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(54) Title: SCREEN

(57) Abstract: The invention relates to a method for the identification of *Mycobacterium* in samples.



Screen

The invention relates to a method for the identification of *Mycobacterium* in samples.

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Whilst the absolute diversity of bacteria in soil is impossible to quantify, recent estimates suggest that a ton of soil could contain some 4 million different taxa (Curtis *et al.*, 2002). This incredible diversity makes the ability to identify a particular genus or species of bacteria in soil samples a complex and laborious task.

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The *Mycobacterium* genus contains about one hundred established species with at least sixty unidentified strains (Wattiau, 2002). Many species are pathogenic, for example *M.tuberculosis*, the closely related *M.bovis*, *M.avium-intracellulare-scofulaceum* complex, *M.paratuberculosis*, *M.ulcerans* and *M.leprae*.

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Current methods of identifying *Mycobacterium* in a sample requires analysis of the bacteria's physical characteristics (e.g acid-fast staining and microscopic detection of bacilli), physiological characteristics (e.g growth on defined media) or biochemical characteristics (e.g membrane lipid composition). However, these methods only
20 identify the species rather than the genus of *Mycobacterium*. These methods also require relatively high concentrations of bacteria in the sample to be detectable may be subjective based on the individual's experience and expertise and are time-consuming. A need therefore exists to be able to accurately, simply and rapidly identify, not only the species of *Mycobacterium* in samples, but the genus
25 *Mycobacterium* particularly in environmental samples, such as soil samples from farmland.

WO 01/44520, and US 5,574,145, both of which are herein incorporated by reference, disclose assays for detecting *Mycobacterium* species in a sample based on the use of
30 specific primers for the 16S rDNA or rRNA in each *Mycobacterium* species.

The ribosome is the site of translation of messenger RNA into protein. It is composed of two subunits. In prokaryotes, the large subunit is called 50S and the small subunit is called 30S. The 30S subunit consists of a single strand of RNA (the 16S rRNA, 1542 bases), and 21 proteins ranging in molecular weight from 9 kD to 61 kD. Some
35

16,000 16S-rRNA molecules have been sequenced and the gene consists of both conserved and divergent regions. Mutations occur in the divergent areas at a slow but constant rate, meaning that these regions can be used to identify different bacterial species.

5

16S rRNA has long been considered as powerful phylogenetic marker molecule and has been widely used to create rRNA-based trees reflecting the relationship among prokaryotes. The current view is that the phylogenetic tree of bacteria contains at least 18 lines of descent, or phyla. Due to the highly conserved nature of 16S rRNA, it would not generally be considered as a candidate gene for distinguishing between bacterial genus. Indeed in WO 01/44520, and US 5,574,145, the oligonucleotide primers disclosed are species specific rather than genus specific, and this overcomes this conservation problem.

15 However, there is a desire to be able to identify the presence of the *Mycobacterium* genus rather than specific *Mycobacterium* species in an environmental sample.

The genus can be divided on the basis of growth rate into the rapid growers and slow growers. The rapid growers, characterised by a growth rate of 1-2 days, are mostly non-pathogenic, e.g *M.phlei*. In comparison the so-called slow growers characterised by a growth rate of several weeks, tend to be pathogenic and are associated with human or animal disease. This sub-group comprises *M.tuberculosis*, the closely related *M.bovis*, *M.avium-intracellulare-scofulaceum* complex, *M.paratuberculosis*, *M.ulcerans* and *M.leprae*.

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Bovine tuberculosis (TB) is a contagious disease of cattle caused by *M.bovis* with cases are escalating world-wide. In the UK and Ireland alone during a nine month period in 2002, some 58,000 cattle test positive by the established BCG tuberculin test, and were slaughtered. Furthermore, cattle considered to have been directly exposed to TB are also compulsorily slaughtered. Once cattle are slaughtered the farmlands are subjected to a clean-up procedure to limit the spread of the bacterium.

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Other *Mycobacterium* species are pathogenic to cattle. For instance *M.paratuberculosis* a subspecies of *M.avium* causes Johne's disease in cattle herds.

Surprisingly we have identified a nucleotide sequence that is conserved in *Mycobacterium* and which enables the identification of both fast and slow growing species in a sample.

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According to an aspect of the invention there is provided a method to determine the presence of the genus *Mycobacterium* in a sample comprising a polymerase chain reaction which allows the amplification of a nucleic acid molecule characteristic of the genus *Mycobacterium*.

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According to an aspect of the present invention there is provided a method for the identification of *Mycobacterium* in a sample comprising;

- i) forming a preparation comprising; a sample to be tested, polymerase chain reaction reagents and at least one oligonucleotide primer pair, characterised in that said oligonucleotide primer pair is selected from the group consisting of;

15

5'tgggaaactgggaaactgggtctaata 3'

5' cccgcacgcccagttaagctgtgag 3'

or;

20

5' cgacgaaggtccgggttctctcggttgac 3'

5' gccatgcaccacctgcacacaggccac 3'

or modifications thereof, which modifications are the addition, deletion or substitution of at least one nucleotide base of at least one oligonucleotide primer of said pair, wherein said oligonucleotide or modified oligonucleotide hybridize to 16S ribosomal nucleic acid;

25

- ii) providing conditions suitable for 16S ribosomal nucleic acid amplification; and optionally;
- iii) detecting 16S ribosomal nucleic acid amplification products.

30 'Oligonucleotides' or primers herein described are designed to hybridize to a target nucleic acid, or its complement, and participate in an *in vitro* nucleic acid amplification reaction. Oligonucleotides are preferably synthesized using well known methods. Oligonucleotides are nucleic acids having generally less than 100 residues,

including polynucleotides in a size range having a lower limit of about 5 to 10 nucleotide residues and an upper limit of about 50 to 99 nucleotide residues. Preferred oligonucleotides are in a size range having a lower limit of about 5 to about 40 nucleotides. "Modifications" as herein described will be apparent to those skilled in the art, and include alterations in the base sequence or length of an oligonucleotide by addition deletion or substitution of nucleic acids. The primers herein described could also be RNA primers.

Typically the primers hybridise to 16SrRNA or 16SrDNA.

10

In a preferred embodiment of the present invention said oligonucleotides contain at least 6nb; 7nb; 8nb; 9nb; 10nb; 11nb; 12nb; 13nb; 14nb; 15nb; 16nb; 17nb; 18nb; 19nb; 20nb; 21nb; 22nb; 23nb; 24nb; 25nb; 26nb; 27nb; 28nb; 29nb; 30nb; 31nb; 32nb; 33nb; 34nb; 35nb; 36nb; 37nb; 38nb; 39nb or 40nb contiguous bases. More preferably still said oligonucleotide is between 26 and 30nb in length.

15

In a more preferred embodiment of the present invention, said oligonucleotide primer pair consists of;

5' tgggaaactgggaaactgggtctaata 3'

20

5' ccgcacgccaagttaagctgtgag 3',

thereby enabling the identification of both fast and slow growing *Mycobacterium* within said sample to be tested.

Alternatively, said oligonucleotide primer pair consists of;

25

5' cgacgaaggtccgggttctctcggattgac 3'

5' gccatgcaccacctgcacacaggccac 3',

thereby enabling the identification of slow growing *Mycobacterium* within said sample to be tested. Examples of slow growing *Mycobacterium* include; *M.bovis*, *M.tuberculosis*, *M.kansasii*, *M.paratuberculosis*, *M.gordonae*, *M.leprae* and *M.celatum*.

30

In an even more preferred embodiment of the present invention *Mycobacterium* species-specific oligonucleotide primer pairs are incorporated into the reaction mix with at least one genus-specific oligonucleotide primer pair or alternatively used in a separate reaction mix when a sample has been tested positive for the genus
5 *Mycobacterium*, in order to confirm the *Mycobacterium* species.

Species-specific oligonucleotide primer pairs for *Mycobacterium* species based around intra- and inter-gene variability in the 16S and 23S rRNA and DNA and are disclosed in WO 01/44520, and US 5,574,145 and are herein incorporated by
10 reference. Specific oligonucleotide pairs can be selected from the group comprising *M.abscessus*, *M.africanum*, *M.asiaticum*, *M.avum*, *M.bovis*, *M.celatum*, *M.chelonae*, *M.flavescens*, *M.fortuitum*, *M.gastri*, *M.gordonae*, *M.haemophilum*, *M.intracellulare*, *M.interjectum*, *M.intermedium*, *M.kansasii*, *M.malmoense*, *M.marinum*, *M.non-chromogenicum*, *M.paratuberculosis*, *M.phlei*, *M.shimodei*, *M.simiae*, *M.smegmatis*,
15 *M.szulgai*, *M.terrae*, *M.trivale*, *M.tuberculosis*, *M.ulcerans* or *M.xenopi*.

Alternatively other species-specific oligonucleotide primer pairs directed towards other genes can be utilised to confirm species, these include the lymphocyte stimulating antigens MPB64, MPB70 and Esat-6 (Gormley *et al.*, 1999), found in the
20 *M.bovis* group. Primer sequences are shown in Fig.1.

Preferably said species-specific oligonucleotide primers pairs are directed towards *M.bovis*.

25 In a preferred embodiment of the present invention said sample is an environmental sample. Preferably said sample is within the farm environment. Even more preferably still this is soil, alternatively said sample may be vegetation (e.g grass, hay, straw) or slurry, water, animal feed, animal waste or other environmental samples.

30 In an alternative preferred embodiment said sample is a sample derived from an animal or human. Samples include, for example, milk, sputum, respiratory tissue or exudates, blood, plasma or serum, cervical swab samples, biopsy tissue, gastrointestinal tissue, gastrointestinal fluids, urine, faeces, semen or other biological samples.

Said animal may be an animal that is symptomatically or asymptotically (ie a carrier) infected with *Mycobacterium*, specifically *M.bovis*.

Preferably said animal is selected from the group consisting of: cow, bulls, steers,
5 oxen, goats, sheep, badgers, deer and opossums. Preferably said animal is bovine.

According to a further aspect of the present invention there is provided an oligonucleotide or a modified oligonucleotide which is modified by addition, deletion or substitution of at least one nucleotide base wherein said oligonucleotide consists of
10 a nucleic acid sequence selected from the group consisting of;

5' tgggaaactgggaaactgggtctaata 3'

5' cccgcacgccaagttaagctgtgag 3'

5' cgacgaaggtccgggtctctcggattgac 3'

5' gccatgcaccacctgcacacaggccac 3'

15 and which oligonucleotide or modified oligonucleotide hybridises to 16S rRNA/DNA nucleic acid.

In a preferred embodiment of the invention said oligonucleotide consists of the nucleic acid sequence 5' tgggaaactgggaaactgggtctaata 3', or part thereof
20

In a further preferred embodiment of the invention said oligonucleotide consists of the nucleic acid sequence 5' cccgcacgccaagttaagctgtgag 3', or part thereof.

In a further preferred embodiment of the invention said oligonucleotide consists of
25 the nucleic acid sequence 5' cgacgaaggtccgggtctctcggattgac 3', or part thereof

In a yet further preferred embodiment of the invention said oligonucleotide consists of the nucleic acid sequence 5' gccatgcaccacctgcacacaggccac 3', or part thereof.

30 In a yet still further aspect of the present invention there is provided a kit for identifying the genus *Mycobacterium* in a sample. Said kit comprising a DNA extraction kit, polymerase chain reaction agents and at least one oligonucleotide

primer pair wherein said pair comprises at least one oligonucleotide according to the invention.

Preferably said oligonucleotide primer pair is;

5' tgggaaactgggaaactgggtctaata 3'

5' cccgcacgccaagttaagctgtgag 3'

or part thereof, which enables identification of both fast and slow growing *Mycobacterium*.

10 Alternatively said oligonucleotide primer pair is;

5' cgacgaaggtccgggttctctcgattgac 3'

5' gccatgcaccacctgcacacaggccac 3'

or part thereof, which enables identification of slow growing *Mycobacterium*.

15 In a even more preferred embodiment, said kit comprises oligonucleotide primer pairs comprising; 5' tgggaaactgggaaactgggtctaata 3' and 5' cccgcacgccaagttaagctgtgag 3' for identification of fast and slow growing *Mycobacterium* and at least one *Mycobacterium* species-specific oligonucleotide pair to enable identification of the species.

20

Alternatively said kit comprises oligonucleotide primer pairs comprising; 5' cgacgaaggtccgggttctctcgattgac 3' and 5' gccatgcaccacctgcacacaggccac 3' for identification of only slow growing *Mycobacterium* and at least one *Mycobacterium* species-specific oligonucleotide pair to enable identification of the species.

25

Preferably said species-specific oligonucleotide primer pair is *M.bovis*.

An embodiment of the invention will now be described by example only and with reference to the following figures:

30

Figure 1 Detection of fast and slow growing *Mycobacterium* in environmental samples

Figure 2 Detection of slow growing *Mycobacterium* in environmental samples

Figure 3 Detection of *M.bovis* in environmental samples using MPB64 specific primers

Figure 4 Detection of *M.bovis* in environmental samples using MPB70 specific primers

Figure 1 : Illustrates a 1% agarose gel showing products obtained using PCR primers targeted specifically to the *Mycobacterium* genus' 16S rRNA genes. Lanes 1) Molecular Markers. 2) Positive Control 3) Warwick Soil.4) Badger Set Soil. 5) Infected Pasture Soil.6) 2A Soil7) 2C Soil. 8) Negative Control;

Fig 2: Illustrates a 1% agarose gel showing products obtained using PCR primers targeted specifically to the slow growing mycobacterial 16S rRNA genes 1, 16) Molecular weight markers, 2, 3) *M. bovis* DNA, 4, 5) *M. bovis* BCG DNA 6, 7, 8, 9, 10) Badger set soil DNA, 11, 12) DNA from soil at the University of Warwick, 13, 14, 15, 17) 2A Soil, 18, 19, 20, 21) Infected Pasture soil;

Fig 3. Illustrates a 1% agarose gel showing products obtained through a PCR targeted to the MPB64 gene 1, 11) Molecular Markers. 2) Negative Control.3) 1A. 4) 1c. 5) 2a. 6) 2c) 7) 3C. 8) Infected Pasture. 9) Badger set. 10) Positive Control;

Fig 4. 1% agarose gel showing products obtained through a PCR targeted to the MPB70 gene 1, 11) Molecular Markers. 2) Negative Control.3,4,5) Positive Control. 6) Badger Set. 7) Infected Pasture. 8) 3C 9) 1A. 10) 1C.

Materials and Methods

Cultures

- 5 25 *Mycobacterium* species used in this experiment were cultured in both liquid and solid media.

For liquid cultures, the organisms were grown in Middlebrook 7H10 broth (Difco) supplemented with OADC enrichment (Difco). Cultures were either incubated at 28°C
10 or 37°C depending on their optimal growth temperatures, and also in static flasks or in an orbital shaker set at 180 rpm. Cultures were grown for between 1 and 6 weeks dependent on the species involved.

For solid medium cultures, mycobacterium species were cultured on Middlebrook
15 7H9 agar plates, again supplemented with OADC enrichment (Difco). Plates were sealed in a damp gas permeable bag and incubated at the above temperatures for between 1 and 6 weeks, depending on the species being cultured.

Soil Sampling

- 20 Soil samples were taken from a farm with a history of bovine tuberculosis located in Ireland. The farm had undergone a herd breakdown approx. 4 months prior to sampling. 8 sampling sites were chosen, soil from entrances to badger sets, soil from pasture-land on which the infected cattle grazed, and adjoining fields, these sites were designated as BS (Badger set soil), IP (Infected pasture soil), and 1A, 1C, 2A, 2C, 3A,
25 and 3C for the remaining fields. Ten x 10cm cores were taken from each site; these were then mixed, and three 2 g sub-samples taken from each. As a comparison, soil

was also taken from a site close to Warwick University, UK. This site had no previously known history of recent use by cattle. To test for background level of the gene in soil, several soils were tested from different geological climate, including soils from Greece, Italy, Cuba, and the Cotswolds and North Wales (UK). For
5 experiments requiring sterilised soil, samples was dried and sieved using a 4 mm mesh, then autoclaved at 121 °C for one hour and this was repeated after 24 h

DNA and RNA extraction

DNA was extracted from soil using the MOBIO ultra-prep soil DNA kit as per the
10 manufacturers instructions. For each extraction 1 g of soil was used. DNA extracted from several *M. bovis* strains isolated from infected cattle and badgers, was supplied by Eamonn Costello.

RNA was extracted using two methods. Firstly, 1 g of soil was mixed with 1g of
15 0.1mm glass beads and 1ml of DEPC water in a sterile microcentrifuge tube. This was then vortexed for 10 min to lyse the cells. 1ml of Catrimox-14 (IOWA biosciences) was added and the tube vortexed for a further 1 min. Samples were then centrifuged at 13,000 x rpm for 5 minutes at 140C. The supernatant was removed and 0.5 ml of 0.5 ml of 2M LiCl was added. Samples were then vortexed for 1 min, and centrifuged for
20 5 min at 13,000 x rpm. The supernatant was removed and the pellet washed with 70% ethanol, dried, and then resuspended in 500 µl of DEPC water. RNA samples were treated with RNase free DNase (Qiagen) as per the manufacturer's instructions. Treated RNA samples were extracted once in chloroform and finally precipitated with ethanol. Finally they were resuspended in 100 µl of DEPC treated water.

Secondly, using the Mobio Microbial RNA extraction kit RNA extractions were visualised on 1% agarose gels and stained with ethidium bromide

PCR Primers

5 *M. bovis* detection

2 sets of PCR primers used in this study were previously published by Gormley *et al.*, 1999. These primers were targeted to three specific genes found only in the *M. bovis* group of organisms. These three antigen genes MPB64, Esat-6 and MPB70 have been shown to be present in only one copy in the genome, of all *M. bovis* strains so far
10 tested. Although an analogue of MPB70 can be found in the related organisms *M. kansasii*, the sequence is different enough to be detected through sequencing.

PCR cycle conditions were the same for all three primer sets (Gormley *et al.*). To test the specificity of these primers in soil, DNA was extracted from a variety of common
15 soil organisms including *Streptomyces coelicolor*, *Streptomyces griseus*, *Micromonospora purpurea*, and *Micromonospora echinospora*. To determine detection limits for the PCR, a dilution series of *M. bovis* BCG (Pasteur) was inoculated into 1 g warwick soil microcosms. DNA was then extracted again using the mobio kit. PCR products were confirmed by cloning using the TA cloning kit
20 (Invitogen) Plasmid DNA was extracted using Qiagen Mini-prep kits, and sequenced using the Applied Biosciences BigDye™ Terminators sequencing protocol.

16S rRNA analysis

Primers were designed for use in the PCR by aligning sequences from Genbank. The
25 first primer set, JSY16S forward and reverse, was designed to be specific for the

Mycobacterium genus. Alignments of 24 mycobacterial type-strains were created using ClustalW and regions of similarity detected by eye. Regions of similarity of between 25 and 35 base pairs in length, and those with 1 or 2 mis-matches to the consensus sequence were chosen as potential primer targets. These regions were then
5 compared to sequences available in the genEmbl databases using the program Blast – N. A forward and reverse primer were then selected showing maximal similarity to Mycobacterial species and minimal similarity to other genera, and also of sufficient distant to give a reasonably-sized product sufficient for accurate identification of the target organisms. The primer sequences are:

10 Forward : 5' tgggaaactgggaaactgggtctaata 3'
Reverse : 5' cccgcacgcccaagttaagctgtgag 3'

A second set of primers JSY16Sslow was also designed using the same method. In this case, however, the forward primer was designed to target the 20 to 25 bp insertion
15 in the 16S rRNA gene, common to the slow growing group of the mycobacteria, but absent from the majority of fast-growing species. This second set of primers was therefore targeted to the slow growing group, in particular those of *the M. tuberculosis/M. bovis* group. The sequences are:

Forward : 5'cgacgaaggtccgggttctctcgattgac3'
20 Reverse : 5'gccatgcaccacctgcacacaggccac3'

To test specificity of the primers, PCR was carried out on DNA extracted from the strains noted above.

PCR cycle conditions for primer set one were, 94°C for 5 min, followed by 35 cycles
25 of 94 °C, 55 °C for 1 min, 65 °C for 1 min, and finally a single extension step of 65 °C

for 5 min. Conditions for primer set 2 were 94°C for 5 min, followed by 35 cycles of 94 °C, 57 °C for 1 min, 67 °C for 1 min, and finally a single extension step of 67 °C for 5 min.

5 The products were then cloned and sequenced using TA cloning kits according to the manufacturers instructions (Invitrogen). Plasmids were then extracted using Mini-prep kits(Qiagen), and sequenced using the Applied Biosciences BigDye™ Terminators sequencing protocol.. 50 clones in total from each sampling site were sequenced for primer set 1, and 25 for primer set 2. Where several sequences were shown to have
10 greater then 99% identity to each other, one representative was chosen.

Cloned sequences were then aligned using ClustalW, and put into the same orientation using BIOEDIT. The sequences were then aligned with sequences obtained from typestrains, from GenBank, and edited, again using BIOEDIT. Distance matrices and
15 trees were created using SEQBOOT, DNADIST, NEIGHBOR, and CONSENSE, from Phylip V3.57. 100 replicates were used to give bootstrap values.

Where no product was obtained from a paticular soil sample, DNA extracted from *M. bovis* BCG was added to the soil DNA, to determine if the lack of product was due to
20 inhibition of the PCR, or to no target DNA being present.

Quantification

PCR products were quantified using the TotalLab suite of programmes. To determine when the PCR reaction remained linear for all primer sets, A PCR was carried out using 35 replicates. One replicate was removed after each PCR cycle. These replicates
25 were then visualized on a 1% agarose gel, and an image of the gel captured. This

image was then analysed for pixel intensity of each band using the TotalLab 1d gel program (Phoretix). A set of standards was created using a dilution series of *M. bovis* BCG (Pasteur) from 10^9 to 10^1 Cells per ml. Each dilution was inoculated into 1 g of sterile Warwick soil, the soil was then dried and the DNA extracted. PCR was carried out and a calibration curve created using TOTAL LAB to determine pixel intensity of each band. To determine the efficacy of the quantification, blind testing was carried out. A culture of *M. bovis* BCG was randomly diluted into 2 ml aliquots. 1 ml of each dilution was added to 1 g of sterile Warwick soil, DNA was then extracted and quantification carried out. The remaining aliquot was used to count cell content using a haemocytometer, the results were then compared. PCR was then carried out on the unknown soil DNA samples from the Irish site. The use of the standards is important to not only standardize each experiment and were used in each separate PCR experiment carried out and also visualised on each gel analysed alongside the unknowns.

15

RT PCR

RT-PCR was carried out using all four primer sets on RNA extracted from the soil samples. The RT step was carried out using Superscript II as per the manufacturers' instructions (Invitrogen)

20

25

Fig 1: Antigen Specific Primers for *M.bovis* (Gormley *et al.*, 1999)

Gene Target	Primer Direction	Sequence	Product Size
MPB64	Forward	acg gca tcg tcg tca gcc ag	543
	Reverse	gtg att ggc ttg cga tag gc	
MPB70	Forward	gaa caa tcc gga gtt gac aa	471
	Reverse	agc acg ctg tca atc atg ta	
Esat-6	Forward	aca tga cag agc agc agt gg	413
	Reverse	tga caa cct ctc aga gtg cg	

Claims

1. A method for the identification of *Mycobacterium* in a sample comprising;

5 forming a preparation comprising; a sample to be tested, polymerase chain reaction reagents and at least one oligonucleotide primer pair, characterised in that said oligonucleotide primer pair is selected from the group consisting of nucleic acid primers comprising;

5' tgggaaactgggaaactgggtctaata 3'

10 5' cccgcacgccaagttaagctgtgag 3'

or;

5' cgacgaaggtccgggtctctcgattgac 3'

5' gccatgcaccacctgcacacagggccac 3'

15 or modifications thereof, which modifications are the addition, deletion or substitution of at least one nucleotide base of at least one oligonucleotide primer of said pair, wherein said oligonucleotide or modified oligonucleotides hybridize to 16S ribosomal nucleic acid;

(ii) providing conditions suitable for 16S ribosomal nucleic acid amplification; and optionally;

20 iii) detecting 16S ribosomal nucleic acid amplification products.

2. A method according to claim 1, wherein said oligonucleotide primer pair consists of;

5' tgggaaactgggaaactgggtctaata 3'

25 5' cccgcacgccaagttaagctgtgag 3',

and wherein said oligonucleotide pair enables the identification of both fast and slow growing *Mycobacterium* within said sample to be tested.

3. A method according to claim 1, wherein said oligonucleotide primer pair consists of;

5' cgacgaagggtccgggttctctcggattgac 3'

5' gccatgcaccacctgcacacaggccac 3',

- 5 and wherein said oligonucleotide primer pair enables the identification of slow growing *Mycobacterium* within said sample to be tested.

4. A method according to claim 2 and 3, wherein said slow growing *Mycobacterium* group comprises; *M.bovis*, *M.tuberculosis*, *M.kansaii*, *M.paratuberculosis*,
10 *M.gordonae*, *M.leprae* and *M.celatum*.

5. A method according to claims 1 to 3, wherein at least one *Mycobacterium* species-specific oligonucleotide primer pair is incorporated into said preparation.

15

6. A method according to claim 5, wherein said one *Mycobacterium* species-specific oligonucleotide primer pair is specific for a *Mycobacterium* species selected from the group comprising; *M.abscessus*, *M.africanum*, *M.asiaticum*, *M.avium*,
20 *M.bovis*, *M.celatum*, *M.chelonae*, *M.flavescens*, *M.fortuitum*, *M.gastri*, *M.gordonae*, *M.haemophilum*, *M.intracellulare*, *M.interjectum*, *M.intermedium*, *M.kansasii*, *M.malmoense*, *M.marinum*, *M.non-chromogenicum*, *M.paratuberculosis*, *M.phlei*, *M.shimodei*, *M.simiae*, *M.smegmatis*, *M.szulgai*, *M.terrae*, *M.trivale*, *M.tuberculosis*, *M.ulcerans* or *M.xenopi*.

25

7. A method according to claim 6, wherein said *Mycobacterium* species is *M.bovis*.

8. A method according to claim 6 or 7, wherein said species-specific oligonucleotide
30 primer pair hybridises with nucleic acid sequences in MPB64, MPB70 or Esat-6.

9. A method according to claim 8, wherein said oligonucleotide primer pairs are selected from;

acg gca tcg tcg tca gcc ag

gtg att ggc ttg cga tag gc

5

or;

gaa caa tcc gga gtt gac aa

agc acg ctg tca atc atg ta

or;

aca tga cag agc agc agt gg

10

tga caa cct ctc aga gtg cg

10. A method according to claim 1-9, wherein said sample is an environmental sample.

- 15 11. A method according to claim 10, wherein said environmental sample is from within a farm environment.

12. A method according to claim 11, wherein said sample is selected from the group consisting of; soil, vegetation, slurry, water, animal feed, animal waste.

20

13. A method according to claim 12, wherein said vegetation is selected from the group consisting of; grass, hay or straw.

14. A method according to claim 1-9, wherein said sample is a cell/tissue/fluid sample.

25

15. A method according to claim 14, wherein said sample is a sample derived from an animal or human.

- 30 16. A method according to claim 14 or 15, wherein said biological sample is selected from the group consisting of; milk, sputum, respiratory tissue, respiratory exudates, blood, plasma, serum, cervical swab samples, biopsy tissue, gastrointestinal tissue, gastrointestinal fluids, urine, feces, semen or other biological samples.

17. A method according to claim 15, wherein said animal is selected from the group consisting of; human, cows, bulls, steers, oxen, goats, sheep, badgers, deer and opossums.

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18. A method according to claim 17, wherein said animal is bovine.

19. A method for the identification of *Mycobacterium* in a sample comprising; forming a preparation to be tested comprising; a sample to be tested, polymerase chain reaction reagents and at least one oligonucleotide primer pair, characterised in that said oligonucleotide primer pair is selected from the group consisting of;

10

5' tgggaaactgggaaactgggtctaata 3'

5' cccgcacgccaagttaagctgtgag 3'

15

and;

5' cgacgaagggtccgggttctctcggttgac 3'

5' gccatgcaccacctgcacacaggccac 3' and;

- ii) providing conditions suitable for 16S nucleic acid amplification; and
- iii) detecting 16S nucleic acid amplification products.

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20. An oligonucleotide or modified oligonucleotide, wherein said oligonucleotide is modified by addition, deletion or substitution of at least one nucleotide base, wherein said oligonucleotide comprises a nucleic acid sequence selected from the group consisting of;

25

5' tgggaaactgggaaactgggtctaata 3'

5' cccgcacgccaagttaagctgtgag 3'

5' cgacgaagggtccgggttctctcggttgac 3'

5' gccatgcaccacctgcacacaggccac 3'

and which said oligonucleotide or modified oligonucleotide hybridises to 16S nucleic acid.

30

21. An oligonucleotide consisting of the nucleic acid sequence 5'
tgggaaactgggaaactgggtctaata^{3'} or part thereof.
22. An oligonucleotide consisting of the nucleic acid sequence 5'
5 cccgcacgccaagttaagctgtgag^{3'} or part thereof.
23. An oligonucleotide consisting of the nucleic acid sequence 5'
cgacgaaggtccgggttctctcgattgac^{3'} or part thereof
- 10 24. An oligonucleotide consisting of the nucleic acid sequence
5' gccatgcaccacctgcacacaggccac^{3'} or part thereof.

25. A kit comprising a DNA extraction kit, polymerase chain reaction
agents and at least one oligonucleotide primer pair according to the invention

15

26. A kit according to claim 25, wherein said oligonucleotides are;

5' tgggaaactgggaaactgggtctaata^{3'}

5' cccgcacgccaagttaagctgtgag^{3'}

20 27. A kit according to claim 25, wherein said oligonucleotides are;

5' cgacgaaggtccgggttctctcgattgac^{3'}

5' gccatgcaccacctgcacacaggccac^{3'}

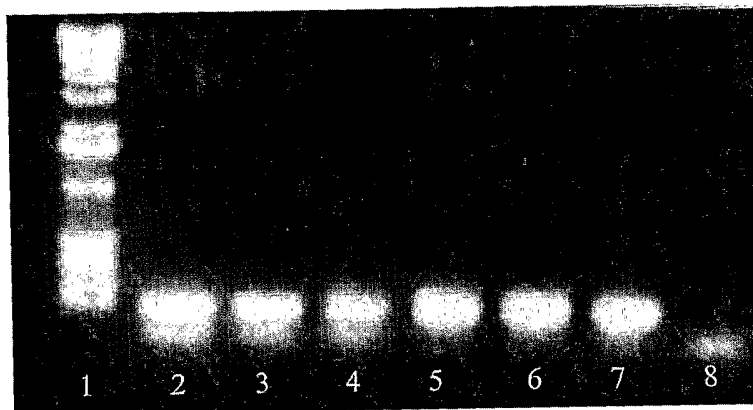
28. A kit according to claim 25, wherein at least one of said oligonucleotide primer
25 pairs are species-specific for *Mycobacterium*.

29. A kit according to claim 28, wherein said species-specific oligonucleotide primer
pair is specific for *M.bovis*.

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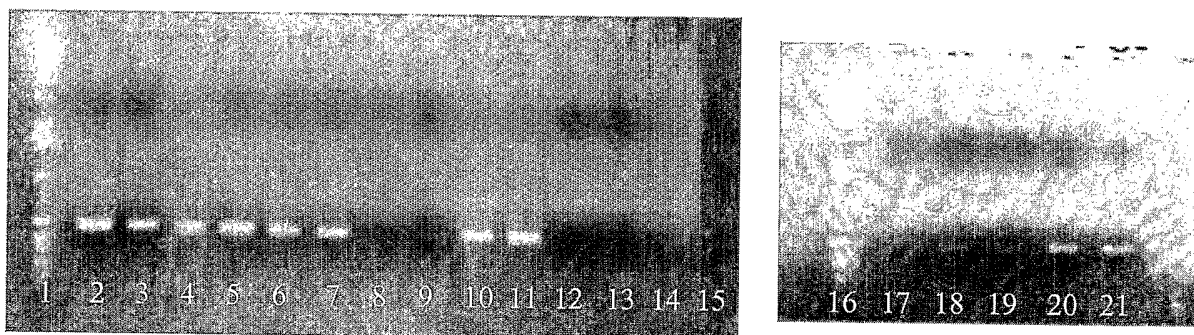
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Figure 1



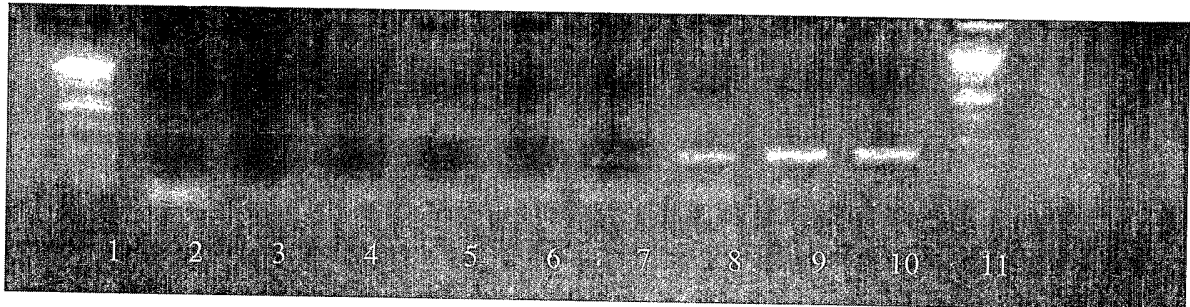
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Figure 2



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Figure 3



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Figure 4

