17	144510001	<i>M.heidelbergense</i>	+	90,85	
18	144390001	<i>M.houstonense</i>	+	91,2	
19	144010001	<i>M.brumae</i>	+	92,38	
20	141460001	<i>M.porcinum</i>	+	90,21	
21	140320001	<i>M.terrae</i>	+	91,63	
22	144110001	<i>M.mageritense</i>	+	90,96	
23	141240001	<i>M.rhodesiae</i>	+	90,78	
24	141100001	<i>M.farcinogenes</i>	+	91,52	
25	144130001	<i>M.triplex</i>	+	91,12	
26	141010001	<i>M.brumense</i>	+	91,83	
27	140410001	<i>M.fortuitum</i>	+	91,86	

28	141560001	<i>M.septicum</i>	+	91,18	
29	144360001	<i>M.brisbanense</i>	+	91,93	
30	144150001	<i>M.conspicium</i>	+	91,51	
31	144090001	<i>M.genavense</i>	+	91,03	
32	141300001	<i>M.phlei</i>	+	90,69	
33	144230001	<i>M.goodii</i>	+	92,18	
34	141500001	<i>M.austroafricanum</i>	+	91,03	
35	140230001	<i>M.scrofulaceum</i>	+	91,54	
36	140090001	<i>M.pinnipedii</i>	+	91,09	
37	141130001	<i>M.malmoense</i>	+	90,9	
38	144190001	<i>M.kubicae</i>	+	90,38	
39	144350001	<i>M.boenickei</i>	+	92,31	
40	141120001	<i>M.marinum</i>	+	91,1	
41	141370001	<i>M.anabanti</i>	+	91,19	
42	141450001	<i>espece non decrite</i>	+	90,9	
43	141520001	<i>M.sphangi</i>	+	88,9	
44	141390001	<i>M.fallax</i>	+	91,67	
45	141240001	<i>M.obuense</i>	+	90,76	
46	141310001	<i>M.thermoresistible</i>	+	91,8	
47	140210001	<i>M.gordonae</i>	+	91,7	
48	141080001	<i>M.asiaticum</i>	+	91,15	
49	144250001	<i>M.novocastrense</i>	+	91,69	
50	141320002	<i>M.agri</i>	+	86,65	90,35
51	144380001	<i>M.chimera</i>	+	92,26	
52	141470001	<i>M.moriokanense</i>	+	90,26	
53	144440001	<i>M.pyrenivorans</i>	+	90,96	
54	144410001	<i>M.palustre</i>	+	88,43	92,01
55	141510001	<i>M.alvei</i>	+	91	
56	141250001	<i>M.neoaurum</i>	+	91,83	
57	144420001	<i>M.parascrofulaceum</i>	+	90,93	
58	140230001	<i>M.flavescens</i>	+	91,04	
59	144370001	<i>M.canariense</i>	+	91,51	
60	144040001	<i>M.madagascariense</i>	+	90,35	

61	144450001	<i>M.saskatchewanense</i>	+	90,35	
62	144170001	<i>M.murale</i>	+	90,13	
63	141210001	<i>M.aurum</i>	+	92,25	
64	144060001	<i>M.celatum</i>	+	91	
65	140430001	<i>M.mucogenicum</i>	+	92,29	
66	144480001	<i>M.cosmeticum</i>	+	90,64	
67	140310001	<i>M.intra</i>	+	92,07	
68	141420001	<i>M.haemophilum</i>	+	89,16	
69	141480001	<i>M.pulveris</i>	+	90,99	
70	144540001	<i>M.fluoranthenvivorans</i>	+	91,43	
71	144280001	<i>M.heckeshornense</i>	+	92,6	
72	140330001	<i>M.triviale</i>	+	91,49	
73	144310001	<i>M.shinsuense</i>	+	77,45	84,76
74	140110026	<i>M.kansasii souche K</i>	+	90,64	
75	141260001	<i>M.chubuense</i>	+	90,35	
76	144220001	<i>M.wolinsky</i>	+	91,3	
77	141200001	<i>M.vaccae</i>	+	77,68	89,55
78	144140001	<i>M.hassiacum</i>	+	78,68	
79	141070001	<i>M.branderi</i>	+	80,37	
80	140110045	<i>M.kansasii Type II</i>	+	82,1	
81	141190001	<i>M.gilvum</i>	+	86,79	
82	140010097	<i>M.tuberculosis</i>	+	88,7	94,73
83	144530001	<i>M.neglectum</i>	+	90,5	
84	141490001	<i>M.poriferae</i>	+	90,9	92,99
85	144490001	<i>M.lacus</i>	+	91,03	
86	144470001	<i>M.holsaticum</i>	+	91,35	
87	144320001	<i>M.florentin</i>	+	91,6	
88	141170001	<i>M.diernhoferi</i>	+	91,7	
89	144020001	<i>M.interjectum</i>	+	92,04	
90	140350001	<i>M.xenopi</i>	+	92,15	
91	144460001	<i>M.vanbaalenii</i>	+	92,67	
92	141290001	<i>M.valentiae</i>	+	92,7	
93	140110032	<i>M.kansasii Type III</i>	+	92,9	

94	144210001	<i>M.bohemicum</i>	+	93,2	
95	140020002	<i>M.bovis</i>	+	77,2	
96	141090001	<i>M.ulcerans</i>	+	77,62	
97	140010017	<i>M.tuberculosis</i>	+	89,03	88,38
98	140040001	<i>M.BCG</i>	+	89,73	94,24
99	140110035	<i>M.kansasii Type I</i>	+	90,96	
100	144260001	<i>M.doricum</i>	+	91,01	
101	140240001	<i>M.szulgai</i>	+	91,34	
102	140060008	<i>M.canetti</i>	+	92,6	
103	140110039	<i>M.kansasii Type IV</i>	+	92,9	
104	141530001	<i>M.tokaiense</i>	+	-	
105	144430001	<i>M.psychrotolerans</i>	-	-	
106	144180001	<i>M.tusciae</i>	-	-	
107	144120001	<i>M.chlorophenolicum</i>	-	-	
108	140340001	<i>M.gastri</i>	-	-	
109	144270001	<i>M.parmense</i>	-	-	
110	144200001	<i>M.botinense</i>	-	-	
111	141280001	<i>M.gadium</i>	-	-	
112	144550001	<i>M.arupense</i>	-	-	
113	141160002	<i>M.chitae</i>	-	-	
114	141540001	<i>M.confluentis</i>	-	-	
115	141180001	<i>M.duvaleii</i>	-	-	
116	141220001	<i>M.parafortuitum</i>	-	-	
117	144070001	<i>M.branderi</i>	-	-	
118	141400001	<i>rhodococcus spec</i>	-	-	

The sodA qPCR assay (Figures 12 and 13) was performed with 2µl of genomic DNA (1ng/µl) prepared from the Institut Pasteur collection of Mycobacteria. A qPCR reaction was regarded as positive when a cp-value below 35 was obtained and when the melting temperature of the obtained PCR product could be clearly estimated. In some cases, two products with different melting temperature may be obtained (product I and product II). Note the negative signal obtained with *Rhodococcus* species, the closest bacterial genus to

mycobacteria.

Table 4

sample	Species direct identified	BstE II	Hae III	Genomes calculated/ 1000 ml	Cfu/ 1000ml
Par5	<i>Mycobacterium cookii</i> type 1	320/115/0	145/0/0	2000	10
Pas2	<i>Mycobacterium terrae</i> type 3	320/115/0	140/60/50	>10000	>100
Pas2	<i>Mycobacterium abscessus</i> type 2	235/210/0	200/70/60	>10000	>100
Drin bef	<i>Mycobacterium cookii</i> type 1	320/115/0	145/0/0	6200	12
Iss	<i>Mycobacterium terrae</i> type 3	320/115/0	140/60/50	>10000	10
Iss	<i>Mycobacterium mucogenicum</i> type 1	320/130/0	140/65/60	>10000	10

Table 4 presents the identified Mycobacterial species in the samples together with the corresponding restriction patterns identified from the gel shown in Figures 18A and B. The table shows also the comparison of the estimated genome numbers with the parallel obtained cfu-values for the cultivated Mycobacteria after decontamination of the sample.

As such, although preferred embodiments of the present invention have been described in detail herein and illustrated in the accompanying drawings, it is to be understood that the invention is not limited to these precise embodiments and that various changes and modifications may be effected therein without departing from the scope or spirit of the present invention.

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WHAT IS CLAIMED IS:

1. A method for isolating mycobacteria from a sample, the method comprising the steps of :
 - 5 a) contacting a sample suspected of containing mycobacteria with magnetic beads coated with an antibody immunologically specific to mycobacteria under three-dimensional motion conditions to form bead-mycobacteria complexes, and
 - 10 b) magnetically recovering the bead-mycobacteria complexes to obtain a sample containing isolated mycobacteria.
- 2 The method of claim 1, further comprising, prior to step a), a step of filtering the sample.
- 15 3. The method according to claim 1 or 2, wherein the sample has a low concentration of mycobacteria.
4. The method of any one of claims 1 to 3, further comprising, after step b), a step of detecting the presence or absence of mycobacteria from
20 the sample containing isolated mycobacteria.
5. The method of claim 4, wherein the step of detecting is by molecular DNA detection.
- 25 6. The method of claim 5, wherein the molecular detection consists of DNA amplification.
7. The method of claim 6, wherein the DNA amplification is PCR.

8. The method of claim 5, wherein the molecular detection consists of nucleic acid hybridization.
9. The method of claim 8, wherein the nucleic acid hybridization is Southern blot.
10. The method of claim 4, wherein the step of detecting is by immuno detection.
11. The method of claim 10, wherein the immuno detection is enzyme-linked immunosorbent assay (ELISA).
12. The method of claim 4, wherein the step of detecting is by mycobacteria-specific staining and/or microscopy observation.
13. A method for detecting mycobacteria in a sample, the method comprising the steps of:
- a) contacting a sample suspected of containing mycobacteria with magnetic beads coated with an antibody immunologically specific to mycobacteria under three-dimensional motion conditions to form bead-mycobacteria complexes;
 - b) magnetically recovering the bead-mycobacteria complexes to obtain a sample containing isolated mycobacteria; and
 - c) determining the presence or absence of a defined mycobacterium strain by the detection of said defined mycobacterium strain.
14. The method of claim 13, wherein the detection of the defined mycobacterium strain is by PCR.
15. The method of claim 14, wherein PCR is performed after a genomic

DNA isolation.

16. A method for detecting viable mycobacteria in a sample, the method comprising the steps of:

5 a) contacting a sample suspected of containing mycobacteria with magnetic beads coated with an antibody immunologically specific to mycobacteria under three-dimensional motion conditions to form bead-mycobacteria complexes;

10 b) magnetically recovering the bead-mycobacteria complexes to obtain a sample containing isolated mycobacteria;

 c) adding a viability determining agent to the sample containing isolated mycobacteria;

 d) extracting genomic DNA from the sample containing isolated mycobacteria of step c); and

15 e) detecting the genomic DNA of a defined mycobacterium strain;

wherein the detection of a predetermined number of mycobacteria genomes is indicative of a viable mycobacterium strain.

20 17. The method of claim 16, wherein the detection of the genomic DNA in step e) is by quantitative PCR.

18. The method of claim 17, wherein the predetermined number of mycobacteria is equal to 1000 calculated mycobacterial genomes per
25 liter of sample.

19. The method of any one of claims 13 to 18, further comprising, prior to step a), a step of filtering the sample.

20. The method of any one of claims 1 to 19, wherein the sample has a volume ranging from 0.5 ml to 5 ml.
21. The method of any one of claims 1 to 20, wherein the sample is a water-sample, a milk sample, a biofilm sample or a sputum sample.
22. The method of any one of claims 1 to 21, wherein mycobacteria are non-tuberculosis mycobacteria (NTM) strains.
23. The method of claim 22 wherein the strain is selected from the group consisting of *M. abscessus*, *M. kansasii*, *M. xenopi*, *M. avium* and *M. goodnae*.
24. The method of any one of claims 1 to 21, wherein mycobacteria are *M. bovis* BCG strain.
25. The method of any one of claims 1 to 24, wherein the antibody immunologically specific to mycobacteria is a polyclonal antibody from a rabbit immunised with lyophilised *M. abscessus* cell wall preparations.
26. The method of any one of claims 7, 14 to 25, wherein the PCR is performed with at least one of the following pairs of primers:
- Pair 1: Tb11 : 5'-ACCAACGATGGTGTGT-3' (SEQ ID NO:1)
Tb12 : 5'-CTTGTCGAACCGCATA-3' (SEQ ID NO:2);
- Pair 2: HSPF3: 5'-ATCGCCAAGGAGATCGAGCT-3' (SEQ ID NO:3)
HSPR4: 5'-AAggTgCCg Cgg ATC TTg TT-3'(SEQ ID NO:4);
- Pair 3: hspSHf: 5'-CTGGTCAAGGAAGGTCTGCG-3' (SEQ ID NO:5)
hspSHr: 5'-GATGACACCCTCGTTGCCAAC-3'(SEQ ID NO:6);

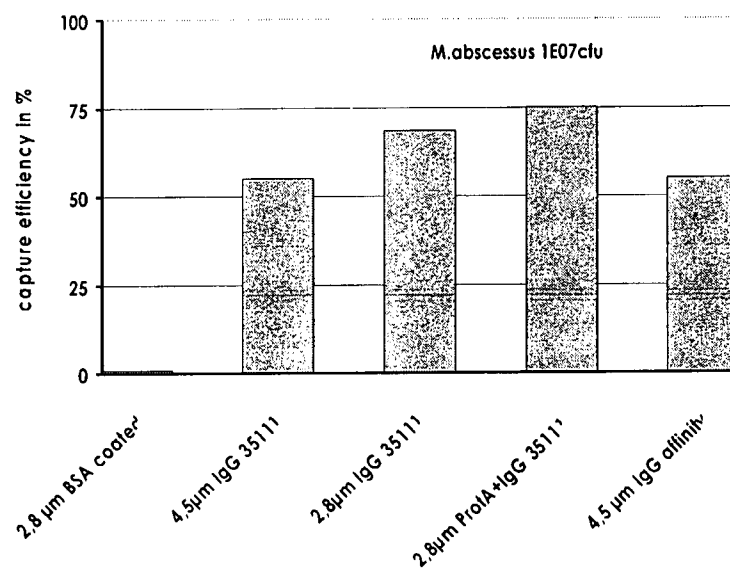
Pair 4: gyrB-f 5-TTCGCCAACACCATCAACAC-3 (SEQ ID NO: 7)
gyrB-r 5-GTGTTGCCCAACTTGGTCTT-3 (SEQ ID NO: 8);

5 Pair 5: inhA-f 5-GCATCAACCCGTTCTTCGAC-3 (SEQ ID NO: 9)
inhA-r 5-ACCGTCATCCAGTTGTACGC-3 (SEQ ID NO: 10);

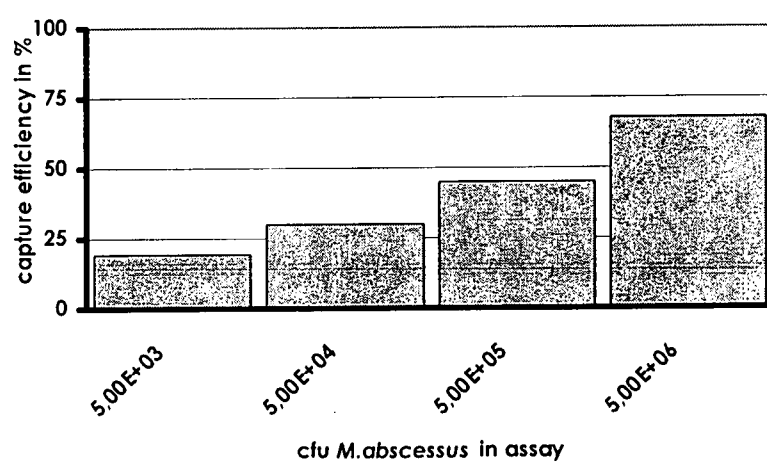
and

10 Pair 6: sodA-f 5-CCACTCGATCTGGTGGAA-3 (SEQ ID NO: 11)
sodA-r 5-TGGTCGTACAGCTGGAAGGT-3 (SEQ ID NO: 12).

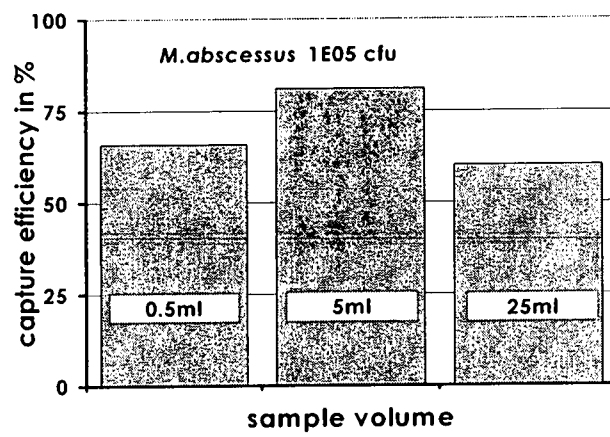
1 / 19

**Figure 1**

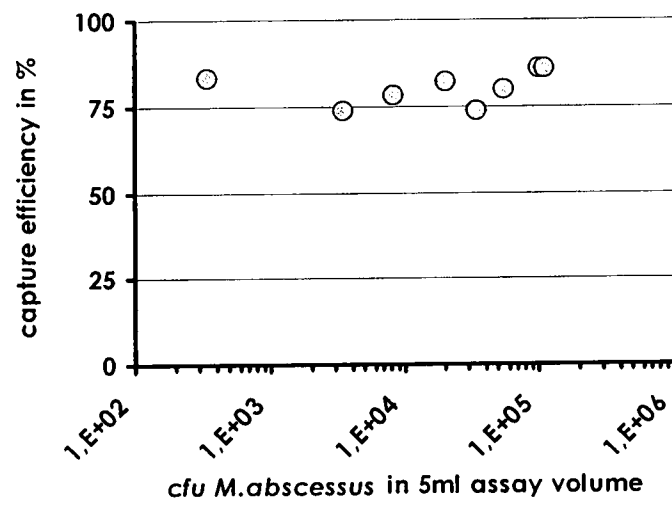
2 / 19

**Figure 2**

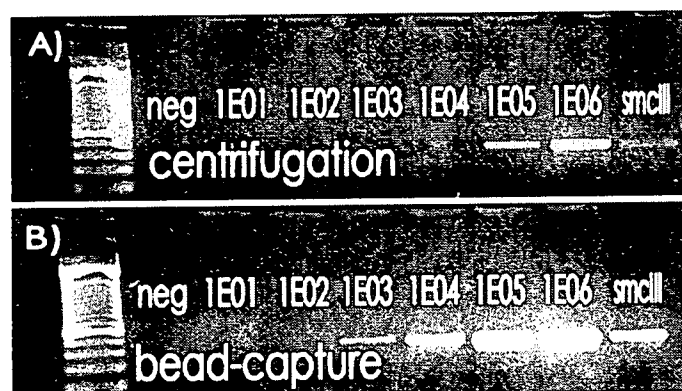
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**Figure 3**

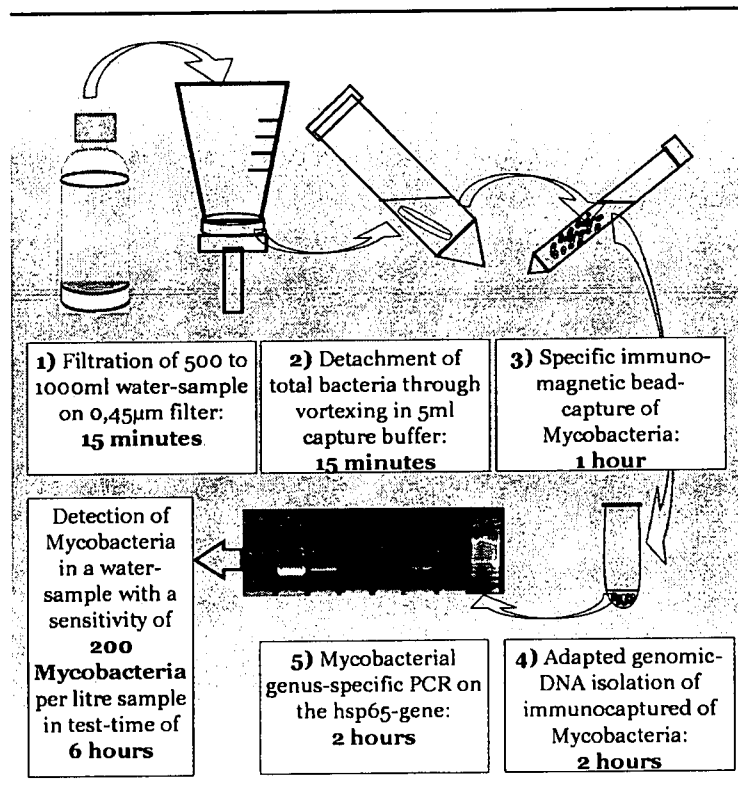
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Figure 4

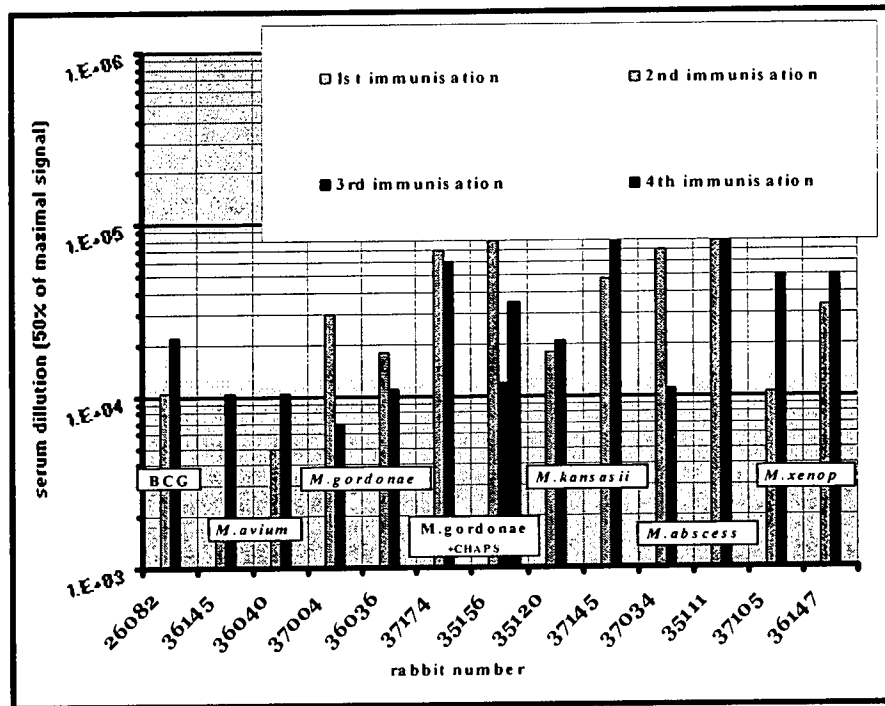
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**Figure 5**

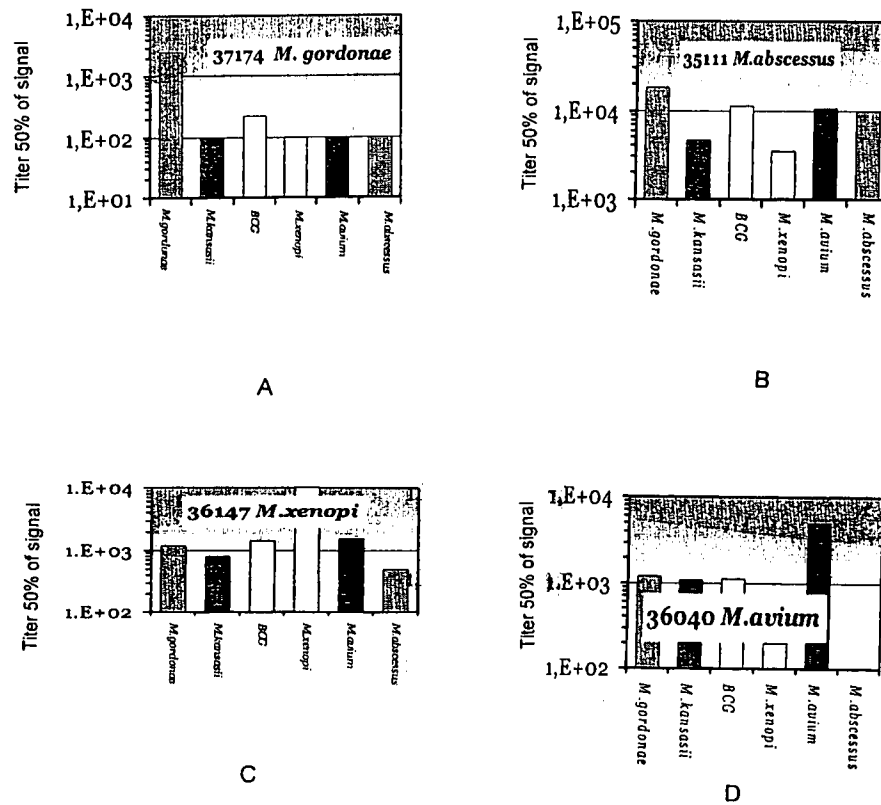
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**Figure 6**

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**Figure 7**

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**Figure 8**

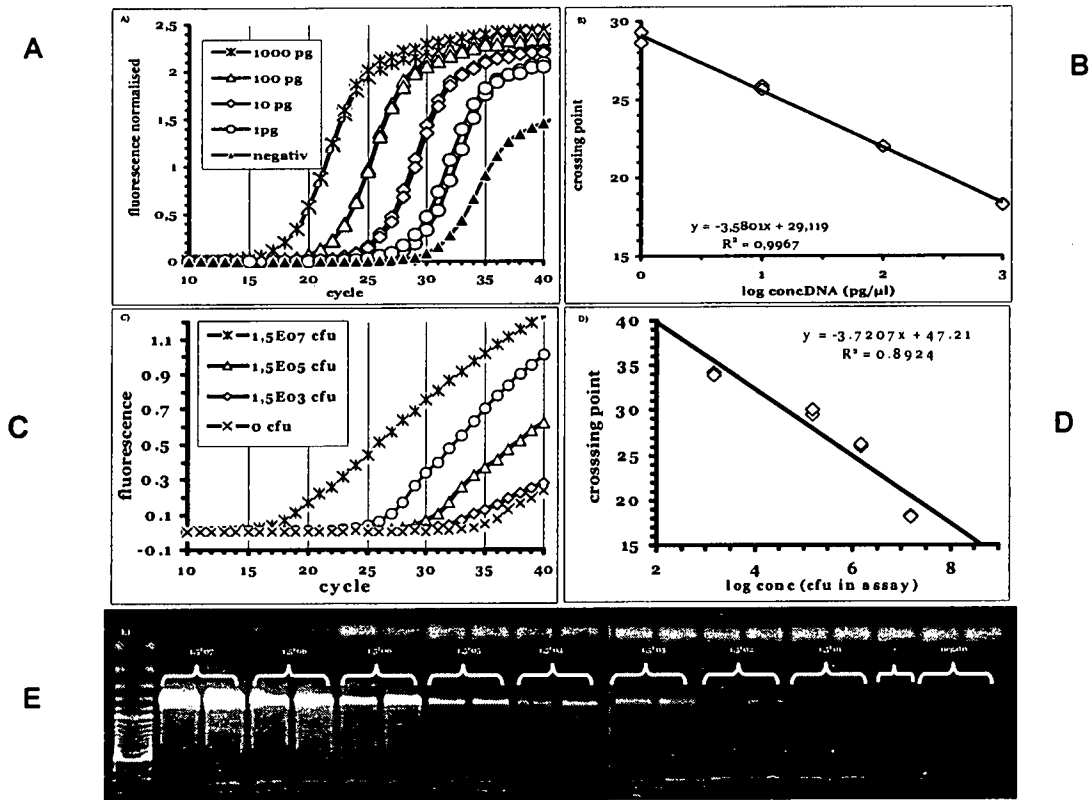
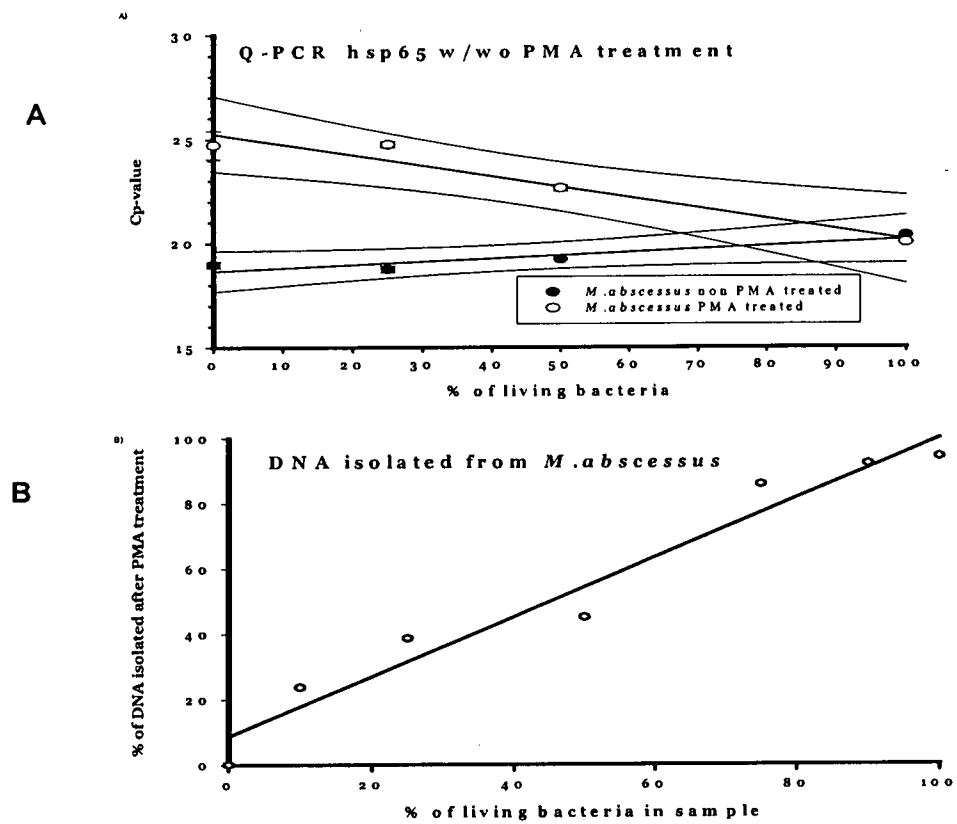
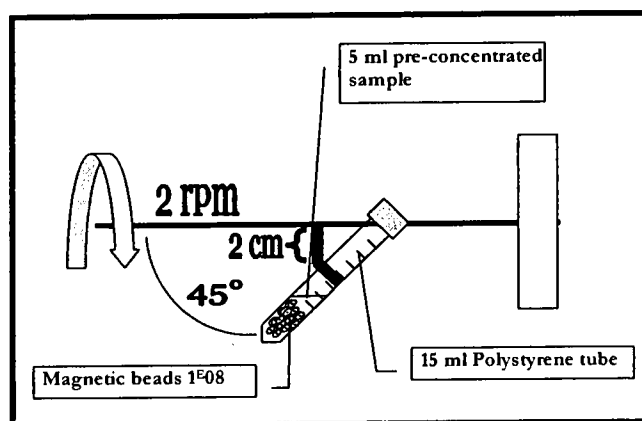


Figure 9

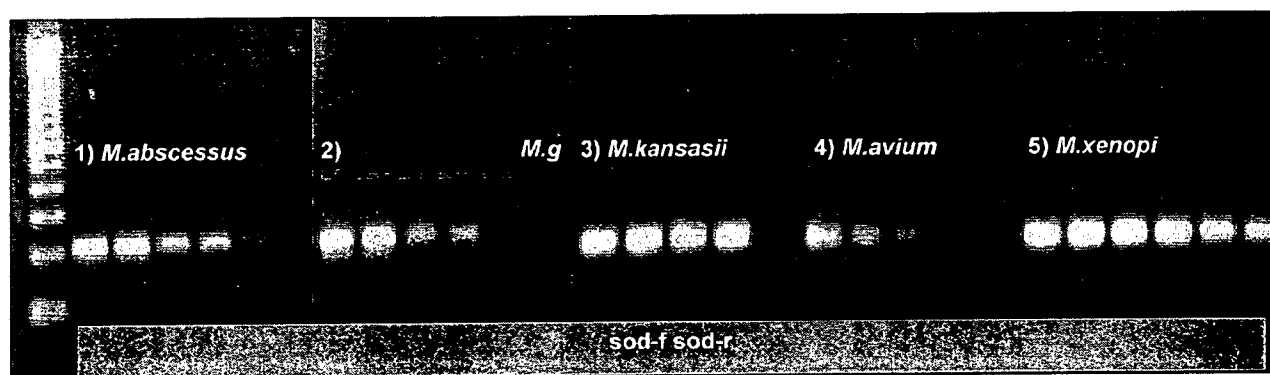
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**Figure 10**

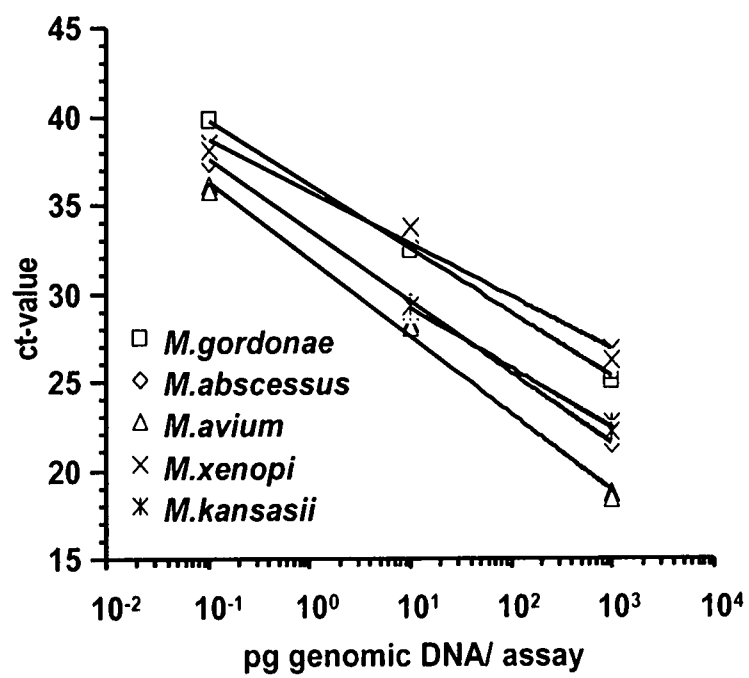
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**Figure 11**

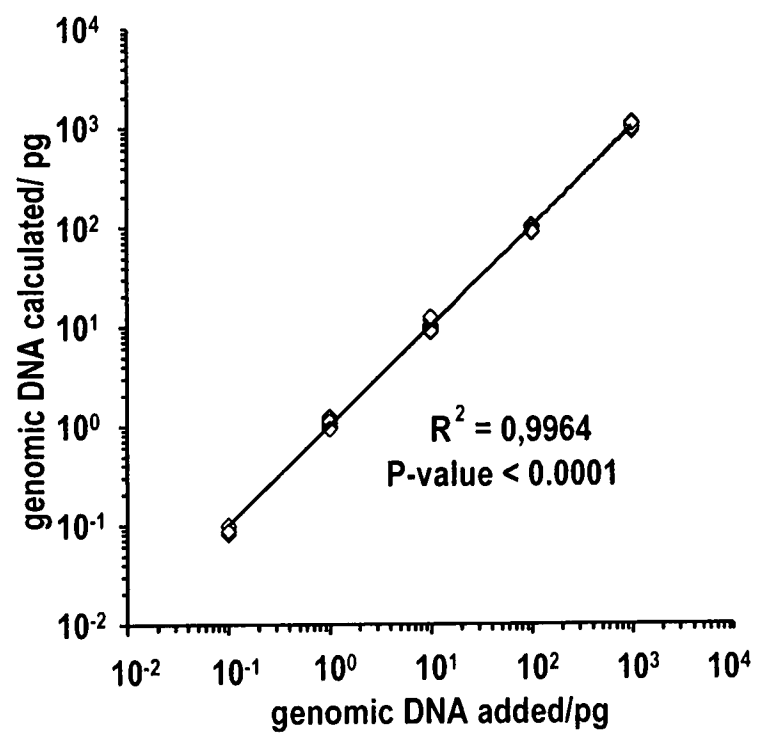
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**Figure 12**

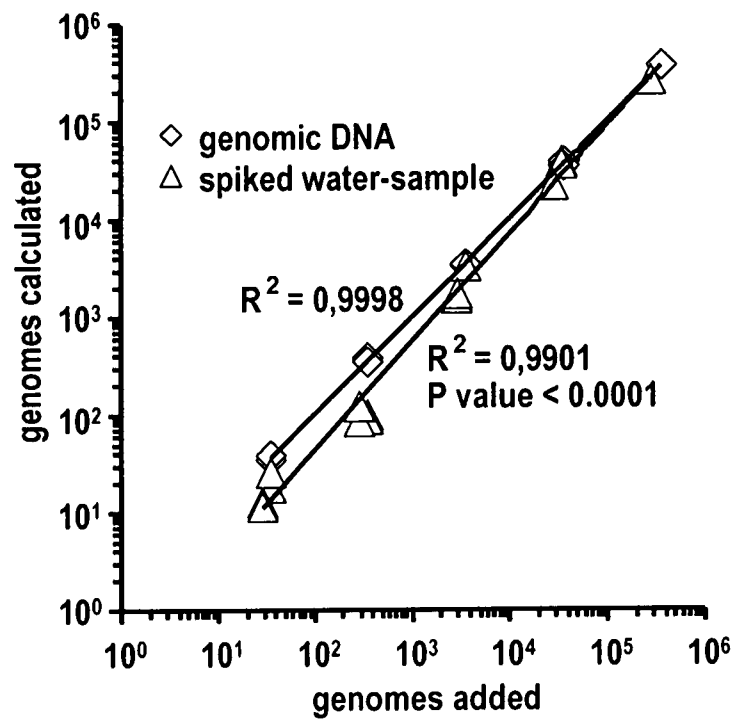
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**Figure 13**

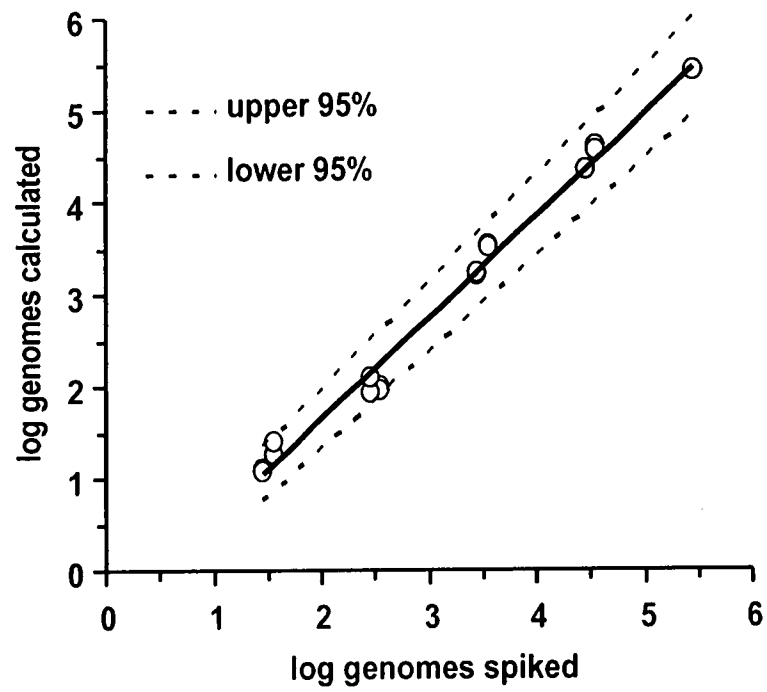
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Figure 14

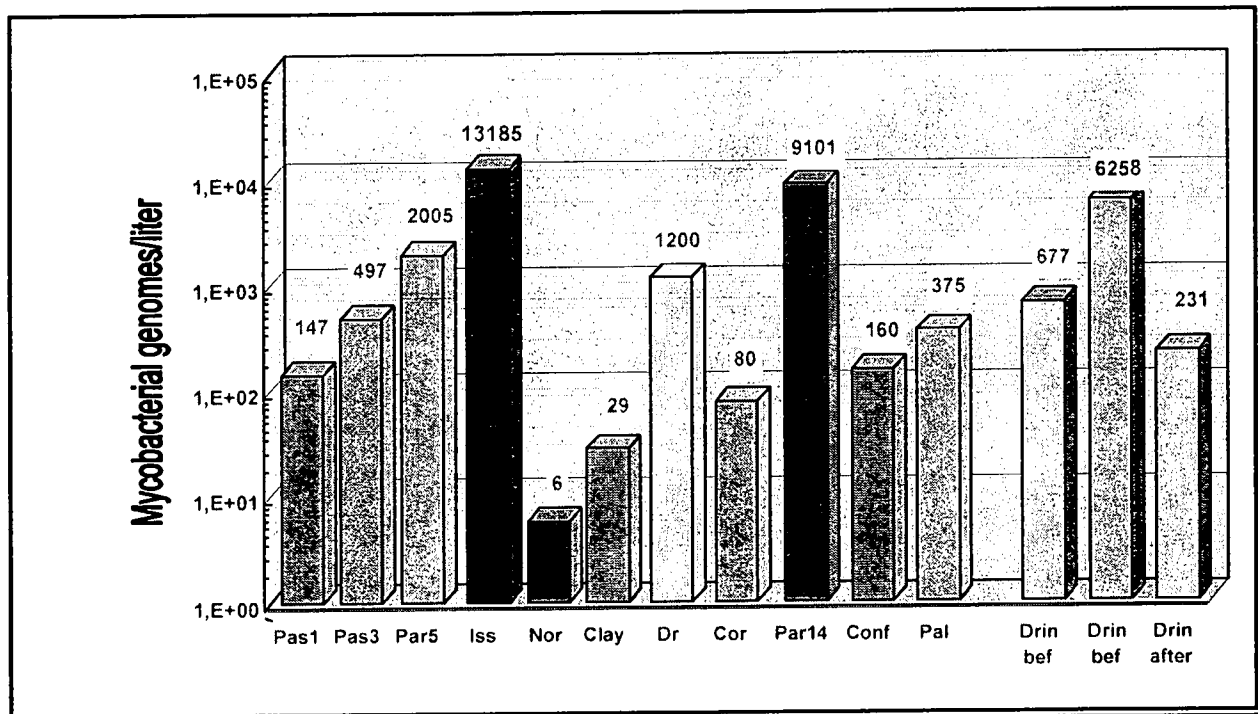
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**Figure 15**

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Figure 16

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**Figure 17**

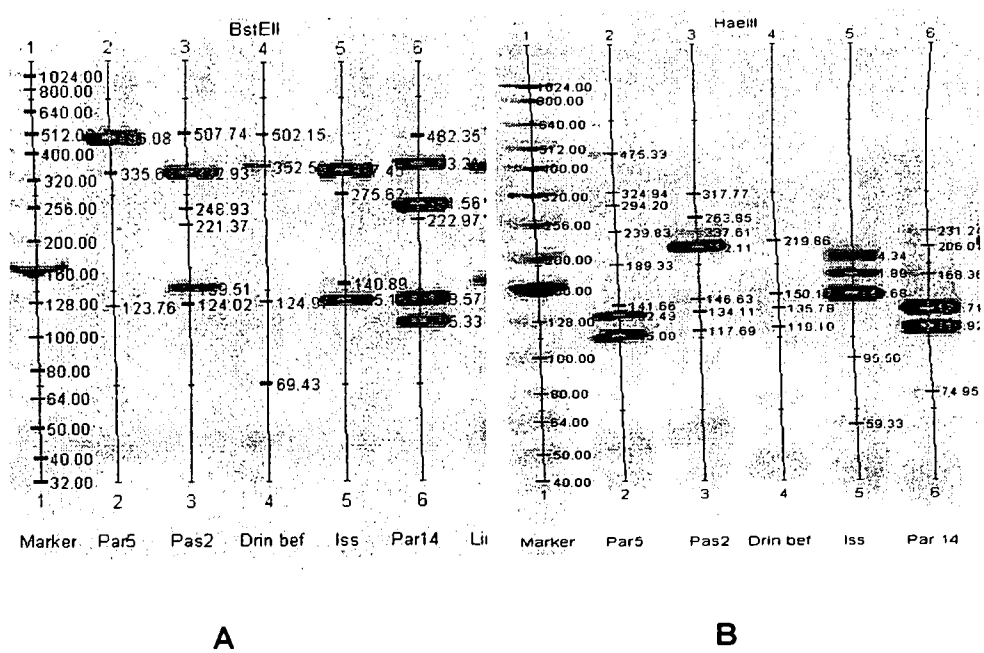


Figure 18

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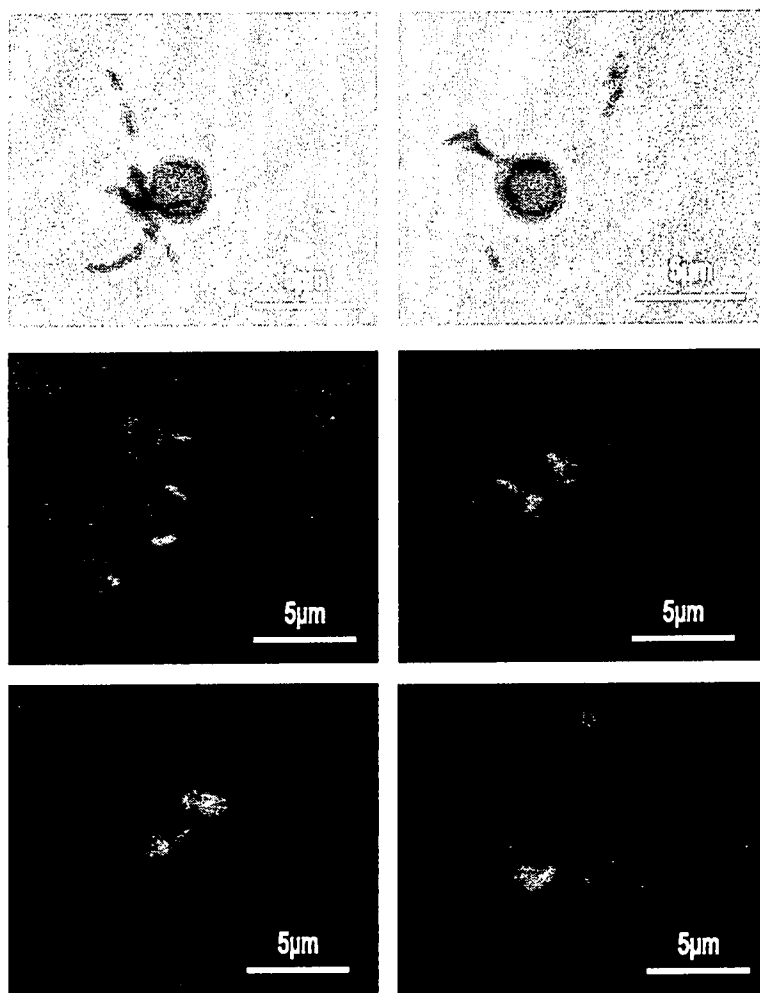


Figure 19