DETECTION OF VOLATILE COMPOUNDS AS MARKERS FOR *MYCOBACTERIA TUBERCULOSIS*

TECHNICAL FIELD

The invention relates to the use of biomarkers for identifying bacterial pathogens. In particular, the invention relates to the detection and identification of certain volatile compounds as markers for *Mycobacterium tuberculosis* and/or *Mycobacterium bovis* in an individual or from a culture by analysis of a gaseous sample.

BACKGROUND

All microorganisms produce by-products as a result of their normal metabolism. The ability of different organisms to metabolise different substrates in order to satisfy their energy and nutritional requirements is fundamental to laboratory microbiology, and forms the basis of many rapid identification tests. The metabolites produced by a single species can vary widely, depending upon the growth substrate, conditions (temperature, oxygen availability), and the age of the culture itself.

Among the many large primary and secondary metabolites produced by microbes, some organic substances are formed which readily volatilise at low temperatures. Microbial volatile organic compounds (MVOCs) have been studied extensively in agriculture and food production. Some MVOCs have important health and economic implications in these fields. For example, MVOCs have been associated with spoilage in stored crops and foodstuffs, where they may be responsible for tainting, "off" flavours, discolouring of products, or toxicity. Profiles of MVOCs are increasingly being found to be unique to the species or strain. This should not seem surprising, as it is the different biochemical ability of different microbes that gives them most of their identifiable and distinguishing traits.

For centuries, pulmonary tuberculosis (TB) has added greatly to the burden of poor health in developing countries. TB now kills one person every 18 seconds worldwide. TB is a difficult disease to diagnose, due mainly to the difficulty in culturing this slow-growing organism in the laboratory. A complete medical evaluation for TB must include a medical history, a chest X-ray, and a physical examination. Diagnosis of TB still

relies on sputum staining for acid-fast bacilli. This method is time consuming. It has high specificity, but poor sensitivity. For more than a century, microscopic examination of a stained sputum smear has been the central, and sometimes only, laboratory tool for the diagnosis of TB available in health clinics of disease endemic countries. An ideal screening test for TB would be highly sensitive and specific as well as rapid, inexpensive and non-invasive. The detection of MVOCs for diagnosing diseases is known, but an effective test for TB using MVOCs has to date remained problematic and elusive.

Detection of acid-fast bacilli (AFB) in stained smears examined microscopically often provides the first bacteriologic evidence of TB in classical laboratory testing for TB. Fluorochrome staining with auramine-rhodamine is the preferred staining method because it is faster than the traditional methods in which Ziehl-Neelsen or Kinyoun (basic fuchsin dye) stains are used. Smear examination is an easy and quick procedure - results should be available within 24 hours of specimen collection. However, smear examination permits only the presumptive diagnosis of TB because the AFB in a smear may be mycobacteria other than *M. tuberculosis*. Furthermore, many TB positive patients have negative AFB smears.

Positive cultures for *M. tuberculosis* confirm the diagnosis of TB. However, TB may also be diagnosed on the basis of clinical signs and symptoms in the absence of a positive culture. Culture examinations should be done on all specimens, regardless of AFB smear results. The BACTEC Radiometric System, or other recently developed liquid medium systems, enables detection of mycobacterial growth in 4 to 14 days.

Once the mycobacteria have been grown in culture, nucleic acid probes can identify the species in 2 to 4 hours. Nucleic acid probes specific for the *M. tuberculosis* complex, for *M. avium*, and for *M. intracellulare* provide a rapid method of species identification. High-performance liquid chromatography (HPLC), which detects differences in the spectrum of mycolic acids in the cell wall, is equally rapid and can identify most pathogenic mycobacterial species. A test for inhibition by r-nitro-a-acetylamino-b-hydroxypropiophenone (NAP or NAP test) can identify *M. tuberculosis* in 3 to 4 days. If a solid medium and conventional biochemical tests are used, the isolation and identification of the organism can take 6 to 12 weeks.

Nucleic acid amplification (NAA) tests, such as PCR and other methods for amplifying DNA and RNA, may facilitate rapid detection of microorganisms. Commercial NAA kits for the identification of *M. tuberculosis* complex have been approved by the Food and Drug Administration (FDA) for use on processed clinical specimens. These tests perform worst where needed most. Specificity is inadequate when applied to smear-negative specimens and sensitivity is inadequate when applied to smear-positive specimens. The test is approved for use in conjunction with cultures for respiratory specimens that are positive for AFB on microscopy and were obtained from untreated patients. When used as approved, a positive NAA test result indicates a high likelihood of TB, but a negative result does not exclude TB. However, a reformulated AMPLIFIED Mycobacterium Tuberculosis Direct (MTD) Test for the detection of *M. tuberculosis* in both smear-positive and smear-negative clinical specimens has recently been approved. This is the first NAA test approved for this indication. Decisions about when and how to use NAA tests for TB diagnosis should be individualised. NAA tests cannot replace clinical judgment or be relied on as the only guide for therapy or isolation practices. The tests may enhance diagnostic certainty, but should be interpreted in a clinical context and on the basis of local laboratory performance.

Follow-up bacteriologic examinations are important for assessing the patient's infectiousness and response to therapy. At a minimum, specimens should be obtained at monthly intervals until the culture results convert to negative. Culture conversion is the most important objective measure of response to treatment. Conversion is documented by the first negative culture in a series of cultures (i.e. all subsequent culture results must remain negative).

The BACTEC radiometric method, which uses a liquid medium, is faster than conventional methods for determining susceptibility to first-line TB medications. Usually, susceptibility results can be obtained within 7-14 days of BACTEC inoculation. Conventional methods, which use solid media for growth, can take as long as 21 days after inoculation.

Certain compounds have been identified, e.g. in extracts of cultures of *Mycobacteria*. For example, Ratledge and Winder (Biochem. J. 84, 1962, 501-506) report that salicylic acid can be identified in the culture medium of *M. smegmatis* cultures, and anisic acid, phenyl acetic acid and salicylic acid can be detected in lipid extracts from *M. tuberculosis*. Edens et al. (Journal of Biological Chemistry, 1944, 587-592) also report

that anisic acid can be detected in lipid extracts from *M. tuberculosis* and Del Rio-Estrada and Patino (J. Bacteriol., 1962, 871-872) report that nicotinic acid is produced by *M. tuberculosis* in culture.

US 3,616,258 describes a diagnostic product for detection of niacin produced by *M. tuberculosis*, which product uses an impregnated paper strip.

The abovementioned problems associated with classical testing and diagnosis of TB have led to some investigations into the detection of MVOCs for diagnosing TB, but no specific markers for TB have yet been found that could form the basis of an effective test for TB.

WO 2006/079846 describes a method of detecting and identifying bacteria comprising the steps of collecting volatile bacterial products, subjecting the volatile products to a gas chromatography system employing a surface acoustic wave detector, and establishing chromatographic profiles for different bacteria. The system is said to be suitable for use in the detection of TB and indicates that mycolic acids could be a potential marker. Example 1 relates to the identification of TB, but this is a prophetic example teaching how this might performed. No specific markers for TB are described.

WO 2003/075745 describes a method for detecting a physiological condition in an animal by measuring a clinical diagnostic marker using an array of sensors to generate a sensor array response profile. The detection and monitoring of TB is described as one possible medical application. However, there is no indication of how TB could be detected, or what markers could be used.

WO 2003/064994 describes a method of collecting and detecting compounds in a human breath sample using a hand held sample collector to absorb at least one breath compound. The collector can then be connected to a breath analyser. The method is described as an easy-to-use diagnostic method for the detection of TB. Further, it is stated that the composition and concentration of volatile compounds emitted from TB-infected cells in the lung is largely unknown, but the detection of tuberculostearic acid (TSA) in TB cultures and in sputum and serum samples of TB patients suggests the presence of characteristic metabolites that might be useful in diagnosing TB.

WO 2005/079669 describes a method and apparatus for diagnosing various medical conditions by analysing breath. The system uses phase measurement of absorption of IR radiation to allow very small concentrations of the diagnostic species to be measured. An example is given of using the system to test ethane in a number of patients. It is merely speculated that this measurement could be a potential diagnostic for TB.

WO 2004/090534 describes a system for breath analysis using asymmetric ion mobility spectrometry. TB is disclosed as a potential condition that could be identified using the system. However, no specific markers for TB are described.

The applicant has now identified a set of compounds which are unique identifiers for TB. The same compounds have not been detected *in vitro* from other fungi and bacteria related to pulmonary diseases. These compounds represent a useful tool for the identification of *Mycobacterium tuberculosis* and *Mycobacterium bovis* from culture samples and also for breath diagnosis of TB.

It is an object of the invention to provide a method of diagnosing TB, or at least to provide a useful alternative to other methods.

SUMMARY OF INVENTION

In one aspect of the invention there is provided a method for detecting a *Mycobacterium* microorganism by analysing a gas mixture for any one or more of methyl phenylacetate, methyl p-anisate, methyl nicotinate, and o-phenylanisole.

In a second aspect, the invention provides a method of detecting a *Mycobacterium* microorganism in a patient comprising:

a) obtaining a sample of breath of the patient;
b) analysing the sample for the presence of any one or more of methyl phenylacetate, methyl p-anisate, methyl nicotinate, and o-phenylanisole; and
c) determining whether a *Mycobacterium* microorganism is present in the patient.

In a third aspect, the invention provides a method of detecting a *Mycobacterium* microorganism in an *in vitro* culture or a biological sample comprising:

a) obtaining a sample from the headspace gas of the *in vitro* culture or biological sample;

b) analysing the sample for the presence of any one or more of methyl phenylacetate, methyl p-anisate, methyl nicotinate, and o-phenylanisole; and

c) determining whether a *Mycobacterium* microorganism is present in the *in vitro* culture or biological sample.

In another aspect, the invention provides a method for detecting a *Mycobacterium* microorganism by analysing a gas mixture for any one or more of phenyl acetic acid, anisic acid (preferably p-anisic acid), nicotinic acid and salicylic acid.

In another aspect, the invention provides a method of detecting a *Mycobacterium* microorganism in a patient comprising:

a) obtaining a sample of breath of the patient;

b) analysing the sample for the presence of any one or more of phenyl acetic acid, anisic acid (preferably p-anisic acid), nicotinic acid and salicylic acid; and

c) determining whether a *Mycobacterium* microorganism is present in the patient.

In yet another aspect, the invention provides a method for detecting a *Mycobacterium* microorganism in an *in vitro* culture or a biological sample comprising:

a) obtaining a sample from the headspace gas of the *in vitro* culture or biological sample;

b) analysing the sample for the presence of any one or more of phenyl acetic acid, anisic acid (preferably p-anisic acid), nicotinic acid and salicylic acid; and

c) determining whether a *Mycobacterium* microorganism is present in the *in vitro* culture or biological sample.

In still another aspect, the invention provides a method for detecting a *Mycobacterium* microorganism by analysing a gas mixture for a derivative of any one or more of phenyl acetic acid, anisic acid (preferably p-anisic acid), nicotinic acid and salicylic acid.

In a further aspect, the invention provides a method of detecting a *Mycobacterium* microorganism in a patient comprising:

a) obtaining a sample of breath of the patient;

b) analysing the sample for the presence of a derivative of any one or more of phenyl acetic acid, anisic acid (preferably p-anisic acid), nicotinic acid and salicylic acid; and

c) determining whether a *Mycobacterium* microorganism is present in the patient.

In another aspect, the invention provides a method for detecting a *Mycobacterium* microorganism in an *in vitro* culture or a biological sample comprising:

(a) obtaining a sample from the headspace gas of the *in vitro* culture or biological sample;

(b) analysing the sample for the presence of a derivative of any one or more of phenyl acetic acid, anisic acid (preferably p-anisic acid), nicotinic acid and salicylic acid; and

(c) determining whether a *Mycobacterium* microorganism is present in the *in vitro* culture or biological sample.

Preferably the derivative is an ester, e.g. an alkyl, alkenyl or alkynyl ester, preferably an alkyl ester, e.g. a lower alkyl ester, e.g. a methyl ester. Alternatively, the derivative of phenyl acetic acid, anisic acid (preferably p-anisic acid), nicotinic acid or salicylic acid may be a silyl derivative, e.g. a trimethylsilyl derivative.

In still another aspect of the invention there is provided a method for detecting a *Mycobacterium* microorganism by analysing a gas mixture for any one or more of methyl phenylacetate, methyl p-anisate, methyl nicotinate, o-phenylanisole, phenyl acetic acid, anisic acid (preferably p-anisic acid), nicotinic acid and salicylic acid.

In another aspect, the invention provides a method of detecting a *Mycobacterium* microorganism in a patient comprising:

a) obtaining a sample of breath of the patient;

b) analysing the sample for the presence of any one or more of methyl phenylacetate, methyl p-anisate, methyl nicotinate, o-phenylanisole, phenyl acetic acid, anisic acid (preferably p-anisic acid), nicotinic acid and salicylic acid; and

c) determining whether a *Mycobacterium* microorganism is present in the patient.

In another aspect, the invention provides a method for detecting a *Mycobacterium* microorganism in an *in vitro* culture or a biological sample comprising:

a) obtaining a sample from the headspace gas of the *in vitro* culture or biological sample;

b) analysing the sample for the presence of any one or more of methyl phenylacetate, methyl p-anisate, methyl nicotinate, o-phenylanisole, phenyl acetic acid, anisic acid (preferably p-anisic acid), nicotinic acid and salicylic acid; and

c) determining whether a *Mycobacterium* microorganism is present in the *in vitro* culture or biological sample.

In some embodiments, the method includes the following step (i), which may optionally be carried out after step a):

(i) carrying out a derivatisation, preferably an *in situ* derivatisation, of the phenyl acetic acid, anisic acid (preferably p-anisic acid), nicotinic acid or salicylic acid to give a derivative of phenyl acetic acid, anisic acid (preferably p-anisic acid), nicotinic acid or salicylic acid;

where the analysing step b) is analysing the sample for the presence of the derivative of phenyl acetic acid, anisic acid (preferably p-anisic acid), nicotinic acid or salicylic acid.

Preferably the derivative is an ester, e.g. an alkyl, alkenyl or alkynyl ester, preferably an alkyl ester, e.g. a lower alkyl ester, e.g. a methyl ester. Alternatively, the derivative of phenyl acetic acid, anisic acid (preferably p-anisic acid), nicotinic acid or salicylic acid may be a silyl derivative, e.g. a trimethylsilyl derivative.

The gas mixture is preferably analysed for both methyl p-anisate and methyl nicotinate. In some embodiments of the invention, it may be preferable to analyse the gas mixture for all of methyl phenylacetate, methyl p-anisate, methyl nicotinate, and o-phenylanisole. In other embodiments of the invention, it may be preferable to analyse the gas mixture for all of methyl phenylacetate, methyl p-anisate, methyl nicotinate, o-phenylanisole, phenyl acetic acid, anisic acid (preferably p-anisic acid), nicotinic acid and salicylic acid. In still other embodiments of the invention, it may be preferable to analyse the gas mixture for all of phenyl acetic acid, anisic acid (preferably p-anisic acid), nicotinic acid and salicylic acid. In still other embodiments of the invention, it may be preferable to analyse the gas mixture for all of methyl phenylacetate, methyl p-anisate and methyl nicotinate. In still other embodiments of the invention, it may be preferable to analyse the gas mixture for one or more of methyl phenylacetate, methyl

p-anisate, methyl nicotinate and o-phenylanisole and one or more of phenyl acetic acid, anisic acid (preferably p-anisic acid), nicotinic acid and salicylic acid. In still other embodiments of the invention, it may be preferable to analyse the gas mixture for one of methyl phenylacetate, methyl p-anisate, methyl nicotinate and o-phenylanisole. In still other embodiments of the invention, it may be preferable to analyse the gas mixture for one of phenyl acetic acid, anisic acid (preferably p-anisic acid), nicotinic acid and salicylic acid.

The *in vitro* culture or biological sample may be a liquid or a solid sample. Typically it is a liquid sample, e.g. sputum, blood, serum or lung fluid.

The biological sample is preferably sputum, but may also be lung fluid, serum or blood, or any other sample capable of analysis according to the invention.

It is further preferred that the gas mixture is analysed using GC-MS, e.g. as described in the Examples.

In another aspect, the invention provides an apparatus for detecting the presence of a *Mycobacterium* microorganism having:

a) a gas sample inlet enabling introduction of a gas sample into the apparatus;

b) a means for analysing the gas sample for the presence of any one or more of methyl phenylacetate, methyl p-anisate, methyl nicotinate, and o-phenylanisole; and

c) a means for displaying information enabling the diagnosis of *Mycobacterium tuberculosis, Mycobacterium africanum* or *Mycobacterium bovis*, or a means for transmitting information to a device for displaying information enabling the diagnosis of *Mycobacterium tuberculosis, Mycobacterium africanum* or *Mycobacterium bovis*.

In yet another aspect, the invention provides an apparatus for detecting the presence of a *Mycobacterium* microorganism having:

a) a gas sample inlet enabling introduction of a gas sample into the apparatus;

b) a means for analysing the gas sample for the presence of any one or more of methyl phenylacetate, methyl p-anisate, methyl nicotinate, and o-phenylanisole; and

c) a means for displaying information enabling the diagnosis of *Mycobacterium tuberculosis* or *Mycobacterium bovis*, or a means for transmitting information to a device for displaying information enabling the diagnosis of *Mycobacterium tuberculosis* or *Mycobacterium bovis*.

In still another aspect, the invention provides a method of diagnosis of a mycobacterial infection in a patient, including detecting a *Mycobacterium* microorganism by any of the methods described above.

In some embodiments, the gas sample is the headspace gas of a *Mycobacterium* culture, a sputum sample from a patient, e.g. a patient suspected of being infected with a mycobacterial infection, a blood sample from a patient, e.g. a patient suspected of being infected with a mycobacterial infection, a serum sample from a patient, e.g. a patient suspected of being infected with a mycobacterial infection or a lung fluid sample from a patient, e.g. a patient suspected of being infected with a mycobacterial infection.

In other examples, the gas sample is a breath sample from a patient, e.g. a patient suspected of being infected with a mycobacterial infection.

Preferably the *Mycobacterium* microorganism is *Mycobacterium tuberculosis*, *Mycobacterium bovis, Mycobacterium smegmatis, Mycobacterium tuberculosis* BCG, *Mycobacterium avium, Mycobacterium leprae, Mycobacterium africanum* or *Mycobacterium ulcerans,* especially *Mycobacterium tuberculosis, Mycobacterium africanum* or *Mycobacterium bovis*, in particular *Mycobacterium tuberculosis.*

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows growth dependent concentration curves of methyl p-anisate (TB5) on Löwenstein-Jensen medium.

Figure 2 shows the detection of methyl nicotinate (after derivatisation) in the headspace of serum samples of *Mycobacterium tuberculosis*-positive (p) and *Mycobacterium tuberculosis*-negative (n) patients.

DETAILED DESCRIPTION

The invention provides a method for detecting a *Mycobacterium* microorganism by analysing a gas mixture for any one or more of methyl phenylacetate, methyl p-anisate, methyl nicotinate, and o-phenylanisole.

The invention also provides a method for detecting a *Mycobacterium* microorganism by analysing a gas mixture for any one or more of nicotinic acid, anisic acid (preferably p-anisic acid), phenyl acetic acid and salicylic acid.

The invention also provides a method for detecting a *Mycobacterium* microorganism by analysing a gas mixture for any one or more of nicotinic acid, anisic acid (preferably p-anisic acid), phenyl acetic acid, salicylic acid, methyl phenylacetate, methyl p-anisate, methyl nicotinate, and o-phenylanisole.

The applicant has determined that the four volatile organic compounds methyl phenylacetate, methyl p-anisate, methyl nicotinate, and o-phenylanisole, either alone or two or more in combination, are unique biomarkers for *Mycobacteria*, e.g. TB, specifically *Mycobacterium complex* which comprises *Mycobacterium tuberculosis* and/or *Mycobacterium bovis*, particularly when detected in gas samples, e.g. a sample of breath of a patient or a sample of the headspace gas of an *in vitro* culture or biological sample.

These four compounds are referred to herein as methyl phenylacetate, methyl p-anisate, methyl nicotinate, and o-phenylanisole, but it will be appreciated that these compounds are also known by other names:

- methyl phenylacetate (CAS RN 101-41-7) is also known as methyl alpha-toluate and phenyl acetic acid methyl ester.

- methyl p-anisate (CAS RN 121-98-2) is also known as methyl para-anisate, methyl anisate and methyl 4-methoxybenzoate.

- methyl nicotinate (CAS RN 93-60-7) is also known as methyl 3-pyridinecarboxylate.

- o-phenylanisole (CAS RN 86-26-0) is also known as 2-methoxybiphenyl.

The acids corresponding to three of the above compounds (nicotinic acid, CAS RN 59-67-6; p-anisic acid, CAS RN 100-09-4; phenyl acetic acid, CAS RN 100-79-2) as well as salicylic acid, CAS RN 69-72-7, either alone or two or more in combination, are also biomarkers for *Mycobacteria*, e.g. TB, specifically *Mycobacterium complex* which comprises *Mycobacterium tuberculosis* and/or *Mycobacterium bovis*, particularly when detected in gas samples, e.g. a sample of breath of a patient or a sample of the headspace gas of an *in vitro* culture or biological sample. It will be appreciated by those skilled in the art that these acids may also be known by other names, corresponding to the other names shown above for their methyl esters.

While the detection of the four markers methyl phenylacetate, methyl p-anisate, methyl nicotinate, and o-phenylanisole is determinative of the presence of *Mycobacterium tuberculosis* or *Mycobacterium bovis,* the detection of only one or two of the markers, or one or more of their corresponding acids, may be sufficient for a positive diagnosis in many circumstances. For example, experimental results indicate that detection of methyl p-anisate in combination with methyl nicotinate is sufficient for TB diagnosis.

The applicant initially analysed various *Mycobacterium* species for the production *in vitro* of volatile organic compounds using Solid Phase Micro Extraction (SPME) and Gas Chromatography/Mass Spectroscopy (GC/MS) from various culture media such as Löwenstein-Jensen, BacT/Alert®MP and sheep blood agar. In addition to the reference strains of *Mycobacterium*, ten wild clinical respiratory strains of *M. tuberculosis* were analysed at least three times with consistent results over the incubation period.

The four compounds methyl phenylacetate, methyl p-anisate, methyl nicotinate, and o-phenylanisole were identified as a set of compounds which are unique for *Mycobacterium*. These compounds have not been detected *in vitro* from other fungi and bacteria related to pulmonary diseases. The compounds are useful for the identification of *Mycobacteria*, e.g. *Mycobacterium complex* which comprises *Mycobacterium tuberculosis* and *Mycobacterium bovis* from culture, and also as useful specific markers for breath diagnosis of TB.

Two compounds (methyl p-anisate and methyl nicotinate) were identified which appear to be unique to *M. tuberculosis* and *M. bovis*. They were consistently detected from all

three media. Methyl phenylacetate was also detected for *M. tuberculosis* and *M. bovis*, and was a volatile by-product of the *M. avium* complex. O-Phenylanisole was only detected on sheep blood agar, but in all *Mycobacteria* species.

Due to the growth requirements of other important respiratory pathogens confirmation of the uniqueness of the TB marker compounds methyl phenylacetate, methyl p-anisate, methyl nicotinate, and o-phenylanisole was carried out on sheep blood agar. At least five strains each of *A. fumigatus, A. flavus, A. niger, A. terreus, Fusarium spp., Rhizopus arrhizus, Scedesporium apiospermum, Candia albicans, Ps. Aeruginosa, B. cepacia, Ps. Fluorescens, S. aureus, E. coli, S. pneumoniae, Moraxella catarrhalis,* and *H. influenza* did not show any traces of methyl phenylacetate, methyl p-anisate, methyl nicotinate, or o-phenylanisole.

The corresponding acids of the three compounds methyl phenylacetate, methyl p-anisate and methyl nicotinate (i.e. nicotinic acid, p-anisic acid and phenyl acetic acid), as well as salicylic acid, are detectable, either as the free acids or after *in situ* derivatisation, in patient samples, e.g. serum and sputum samples of patients suspected of being infected with a mycobacterial infection. These acids are detectable, either as the free acids or after *in situ* derivatisation, in the headspace gas of samples of bodily fluid.

These four acids (i.e. nicotinic acid, p-anisic acid, phenyl acetic acid and salicylic acid) are therefore also markers that are detectable in the headspace gas of samples of bodily fluid, e.g. sputum, lung fluid, serum, blood, of patients suspected of being infected with a mycobacterial infection, e.g. *Mycobacterium tuberculosis* or *Mycobacterium bovis*.

The acids can be converted to a variety of derivatives for detection as biomarkers.

It is preferred that this derivatisation is carried out *in situ.* A sample may be taken from an *in vitro* culture of a *Mycobacterium* microorganism, e.g. as described in the Examples section, or from a biological sample such as a sputum sample from a patient, e.g. a patient suspected of being infected with a mycobacterial infection; a blood sample from a patient, e.g. a patient suspected of being infected with a mycobacterial infection; a serum sample from a patient, e.g. a patient suspected of being infected with a mycobacterial infection; or a lung fluid sample from a patient, e.g. a patient suspected

of being infected with a mycobacterial infection. The sample, e.g. the sample taken from an *in vitro* culture of a *Mycobacterium* microorganism or the biological sample, may be prepared for sampling by placing in a suitable sample vial for collection of a sample of the headspace gas. Alternatively, a sample of breath may be taken from a patient, e.g. a patient suspected of being infected with a mycobacterial infection. The breath or the headspace gas from the culture or biological sample can be sampled, for example with a conditioned Solid Phase Micro Extraction (SPME) fibre or via another absorbent trap, such as Tenax. The sampled potion, whether it be the breath sample or the headspace gas sample, is contacted with a suitable derivatising agent, e.g. an alkylating agent, e.g. trimethyl sulfonium hydroxide (TMSH) or trimethyl anilinium hydroxide (TMAH) or a silylating agent, e.g. N,O-bis(trimethylsilyl)acetamide (BSA) or N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA). The sample may be contacted with the derivatising agent while on the SPME fibre or on the absorbent trap. The fibre is exposed directly into the spectrometer, e.g. into the hot injector of a gas chromatograph, e.g. as described in the Examples section. Any of the free acids are converted *in situ* to the corresponding derivative, e.g. to the methyl ester or to the trimethylsilyl derivative. The derivatised compounds are detectable by a variety of methods, such as GC-MS.

This *in situ* derivatisation can give ester derivatives, e.g. alkyl ester derivatives such as methyl ester derivatives. The acids can be converted as described above and in the Examples section, using a suitable alkylating agent such as TMSH or TMAH, to their corresponding alkyl esters, e.g. methyl esters. Other alkylating agents can be used, such as, for example, diazomethane or methanolic BF_3.

Alternative methods of derivatising the free acids for detection as biomarkers will be apparent to those skilled in the art. These can be carried out using standard techniques, where the derivatising agent, e.g. the alkylating agent or the silylating agent, is contacted with a headspace gas or breath sample prior to analysis, thereby converting one or more of the four acids (nicotinic acid, p-anisic acid, phenyl acetic acid or salicylic acid) present in the headspace gas or breath sample to its derivative.

For analysis of patient breath samples, these samples can be delivered to the inlet of the spectrometer instrument. Alternatively, a breath sample can be collected, e.g. in a sample bag or other suitable container, for later delivery to the spectrometer instrument.

It will also be appreciated by those skilled in the art that a number of different techniques are suitable for analysing the gas samples in the methods of the invention. One preferred technique is GC-MS, as described in the Examples.

Other techniques for analysing the gas samples include ion mobility spectroscopy, SIFT-MS, PTR-MS, Laser Spectroscopy, Quantum Cascade Laser-Based Gas Sensors, electronic noses or sensitive moth antenna/sensilla (Y. Kuwana, S. Nagasawa, I. Shimoyama, R. Kanzai, Biosensors & Bioelectronics, 14, 1999, 195-202) and electroantennogram.

PTR-MS can be used to analyse headspace gas samples of cell cultures of *Pseudomonas aeruginosa* and *Steptococcus milleri*. The same technique can also be used to analyse patient breath samples for drug monitoring, as can SIFT-MS (A. Critchley, T. S. Elliott, G. Harrivson, C. A. Mayhew, J. M. Thompson and T. Worthington, International Journal of Mass Spectrometry, 239, 2004, 235-241). The technique may therefore be used to analyse headspace samples for the presence of volatile compounds, and thus finds application in the present methods for detecting the above-described biomarkers.

SIFT-MS can also be used to detect small molecules in patient breath samples (S. M. Abbott, J. B. Elder, P. Spanel, D. Smith, International Journal of Mass Spectrometry, 228, 2003, 655-665).

More recent reports indicate that techniques such as laser absorption spectroscopy have application in the analysis of breath samples for small molecules (M. R. McCurdy, Y. Bakhirkin, G. Wysocki, R. Lewicki, F. K. Tittel, J. Breath Res. 1, 2007, R1-R12). Such techniques are also contemplated for use in the methods of the present invention.

The development of devices such as electronic noses also provides potential for these devices to be used in the detection of small molecules for disease, such as TB (A. K. Pavlou, A. P. F. Turner, Clin. Chem. Lab. Med. 2000, 38(2):99-112). Such devices are also contemplated for use in the methods of the present invention.

It will be apparent to those skilled in the art that these other analytical techniques find application in the analysis of breath and/or headspace gas samples.

The specific volatile biomarkers, nicotinic acid, p-anisic acid, phenyl acetic acid, salicylic acid, methyl phenylacetate, methyl p-anisate, methyl nicotinate, and o-phenylanisole, enable the speedy diagnosis of *M. tuberculosis* and *M. bovis* relative to time consuming existing diagnostic methods. They provide increased sensitivity and specificity for these organisms. The biomarkers enable the identification of *M. tuberculosis, M.africanum* and *M. bovis* not only from culture samples, but also from sputum, lung fluid, serum and even blood and other body fluids. The detection of the biomarkers by analysis of the headspace over these materials makes this method ideally suited for breath diagnosis of TB. With the advent of instrumentation capable of testing for trace amounts of volatile compounds in a gaseous sample, such as those described above, e.g. electronic noses, the diagnosis of TB by testing for the biomarkers on the breath of patients in both clinical and field environments is made possible by this invention.

The term "patient" as used herein includes both human and animal patients.

The term "alkyl" as used herein is intended to include both straight- and branched-chain alkyl groups. The term "lower alkyl" means C_1-C_6 alkyl. Examples of alkyl groups include methyl group, ethyl group, n-propyl group, i-propyl group, n-butyl group, sec-butyl group i-butyl group, t-butyl group, n-pentyl group, 1,1-dimethylpropyl group, 1,2-dimethylpropyl group, 2,2-dimethylpropyl group, 1-ethylpropyl group, 2-ethylpropyl group, *n*-hexyl group and 1-methyl-2-ethylpropyl group. Any alkyl group may be substituted or unsubstituted. Preferably it is unsubstituted. More preferably any alkyl group is an unsubstituted lower alkyl group.

The term "alkenyl" as used herein is intended to include both straight- and branched-chain alkenyl groups. The term "lower alkenyl" means C_2-C_6 alkenyl. Examples of alkenyl groups include straight- or branched-chain enthenyl, propenyl, butenyl, pentenyl and hexenyl groups. Any alkenyl group may be substituted or unsubstituted. Preferably it is unsubstituted. More preferably any alkenyl group is an unsubstituted lower alkenyl group.

The term "alkynyl" as used herein is intended to include both straight- and branched-chain alkynyl groups. The term "lower alkynyl" means C_2-C_6 alkynyl. Examples of alkynyl groups include straight- or branched-chain ethynyl, propynyl, butynyl, pentynyl

and hexynyl groups. Any alkynyl group may be substituted or unsubstituted. Preferably it is unsubstituted. More preferably any alkynyl group is an unsubstituted lower alkynyl group.

The term "headspace gas" as used herein is intended to include the gas phase contained in a sample container, e.g. a chromatography sample vial, which also contains a liquid or solid sample.

ABBREVIATIONS

MS	Mass Spectrometry
GC-MS	Gas Chromatography Mass Spectrometry
PTR-MS	Proton Transfer Reaction Mass Spectrometry
SPME	Solid Phase Micro Extraction
SIFT-MS	Selected Ion Flow Tube Mass Spectrometry
EI	Electron Impact
TMSH	Trimethyl sulfonium hydroxide
TMAH	Trimethyl anilinium hydroxide
BSA	N,O-bis(trimethylsilyl)acetamide
BSTFA	N,O-bis(trimethylsilyl)trifluoroacetamide

EXAMPLES

The invention is further described with reference to the following examples. It is to be appreciated that the invention is not limited to these examples.

Strains and culture conditions

Clinical isolates and reference cultures of *Mycobacterium tuberculosis* (ATCC 25177, strain H37Ra), *Mycobacterium bovis* (ATCC 19210, NCTC 10772), BCG Vaccine SSI (Danish strain 1331), *Mycobacterium fortuitum* (ATCC 6841, NCTC 10394), *Mycobacterium chelonae* (ATCC 35752, NCTC 946) and *Mycobacterium abcsessus* (ATCC 35751, TMC 1542) were used. Organisms were grown on Lowenstein Jensen/Glycerol, sheep blood agar and BacT/Alert®MP within sterile glass vials stoppered with airtight aluminium caps incorporating a teflon-coated rubber septum. MB/BacT® (0.5 mL) reconstitution fluid (Biomerieux, Inc., Marcy l'Etoile, France) was added to each vial.

Strain preparation

Freeze dried cultures were revived as recommended by the New Zealand Reference Culture Collection, Medical Section, and transferred onto the medium. Each medium was then incubated at 37 °C for up to four weeks. For qualitative identification work, a suspension was made to 0.5 McFarland standard. A $1:10^6$ dilution (equivalent to approximately 10^3 organisms) of this suspension (500 µL) was introduced into the sealed culture vial by injecting through the septum onto the culture medium. The medium was then incubated at 37 °C for up to four weeks or until there was visibly identifiable growth. Headspace probing was carried out at weekly intervals. Verification of fully grown cultures was provided independently by the Microbiology Unit of the Canterbury Health Laboratories, Christchurch, New Zealand, using Fuchsin acid-fast stains (Ziehl-Neelsen) and microscopy. For time dependence of metabolite production, six dilutions (1:10 to $1:10^6$) of a 0.5 McFarland standard of the reference strain of *Mycobacterium tuberculosis* were prepared. Each dilution (500 µL) was injected through the septum onto all three media. The vials were incubated at 37 °C and sampling was carried out every three days for four weeks.

Sputum and Serum Samples

Serum and sputum samples were obtained from the WHO Tuberculosis Specimen Bank in lots of 20 sputum and serum samples. The lots consisted of clinically well characterised TB positive and TB negative material (n=10 respectively).

Headspace analysis of in vitro culture samples and of serum and sputum samples was carried out as described herein, and in M. Syhre and S. T. Chambers, *Tuberculosis* 88, 317 (2008).

SPME Gas Chromatography - Mass Spectrometry (GC-MS)

A conditioned SPME fibre was exposed into culture vials for 12 hours and then desorbed directly in the injection port for 15 min. The temperatures of the injector, ion trap, manifold and transfer line were 250, 200, 60 and 250 °C, respectively. A ZB-624 column was used. The oven program commenced at 60 °C for 2 min, was raised to 260 °C at a rate of 10 °C/min, and maintained at this temperature for a further 2 min. Helium flow was set at a constant rate of 1.2 mL/min. The split vent was opened to a ratio of 1:50 after 1 min. Fragmentation was performed in the EI-mode as full scan

which gave additional certainty. Further MS/MS fragmentation was used to increase sensitivity.

Results – Detection of Biomarkers in Culture

Detection results for *Mycobacterium* species are shown in Table 1.

Table 1: Identity and detection of compounds on three different growth media

name	methyl phenylacetate	methyl p-anisate	methyl nicotinate	o-phenylanisole†
M. tuberculosis	+	+	+	+
M. bovis	+	+	+	+
M. bovis BCG	nd	nd	nd	+
M. africanum	+	+	+	-
M. avium c.	+	nd	nd	+
M. abcsessus	nd	nd	nd	+
M. chelonae	nd	nd	nd	+
M. fortuitum	nd	nd	nd	+

+ = detected

nd = not detected

† = only detected on blood agar

- = not measured

The results of time dependence studies are shown in Figure 1. The detection of methyl p-anisate was measured against growth of *M. tuberculosis* colonies. Positive detection of the 4 compounds was observed up to 4 days before visual appearance of colonies on the media. Concentrations are shown in CFU/500 µL inoculum with highlighted data points indicating the visual appearance of *M. tuberculosis* colonies on the media.

Results – Detection of Biomarkers in Patient Samples

SPME fibres (colour code grey from Supelco, Bellefonte) were conditioned and then exposed via septa into the headspace of a 2 mL vial containing serum (0.3 mL) or sputum (0.5 mL) and sampled for up to 10 hours.

A derivatisation step converted the free acid biomarkers present in the serum and sputum samples into their corresponding methyl esters. Derivatisation was achieved *in situ* by exposing the fibre after the sampling step into the headspace of 0.2 M trimethyl sulfonium hydroxide (TMSH) for one minute. The fibre was then retracted and again exposed into the hot injector of the gas chromatograph at 250°C and analysed by GC-MS as described above and in M. Syhre and S. T. Chambers, *Tuberculosis* 88, 317 (2008). In the hot injector the derivatisation took place *in situ* and the resulting methyl esters methyl phenylacetate, methyl p-anisate and methyl nicotinate were detected.

Methyl phenylacetate and methyl p-anisate were detected in the headspace of sputum samples after derivatisation. Methyl p-anisate and methyl nicotinate were detected in the headspace of serum samples. Figure 2 shows the detection of methyl nicotinate (after derivatisation) in the headspace of serum samples of *Mycobacterium tuberculosis*-positive and *Mycobacterium tuberculosis*-negative patients.

Although the invention has been described by way of example, it should be appreciated that variations and modifications may be made without departing from the scope of the invention. Furthermore, where known equivalents exist to specific features, such equivalents are incorporated as if specifically referred to in the specification.

INDUSTRIAL APPLICABILITY

The invention relates to the use of biomarkers for identifying bacterial pathogens. The invention provides a method for detecting a *Mycobacterium* microorganism.

CLAIMS

1. A method for detecting a *Mycobacterium* microorganism by analysing a gas mixture for any one or more of methyl phenylacetate, methyl p-anisate, methyl nicotinate, and o-phenylanisole.

2. A method of detecting a *Mycobacterium* microorganism in a patient comprising:
   a) obtaining a sample of breath of the patient;
   b) analysing the sample for the presence of any one or more of methyl phenylacetate, methyl p-anisate, methyl nicotinate, and o-phenylanisole; and
   c) determining whether a *Mycobacterium* microorganism is present in the patient.

3. A method of detecting a *Mycobacterium* microorganism in an *in vitro* culture or a biological sample comprising:
   a) obtaining a sample from the headspace gas of the *in vitro* culture or biological sample;
   b) analysing the sample for the presence of any one or more of methyl phenylacetate, methyl p-anisate, methyl nicotinate, and o-phenylanisole; and
   c) determining whether a *Mycobacterium* microorganism is present in the *in vitro* culture or biological sample.

4. A method for detecting a *Mycobacterium* microorganism by analysing a gas mixture for any one or more of phenyl acetic acid, p-anisic acid, nicotinic acid and salicylic acid.

5. A method of detecting a *Mycobacterium* microorganism in a patient comprising:
   a) obtaining a sample of breath of the patient;
   b) analysing the sample for the presence of any one or more of phenyl acetic acid, p-anisic acid, nicotinic acid and salicylic acid; and
   c) determining whether a *Mycobacterium* microorganism is present in the patient.

6. A method for detecting a *Mycobacterium* microorganism in an *in vitro* culture or a biological sample comprising:

a) obtaining a sample from the headspace gas of the *in vitro* culture or biological sample;

b) analysing the sample for the presence of any one or more of phenyl acetic acid, p-anisic acid, nicotinic acid and salicylic acid; and

c) determining whether a *Mycobacterium* microorganism is present in the *in vitro* culture or biological sample.

7. A method for detecting a *Mycobacterium* microorganism by analysing a gas mixture for a derivative of any one or more of phenyl acetic acid, p-anisic acid, nicotinic acid and salicylic acid.

8. A method of detecting a *Mycobacterium* microorganism in a patient comprising:

a) obtaining a sample of breath of the patient;

b) analysing the sample for the presence of a derivative of any one or more of phenyl acetic acid, p-anisic acid, nicotinic acid and salicylic acid; and

c) determining whether a *Mycobacterium* microorganism is present in the patient.

9. A method for detecting a *Mycobacterium* microorganism in an *in vitro* culture or a biological sample comprising:

a) obtaining a sample from the headspace gas of the *in vitro* culture or biological sample;

b) analysing the sample for the presence of a derivative of any one or more of phenyl acetic acid p-anisic acid, nicotinic acid and salicylic acid; and

c) determining whether a *Mycobacterium* microorganism is present in the *in vitro* culture or biological sample.

10. A method as claimed in claim 8 or claim 9, including step (i), carried out after step a):

(i) derivatisating the phenyl acetic acid, p-anisic acid, nicotinic acid or salicylic acid *in situ* to give a derivative of phenyl acetic acid, p-anisic acid, nicotinic acid or salicylic acid.

11. A method as claimed in any one of claims 7 to 10, wherein the derivative is an alkyl, alkenyl or alkynyl ester of phenyl acetic acid, p-anisic acid, nicotinic acid or salicylic acid.

12. A method as claimed in any one of claims 1 to 3 or 7 to 11, wherein the gas mixture is analysed for both methyl p-anisate and methyl nicotinate.

13. A method as claimed in any one of claims 1 to 3, wherein the gas mixture is analysed for all of methyl phenylacetate, methyl p-anisate, methyl nicotinate, and o-phenylanisole.

14. A method as claimed in any one of claims 1, 2, 4, 5, 7, 8, 10, 11, 12 or 13, wherein the gas mixture is a sample of breath of a patient.

15. A method as claimed in any one of claims 1, 3, 4, 6, 7, 9, 10, 11, 12 or 13 wherein the gas mixture is a sample of the headspace gas of an *in vitro* culture or biological sample.

16. A method as claimed in claim 15 wherein the biological sample is sputum, lung fluid, serum or blood.

17. A method as claimed in any one of claims 1 to 16 wherein the microorganism is *Mycobacterium tuberculosis*, *Mycobacterium bovis, Mycobacterium smegmatis, Mycobacterium tuberculosis* BCG, *Mycobacterium avium, Mycobacterium leprae, Mycobacterium africanum* or *Mycobacterium ulcerans.*

18. A method as claimed claim 17 wherein the microorganism is *Mycobacterium tuberculosis* or *Mycobacterium bovis.*

19. An apparatus for detecting the presence of a *Mycobacterium* microorganism having:

a) a gas sample inlet enabling introduction of a gas sample into the apparatus;

b) a means for analysing the gas sample for the presence of any one or more of methyl phenylacetate, methyl p-anisate, methyl nicotinate, and o-phenylanisole; and

c) a means for displaying information enabling the diagnosis of *Mycobacterium tuberculosis* or *Mycobacterium bovis*, or a means for

transmitting information to a device for displaying information enabling the diagnosis of *Mycobacterium tuberculosis* or *Mycobacterium bovis*.

20. A method of diagnosis of a mycobacterial infection in a patient, including detecting the presence of a *Mycobacterium* microorganism by the method as claimed in any one of claims 1 to 18.

21. A method as claimed in claim 21 wherein the mycobacterial infection is caused by *Mycobacterium tuberculosis* or *Mycobacterium bovis*.