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(54) Title: DIAGNOSTIC METHOD AND KIT

(57) Abstract: Provided are methods of and kits for diagnosis of tuberculosis in an animal comprising determining the presence or absence of an Rv3616 antigen, an MPB70 antigen, an MPB70 linear epitope, an MPB83 antigen, and at least one of (a) a CFPlO antigen and (b) an ESAT 6 antigen. The animal may be a bovid. Also provided are methods and kits which may be used to identify tuberculosis infection in animals such as badgers, cervids and wild boar.

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"Diagnostic Method and Kit"

Field of the Invention

The invention relates to methods and kits for the diagnosis and/or herd profiling of Mycobacterium infected or contaminated animals.

Background to the Invention

Tuberculosis (TB), one of the most widespread infectious diseases, is the leading cause of death due to a single infectious agent among adults in the world. Mycobacterium tuberculosis is the most common cause of human TB.

In order to monitor and control the disease, herd profiling is necessary. However, the methods currently used to monitor tuberculosis in animals suffer from a number of drawbacks. Nowadays, the disease control programmes for bovine TB carried out in most countries are based on a test and removal strategy utilizing the intradermal skin test, which relies on PPD, a purified protein derivative of *M. bovis* strain AN5, to elicit an immune response in infected cattle.

In cattle, the intradermal skin tests currently used are the Caudal-fold Tuberculin Test (CFT) and the Comparative cervical tuberculin test (CCT). The

Caudal-fold Tuberculin Test (CFT) is the primary screening test used to identify cattle herds potentially infected with bovine tuberculosis. It measures the immune response to Purified Protein Derivative (PPD) tuberculin (M. *bovis* AN5) . However, in 5% of cases, the CFT test may result in false-positive test results or, in 15% of cases, in false-negative test results. Disease control based on the skin test can be complemented by the gamma-interferon test, which measures an animal's T cell response when exposed to PPD material. The gamma-interferon test is utilised as a second line diagnostic for 'skin test positive' animals.

One of the problems associated with the complex antigenic nature of mycobacteria is the definition of those proteins which are important targets of the immune system and are thus likely present in large numbers of field samples. It is also important to recognize that there is regional variation in the infectious mycobacterial strains, for example *M. bovis* strains.

An antibody assay developed using strain specific proteins could resolve both specificity and sensitivity issues.

A number of methods of discriminating between strains of tuberculosis have been suggested. US 6,686,166B, WO 2004/083448A, US2004/00 63923A, and US 6,291,19OB describe 129 genetic marker sequences which are suggested for use in the identification of

strains of mycobacteria. However, many markers are not expressed sufficiently to reliably be used in the identification of a strain. Further, as described further below, the amount of expression of individual markers varies considerably, not only between strains, but within strains geographically and within strains dependent on the stage of infection .

US2005/0272104A suggests the use of the PPD antigens ESAT-6 and CFP-IO in the detection of Mycobacterium tuberculosis in humans. In general however, antibody (Ab) tests based upon PPD tuberculins are characterised by a low discriminating power, with the distribution of the antibody titers between infected and non-infected animals being widely overlapping (Amadori *et al*. 1998).

US 7,192,721 describes a test kit for detection of TB in different non-primate mammals, the kit comprising a mycobacterial antigen cocktail comprising ESAT-6/CFP10 and 16kDa/MPB83 fusion proteins. In a sample of 85 cattle, the sensitivity demonstrated was 79%; however, in 513 cattle, the sensitivity was 73%.

Due to the heterogeneity of the disease, no single antigen is present in all cases of infection. However, although a great many antigens associated with tuberculosis infection have been identified in the prior art, the need remains for particular combinations which may be used in a test with

improved levels of sensitivity and specificity to avoid the many false positive and false negative test results associated with conventional TB testing.

Although many antigens have been identified, by simply increasing the number of antigens in a test kit, the sensitivity and specificity of the test may not necessarily be improved. It is recognised in the art that, where diagnostic kits comprise too many antigenic additional antigens, the specificity and sensitivity of the kit may in fact be reduced. For example, US 792,721 describes a decrease in sensitivity and specificity associated with increasing the number of antigens used in the kits described therein. Thus, crucial to the design of an effective kit is the selection of antigens to be used therein.

As well as the problems discussed above with respect to identification of antigens for reliable diagnosis of tuberculosis infection, the formats of many diagnostic kits currently used may contribute to the lack of reliability. Cumulative results from animals experimentally infected with *Mbv (M. bovis)* have shown there may be a transient antibody response to some antigens that occur at different stages of infection, further complicating development of a diagnostic assay (Amadori,et al 2002. Vet. Microbiol. 85:379-389). The advances that have been made show that some of the difficulties in developing an optimal assay can be overcome by

including multiple antigens in the assay (Aagaard, et al 2006. J. Clin. Microbiol. 44:4326-4335., Amadori,et al 2002. Vet. Microbiol. 85:379-389). Although some success has also been made in identifying antigens potentially useful in multiplexed diagnostic assays (Aagaard, et al 2006. J. Clin. Microbiol. 44:4326-4335., Koo et al 2005. J. Clin. Microbiol. 43:4498-4506, Lightbody, et al 2000. Vet. Microbiol. 75:177-188, McNair et al 2001. Scand. J. Immunol. 53:365-371., Pollock, J. M. and P. Andersen. 1997. Infect. Immun. 65:2587-2592, Pollock, J. M. and P. Andersen. 1997. J. Infect. Dis. 175:1251-1254, Pollock, et al 2000. Vet. Rec. 146:659-665, Wiker et al 1998. Infect. Immun. 66:1445-1452), the necessity of using multiple antigens in an assay, has, however, introduced another challenge. Evaluation of the standard type of ELISA has shown sensitivity and specificity are reduced when multiple antigens are combined for analysis in a single well thus limiting the way a conventional ELISA can be used (Lyashchenko, et al 2000. J. Immunol. Methods 242:91-100). Fluorescence polarization has been evaluated as an alternative platform (Lin et all996. Clin. Diag. Lab. Immunol. 3:438-443, Surujballiet al 2002. Vet. Microbiol. 87:149-157). It has been successfully used in testing for *Mbv* in cattle, elk, llamas, and bison with a single antigen. A further improvement over an ELISA-based system has been the use of lateral flow technology (Koo et al 2005. J. Clin. Microbiol. 43:4498-4506, Lyashchenko et al 2000. J. Immunol. Methods 242:91-100). This methodology has been used

successfully for multiplexing antigens for visual analysis of patterns of antibody activity at the single animal level (Greenwald, et al 2003. Diagn. Microbiol. Infect. Dis. 46:197-203, Lyashchenko, et al 2006. Clin. Vaccine Immunol. 13:722-732, Waters, et al 2006. Clin. Vaccine Immunol. 13:648-654, Waters, et al 2006. Clin. Vaccine Immunol. 13:611-619, Wernery et al 2007. Vet. Microbiol. 122:108-115.) . Other areas that have not been fully explored are electrochemiluminescence (Lu, Y. et al 2006. J. Immunol. Methods 314:74-79, Yan, et al. 2004. J. Immunol. Methods 288:47-54) and chemiluminescence (Liew et al 2007. Biotechniques 42:327-333). Commercial imaging systems using these technologies have been developed for multiplexing and analysis of antibody activity to multiple antigens (Liew et al 2007. Biotechniques 42:327-333, Lu, Y. et al 2006. J. Immunol. Methods 314:74-79, Yan, et al. 2004. J. Immunol. Methods 288:47-54). The platforms are designed for rapid processing and analysis of large numbers of samples. However, a number of problems persist with many of these methods .

There thus remains the need for an improved test which can accurately and reliably diagnose the presence of tuberculosis infection with satisfactory sensitivity and specificity across different populations of animals.

PCT/GB2008/050721, which shares the same applicants as the present application) describes particular

combinations of mycobacterial antigens which may be used in the diagnosis of TB with high levels of reliability.

Summary of the Invention

As described herein, the present inventors have identified a number of polypeptides, which together are useful for the sensitive specific diagnosis and profiling of Mycobacterium in a number of different families of mammals.

Each of the specific polypeptides is capable of eliciting a strong immune response in the absence of adjuvant. The characterized proteins/fragments which are immunogenic (or early antigens) and specific to one or other mycobacterial strains can be used reliably for the diagnosis of tuberculosis infection. As described in the examples, such polypeptides elicit a strong immune response in serum from animals immunised with *Mycobacterium bovis.* Significantly, the inventors have found that, when particular combinations of these polypeptides are used together in an assay, the sensitivity of tuberculosis testing can be vastly improved over the sensitivity obtained using prior art methods.

The polypeptides which may be used in the invention are SEQ ID NOs: 1-13 and variants and fragments thereof.

In one embodiment of the invention, the polypeptides which may be used in the invention comprise peptides

having amino acid sequences comprising the amino acid sequences shown as one or more of SEQ ID NOs: 1-13.

SEQ ID NO: 1 corresponds to Rv3616C (full length)

MSRAFIIDPT ISAIDGLYDL LGIGIPNQGG ILYSSLEYFE
KALEELAAAF PGDGWLGSAA DKYAGKNRNH VNFFQELADL
DRQLISLIHD QANAVQTTRD ILEGAKKGLE FVRPVAVDLT
YIPVVGHALS AAFQAPFCAG AMAVVGGALA YLVVKTLINA
TQLLKLLAKL AELVAAAIAD IISDVADI IK GTLGEVWEFI
TNALNGLKEL WDKLTGWVTG LFSRGWSNLE SFFAGVPGLT
GATSGLSQVT GLFGAAGLSA SSGLAHADSL ASSASLPALA
GIGGGSGFGG LPSLAQVHAA STRQALRPRA DGPVGAAAEQ
VGGQSQLVSA QGSQGMGGPV GMGGMHPSSG ASKGTTTKKY
SEGAAAGTED AERAPVEADA GGGQKVLVRN VV

SEQ ID NO: 2 corresponds to residues 300-370 of Rv3616c (Mtb40) :

ASTRQALRPRADGPVGAAAEQVGGQSQLVSAQGSQGMGGPVGMGGMHPSSGA
SKGTTTKKYSEGAAAGTED

SEQ ID NO: 3 corresponds to residues 35-94 of Rv3616c:

SLEYFEKALEELAAAFPGDGWLGSAADKYAGKNRNHVNFFQELADLDRQLIS
LIHDQANA

SEQ ID NO: 4 corresponds to residues 1-34 of Rv3616c:

MSRAFIIDPTISAIDGLYDLLGIGIPNQGGILYS

SEQ ID NO: 5 corresponds to residues 95-392 of Rv3616c:

VQTTRDILEGAKKGLEFVRPVAVDLTYIPVVGHALSAAFQAPFCAGAMAVVG
GALAYLVVKTLINATQLLKLLAKLAELVAAAIADIISDVADIIKGTLGEVWE
FITNALNGLKELWDKLTGWVTGLFSRGWSNLESFFAGVPGLTGATSGLSQVT
GLFGAAGLSASSGLAHADSLASSASLPALAGIGGGSGFGGLPSLAQVHAAST
RQALRPRADGPVGAAAEQVGGQSQLVSAQGSQGMGGPVGMGGMHPSSGASKG
TTTKKYSEGAAAGTEDAERAPVEADAGGGQKVLVRNVV

SEQ ID NO: 6 corresponds to MPB70

MKVKNTIAATSFAAAGLAALAVAVSPPAAAGDLVGPGCAEYAAANPTGPASV
QGMSQDPVAVAASNNPELTTLTAALSGQLNPQVNLVDTLNSGQYTVFAPTNA
AFSKLPASTIDELKTNSSLLTSILTYHVVAGQTSPANVVGTRQTLQGASVTV
TGQGNSLKVGNADVVCGGVSTANATVYMIDSVLMPPA

SEQ ID NO: 7 corresponds to PEP1(MPB70):

KGSGS SVQGMSQDPVAVAASNNPEL

SEQ ID NO: 8 corresponds to MPB83

MINVQAKPAAAASLAAIAIAFLAGCSSTKPVSQDTSPKPATSPAAPVTTAAM
ADPAADLIGRGCAQYAAQNPTGPGSVAGMAQDPVATAASNNPMLSTLTSALS
GKLNPDVNLVDTLNGGEYTVFAPTNAAFDKLPAATIDQLKTDAKLLSSILTY
HVIAGQASPSRIDGTHQTLQGADLTVIGARDDLMVNNAGLVCGGVHTANATV
YMIDTVLMPPAQ

SEQ ID NO: 9 corresponds to Rv3875 (ESAT6) :

TEQQWNFAGIEAAASAIQGNVTSIHSLLDEGKQSLTKLAAAWGGSGSEAYQG
VQQKWDATATELNNALQNLARTISEAGQAMASTEGNVTGMFA

SEQ ID NO: 10 corresponds to Rv3874 (CFP10) :

AEMKTDAATLAQEAGNFERISGDLKTQIDQVESTAGSLQGQWRGAAGTAAQA
AVVRFQEAANKQKQELDEISTNIRQAGVQYSRADEEQQQALSSQMGF

SEQ ID NO: 11 corresponds to PEP4 (CFP10)

KGSGS MAEMKTDAATLAQEAGN

SEQ ID NO: 12 corresponds to PEP9 (CFP10)

KGSGS NIRQAGVQYSRADEEQQQ

SEQ ID NO: 13 corresponds to PEP 17 (CFP10)

VVRFQEAANKQKQELDE I

Other polypeptides which may be used in the invention comprise peptides having amino acid sequences comprising the amino acid sequences shown as one or more of SEQ ID NOs: 14-16.

SEQ ID NO: 14 corresponds to Rvl573:
MTTTPARFNHLVTVTDLETGDRAVCDRDQVAETIRAWFPDAPLEVREALVRL
QAALNRHEHTGELEAFLRISVEHADAAGGDECGPAILAGRSGPEQAAINRQL
GLAGDDEPDGDDTPPWSRMIGLGGGSPAEDER

SEQ ID NO: 15 corresponds to Rvl580c:
MAETPDHAELRRRIADMAFNADVGMATCKRCGDAVPYI ILPNLQTGEPVMGV
ADNKWKRANCPVDVGKPCPFLIAEGVADSTDDTIEVDQ

SEQ ID NO: 16 corresponds to Rvl585c:
MSRHHNIVIVCDHGRKGDGRIEHERCDLVAPI IWVDETQGWLPQAPAVATLL
DDDNQPRAVIGLPPNESRLRPEMRRDGWVRLHWEFACLRYGAAGVRTCEQRP
VRVRNGDLQTLCENVPRLLTGLAGNPDYAPGFAVQS DAVVVAMWLWRTLCE S
DTPNKLRATPTRGSC

Underlined sequences, as shown above for some peptides, were added to enhance the hydrophilicity of the peptides for binding to the surface. Where reference is made herein to use of such peptides, it should be understood that such peptides may be employed with or without the hydrophilicity enhancing sequence.

The inventors have found that the peptides having amino acid sequences SEQ ID NO: 1 to SEQ ID NO: 13

each elicit a strong immune response in serum from animals challenged with mycobacterial strains. Significantly, the inventors have found that by employing one of two combinations of only five of these antigens, the presence of tuberculosis may be identified in animals with a degree of sensitivity hitherto not obtainable. Furthermore the inventors have also shown that a diagnostic test for tuberculosis infection demonstrating both high sensitivity and specificity may be based on either of these combinations.

Accordingly, in a first aspect of the invention, there is provided a method of diagnosis of tuberculosis in an animal, the method comprising the steps :

providing a biological sample from said animal; and determining the presence or absence of a group of antigens, or the presence or absence of an immune response to each member of said group of antigens, wherein said group of antigens comprises the group consisting of (i)an Rv3616 antigen, (ii) an MPB70 antigen, (iii) an MPB70 linear epitope, for example PEPl (MPB70) (iv) an MPB83 antigen, and (v) at least one of (a) a CFPlO antigen and (b) an ESAT 6 antigen; wherein the identification of the presence of two or more of said antigens of said group of antigens, or of an immune response thereto, is indicative of the presence of tuberculosis infection in the animal.

In a second aspect, the present invention provides an assay for the detection of the presence of Mycobacterium, for example *Mycobacterium bovis,* in a biological sample, said method comprising the steps:

providing a biological sample from said animal; and determining the presence or absence of a group of antigens, or the presence or absence of an immune response to each member of said group of antigens, wherein said group of antigens comprises the group consisting of (i) an Rv3616 antigen, (ii) an MPB70 antigen, (iii) an MPB70 linear epitope, for example PEPl (MPB70) (iv) an MPB83 antigen, and (v) at least one of (a) a CFPlO antigen and (b) an ESAT 6 antigen;

wherein the identification of the presence of two or more of said antigens or of an immune response thereto is indicative of the presence of Mycobacterium in the biological sample.

In one embodiment of the first and second aspects of the invention, the method does not comprise the determination of the presence or absence of any mycobacterial antigen, or an immune response thereto, other than (i) an Rv3616 antigen, (ii) an MPB70 antigen, (iii) an MPB70 linear epitope, for example PEPl (MPB70) (iv) an MPB83 antigen, and (v) at least one of (a) a CFPlO antigen and (b) an ESAT 6 antigen.

In one embodiment of the first or second aspect of the invention, the group of antigens comprises (a) a

CFPlO antigen but does not comprise (b) an ESAT-6 antigen .

In another embodiment of the first or second aspect of the invention, the group of antigens comprises an ESAT-6 antigen but does not comprise a CFPlO antigen .

Unless the context demands otherwise, where reference is made to a CFPlO antigen, such reference should be understood to encompass one or more of a CFPlO antigen having the sequence shown as SEQ ID NO: 10, a pep4 (CFPlO) antigen having the sequence shown as SEQ ID NO: 11, a pep9 (CFPlO) antigen having the sequence shown as SEQ ID NO: 12, or a pepl7 (CFPlO) antigen having the sequence shown as SEQ ID NO: 13.

However, in one particular embodiment, the CFPlO antigen has the sequence shown as SEQ ID NO: 10.

Likewise where reference is made to a Rv3616c antigen, such reference should be understood to encompass one or more of a Rv3616c antigen having the sequence shown as SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, or SEQ ID NO: 5.

The present inventors have established that, even if an animal has been exposed to bovine PPD, the detectable immune response to the PPD test, in many cases, decreases rapidly after the initial exposure. Thus, although exposure to one or more of these

antigens may be detectable in an animal uninfected with tuberculosis but which has been tested with bovine PPD within 2 weeks of the PPD test, within one month or so, the antigenic response will, in many cases, be undetectable. Thus, in general, unless a particular herd has been tested with PPD within one month preceding testing the animals with the method or kit of the present invention, the identification of the presence of an immune response to one or more of these antigens should be indicative of infection with *Mycobacterium bovis* and not merely exposure to bovine PPD.

However, the antigens having SEQ ID NO: 14, SEQ ID NO: 15 and SEQ ID NO: 16 (rvl573, Rvl580c and Rvl585c) are not expressed in bovine PPD and thus may be used to confirm that the presence of a positive result is indicative of infection with bovine tuberculosis and not merely of past exposure to PPD via e.g. the intradermal skin test.

Accordingly, in one embodiment of the first and second aspects of the invention, the method further includes determining the presence or absence of at least one of the antigens, or an immune response to at least one of the antigens, of the group consisting of an Rvl573 antigen, an Rvl580c antigen and an Rvl585c antigen. The identification of the presence of one or more of the Rvl573 antigen, the Rvl580c antigen and the Rvl585c antigen or of an immune response thereto is confirmatory of the presence of, or infection with, *Mycobacterium bovis.*

The methods of the invention may be used with any animal, for example any mammal, including humans, or bird. In one embodiment, the animal is a mammal. In one particular embodiment, the mammal is a non-human mammal .

In a particular embodiment of the invention, the mammal is a domesticated animal, such as a farm animal. In a particular embodiment the mammal is of the family bovidae. The bovidae include cattle, goats, sheep, antelopes and gazelles. In one embodiment, the mammal is a cow. In another embodiment, the mammal is a goat.

In a particular embodiment of the invention, the mammal is a goat and the group of antigens comprises (i) an Rv3616 antigen, (ii) an MPB70 antigen, (iii) an MPB70 linear epitope, for example PEPl (MPB70) (iv) an MPB83 antigen, and (v) a CFPlO antigen.

In another particular embodiment of the invention, the mammal is a goat and the group of antigens consists of (i) an Rv3616 antigen, (ii) an MPB70 antigen, (iii) an MPB70 linear epitope, for example PEPl (MPB70) (iv) an MPB83 antigen, and (v) a CFPlO antigen .

In a further embodiment of the invention the mammal is a cervid.

In a further embodiment of the invention the mammal is a suidae mammal, for example pig, swine ,hog or wild boar. In one particular embodiment, the suidae mammal is a wild boar.

Moreover, as described in the Examples, the inventors have established that tuberculosis can be identified with high sensitivity and selectivity in cervids and wild boar by determining the presence or absence of a group of four (of the five antigens used in the first and second aspects of the invention) , or the presence or absence of an immune response to each member of said group of four antigens. The group of antigens consists of (i) an MPB70 antigen, (ii) an MPB83 antigen, and (iii) an Rv3616c antigen and (iv) a CFPlO antigen.

Accordingly, the invention further extends in a third aspect to a method of diagnosis of tuberculosis in a cervid or a suidae mammal, the method comprising the steps:
providing a biological sample from said cervid or suidae mammal; determining the presence or absence of, or an immune response to each of an Rv3616 antigen, an MPB70 antigen, an MPB83 antigen, and a CFPlO antigen; wherein the identification of the presence of one or more, for example two or more, of said antigens of said group of antigens, or of an immune response thereto, is indicative of the presence of tuberculosis infection in the cervid or suidae mammal .

The invention also extends in a fourth aspect to an assay method for the detection of the presence of Mycobacterium, for example *Mycobacterium bovis,* in a biological sample from a cervid or a suidae mammal, the method comprising the steps:
providing a biological sample from said cervid or suidae mammal; determining the presence or absence of, or an immune response to each of an Rv3616 antigen, an MPB70 antigen, an MPB83 antigen, and a CFPlO antigen; wherein the identification of the presence of one or more, for example two or more, of said antigens, or of an immune response to one or more, for example two or more, of said antigens, is indicative of the presence of tuberculosis infection in the cervid or suidae mammal.

In one embodiment of the third or fourth aspects of the invention, the method does not comprise the determination of the presence or absence of any mycobacterial antigen, or an immune response thereto, other than of or to an Rv3616 antigen, an MPB70 antigen, an MPB83 antigen, and a CFPlO antigen .

In one embodiment of the third or fourth aspect of the invention, wherein the animal is a cervid, the identification of the presence of one of said antigens of said group of antigens, or of an immune response thereto, is indicative of the presence of tuberculosis infection in the cervid.

In one embodiment of the third or fourth aspect of the invention wherein the animal is a suidae mammal, the identification of the presence of two of said antigens of said group of antigens, or of an immune response thereto, is indicative of the presence of tuberculosis infection in the suidae mammal.

In a particular embodiment of the third or fourth aspects of the invention, the animal is a wild boar.

A number of wild mammals are considered reservoirs for TB infection. For example, badgers are considered as a significant source of TB infection for cattle and, as a result, are often culled to prevent TB spread to cattle. However, this means of control of TB infection is controversial. A test which could reliably identify TB infection in badgers (and other host animals, which may act as reservoirs of infection) would therefore be invaluable in establishing whether or not individual animals, sets or populations thereof are indeed a threat to other species and thus save unnecessary culling. To date, however, a test which can reliably identify infection in animals such as badgers is not available. Typical tests have a reported sensitivity of less than 50% when assessing field samples (Chambers et al J Clin Microbiol. 2008 Apr;46 (4) :1498-500) .

The methods of the present invention achieve considerably higher sensitivity than known tests for use in diagnosis of TB infection in animals such as

badgers. Thus, in one embodiment of the invention, the mammal is a "reservoir" animal , for example a badger. In the context of the present invention, a "reservoir" animal is any non-human animal which may carry or be infected with tuberculosis and which may act as a source of infection of tuberculosis to domesticated livestock. Such reservoir animals include, in addition to badgers, white tail deer, possums, elephant and zoo/safari park animals.

Moreover, the inventors have determined that a considerable improvement in sensitivity may be achieved over known reservoir animals, e.g. badger, TB diagnostic assays by testing for the presence or absence of, or an immune response to each of a MPB70 antigen, an Rv3616c antigen and a MPB83 antigen.

Accordingly, in a fifth aspect of the invention, there is provided a method of diagnosis of tuberculosis in a mammal, the method comprising the steps :
providing a biological sample from said mammal; and
determining the presence or absence of, or an immune response to an Rv3616 antigen, an MPB70 antigen, and an MPB83 antigen, wherein the identification of the presence of one or more of said antigens of said group of antigens, or of an immune response thereto, is indicative of the presence of tuberculosis infection in the mammal.

In a particular embodiment of this aspect of the invention, the method does not comprise the

determination of the presence or absence of any mycobacterial antigen, or an immune response thereto, other than Rv3616 antigen, an MPB70 antigen, and an MPB83 antigen. In another embodiment of this aspect of the invention, the method employs determining the presence or absence of CFP-IO, optionally in the absence of determination of the presence or absence of any other mycobacterial antigen, or an immune response thereto, other than Rv3616 antigen, an MPB70 antigen, an MPB83 antigen, and a CFP-IO antigen.

In one embodiment of the fifth aspect of the invention, the mammal is a "reservoir" mammal. In a particular embodiment, the mammal is a badger.

In the present application, unless the context demands otherwise, references to tuberculosis should be taken to refer to any tuberculosis, for example human tuberculosis, non-human animal tuberculosis, avian tuberculosis or a para-tuberculosis disease, such as Johne's disease.

Unless the context demands otherwise (for example where reference is made to an MPB70 antigen and a pepl (MPB70) antigen within the same context), reference to an MPB70 antigen should be understood to encompass an MPB70 antigen having the sequence shown as SEQ ID NO: 6 or a pepl (MPB70) antigen having the sequence shown as SEQ ID NO: 7, or both. In one embodiment of the invention, the presence or absence of an immune response to both an MPB70

antigen having the sequence shown as SEQ ID NO: 6 and a pepl (MPB70) antigen having the sequence shown as SEQ ID NO: 7 are determined. The combination of use of the pepl MPB70 peptide together with the recombinant MPB70 protein allows the identification of antibodies to both linear and conformational epitopes .

Unless the context demands otherwise, reference to Mycobacterium should be taken to refer to any Mycobacterium. In one embodiment, the Mycobacterium is *Mycobacterium tuberculosis* . In another embodiment the Mycobacterium is *Mycobacterium bovis.* In another embodiment, the Mycobacterium is *Mycobacterium avium.* In another embodiment, the Mycobacterium is *Mycobacterium paratuberculosis* . In another embodiment, the Mycobacterium is *Mycobacterium caprae.*

Any suitable biological sample may be used in the methods of the invention. Suitable biological samples include but are not limited to whole blood, serum, plasma, saliva, cerebrospinal fluid, urine and tissue samples.

In the methods of the invention, the presence of particular antigens or an immune response thereto may be determined using any means known in the art, either directly or indirectly. For example, in one embodiment, the presence of the antigen in the sample is determined; alternatively or additionally the presence of an antibody specific to said antigen

is determined; alternatively, or additionally, the presence of a nucleic acid encoding said antibody or said antigen is determined.

The identification by the inventors of the particular selection of mycobacterial antigens which are particularly immunogenic, which are widely prevalent across geographical areas, and which moreover, cover each of the different stages of the TB life cycle enables the use of these polypeptides in the preparation of novel vaccines against tuberculosis. Such vaccines should provide a vaccinated animal with a sustained resistance to infection .

Accordingly, in a sixth aspect of the present invention, there is provided a vaccine or immunomodulator for use in the vaccination of mammals , wherein the vaccine comprises (i)an Rv3616 antigen, (ii) an MPB70 antigen, (iii) an MPB70 linear epitope, for example PEPl (MPB70) (iv) an MPB83 antigen, and (v) at least one of (a) a CFPlO antigen and (b) an ESAT 6 antigen.

In one embodiment of the sixth aspect of the invention, the vaccine does not comprise an ESAT 6 antigen. Optionally, the vaccine does not comprise a mycobacterial antigen other than the Rv3616 antigen, MPB70 antigen, MPB70 linear epitope, for example PEPl (MPB70), MPB83 antigen, and CFPlO antigen and/or ESAT 6 antigen.

Given the demonstration of the high sensitivity of detection of TB infection in reservoir animals such as badgers when testing for the presence or absence of, or an immune response to, MPB70, Rv3616c and MPB83, it is believed that vaccines comprising at least these three antigens would be expected to have a significant effect on protecting against TB infection in such reservoir animals.

Thus, in a seventh aspect of the present invention, there is provided a vaccine or immunomodulator for reservoir animals, said vaccine or immunomodulator comprising an Rv3616 antigen, an MPB70 antigen, and an MPB83 antigen.

In one embodiment, the vaccine does not comprise a mycobacterial antigen other than the Rv3616 antigen, the MPB70 antigen, and the MPB83 antigen.

In an alternative embodiment, the vaccine further comprises a CFPlO antigen. In such an embodiment, the vaccine optionally does not comprise a mycobacterial antigen other than an Rv3616 antigen, an MPB70 antigen, an MPB83 antigen and a CFPlO antigen.

In one embodiment of the sixth or seventh aspects of the invention, the vaccine is a vaccine against tuberculosis .

In an alternative embodiment of the sixth or seventh aspects of the invention, one or more of said polypeptides are used in a vaccine as an adjuvant.

In a eighth aspect of the invention, there is provided a method of providing immunity in an animal against tuberculosis, the method comprising administering to said animal a vaccine according to the sixth aspect or the seventh aspect of the invention.

In a ninth aspect of the invention, there is provided (i)an Rv3616 antigen, (ii) an MPB70 antigen, (iii) an MPB70 linear epitope, for example PEPl (MPB70) (iv) an MPB83 antigen, and (v) at least one of (a) a CFPlO antigen and (b) an ESAT 6 antigen for use in a vaccine against tuberculosis.

In a tenth aspect of the invention, there is provided the use of (i)an Rv3616 antigen, (ii) an MPB70 antigen, (iii) an MPB70 linear epitope, for example PEPl (MPB70) (iv) an MPB83 antigen, and (v) at least one of (a) a CFPlO antigen and (b) an ESAT 6 antigen in the preparation of a vaccine against tuberculosis.

In an eleventh aspect of the invention, there is provided an Rv3616 antigen, an MPB70 antigen, an MPB83 antigen, and a CFPlO antigen for use in a vaccine against tuberculosis.

In a twelfth aspect of the invention, there is provided the use of an Rv3616 antigen, an MPB70 antigen, an MPB83 antigen, and a CFPlO antigen in the preparation of a vaccine against tuberculosis.

In each of the ninth, tenth, eleventh and twelfth aspects of the invention, the vaccine optionally comprises no other mycobacterial antigens.

In an thirteenth aspect, the invention provides a diagnostic kit for the diagnosis of the presence of tuberculosis in a subject, said kit comprising: (i)an Rv3616 antigen, (ii) an MPB70 antigen, (iii) an MPB70 linear epitope, for example PEPl (MPB70) (iv) an MPB83 antigen, and (v) at least one of (a) a CFPlO antigen and (b) an ESAT 6 antigen.

In an embodiment of the thirteenth aspect, the kit further comprises at least one of an Rvl573 antigen, an Rvl580c antigen, and an Rvl585c antigen.

In a fourteenth aspect of the invention, there is provided a diagnostic kit for the diagnosis of the presence of tuberculosis in a biological sample, said kit comprising:
(i) an antibody molecule with binding specificity for an Rv3616 antigen, (ii) an antibody molecule with binding specificity for an MPB70 antigen, (iii) an antibody molecule with binding specificity for an MPB70 linear epitope, for example PEPl (MPB70) (iv) an antibody molecule with binding specificity for an MPB83 antigen, and (v) at least one of (a) an

antibody molecule with binding specificity for a CFPlO antigen and (b) an antibody molecule with binding specificity for an ESAT 6 antigen.

In one embodiment of the fourteenth aspect, the kit does not comprise an antibody molecule with binding specificity for any mycobacterial antigen other than for a Rv3616 antigen, an MPB70 antigen, an MPB70 linear epitope, for example PEPl (MPB70), an MPB83 antigen, a CFPlO antigen or an ESAT 6 antigen.

In an embodiment of the fourteenth aspect, wherein the kit comprises (a) an antibody molecule with binding specificity for a CFPlO antigen, but does not comprise (b) an antibody molecule with binding specificity for an ESAT-6 antigen.

In an embodiment of the fourteenth aspect, the kit further comprises at least one of the antibody molecules selected from the group consisting of an antibody molecule with binding specificity for an Rvl573 antigen, an antibody molecule with binding specificity for an Rvl580c antigen, and an antibody molecule with binding specificity for an Rvl585c antigen.

In a fifteenth aspect of the invention, there is provided a diagnostic kit for the diagnosis of the presence of tuberculosis in a reservoir animal such as a badger, said kit comprising an Rv3616 antigen, an MPB70 antigen, and an MPB83 antigen.

In one embodiment, the diagnostic kit further comprises a CFPlO antigen. In another embodiment, the kit does not comprise another mycobacterial antigen .

In a sixteenth aspect of the invention, there is provided a diagnostic kit for the diagnosis of the presence of tuberculosis in a biological sample from a reservoir animal such as a badger, said kit comprising: an antibody molecule with binding specificity for an Rv3616 antigen, an antibody molecule with binding specificity for an MPB70 antigen, and an antibody molecule with binding specificity for an MPB83 antigen.

In one embodiment of the sixteenth aspect of the invention, the diagnostic kit further comprises an antibody molecule with binding specificity for CFPlO antigen. In another embodiment, the kit does not comprise an antibody molecule with binding specificity for a mycobacterial antigen other than for a Rv3616 antigen, a MPB70 antigen, or a MPB83 antigen. In another embodiment, the kit does not comprise an antibody molecule with binding specificity for a mycobacterial antigen other than for a Rv3616 antigen, a MPB70 antigen, a MPB83 antigen, or a CFP-IO antigen.

Preferred features of each aspect of the invention are as for each of the other aspects *mutatis mutandis* .

Detailed Description

Polypeptides, Variants and Fragments

As described above, the inventors have identified a number of tuberculosis antigens which may be used in the diagnosis of tuberculosis, the differentiation between strains of Mycobacteria, and/or preparation of vaccines. The polypeptide sequences for each of these antigens are provided in Table 1. In one embodiment of the invention, the full length antigen sequences are used.

In one embodiment of the invention, the antigens for use in the invention consist of polypeptides consisting of the amino acid sequence shown as any one of SEQ ID N0:l to SEQ ID NO: 16.

However, the present invention is not limited to the use of polypeptides having such specific sequences of the polypeptides or disclosed herein but also extends to fragments or variants of such full length polypeptides. Thus, unless the context demands otherwise, where reference is made to an Rv3616c antigen, it should be understood to encompass an Rv3616c antigen consisting of the amino acid sequence shown as SEQ ID NO: 1, or a variant or fragment thereof, where reference is made to an MPB70 antigen, it should be understood to encompass an MPB70 antigen consisting of the amino acid sequence shown as SEQ ID NO: 7, or a variant or fragment thereof, where reference is made to an

MPB83 antigen, it should be understood to encompass an MP83 antigen consisting of the amino acid sequence shown as SEQ ID NO: 8, or a variant or fragment thereof, where reference is made to an ESAT6 antigen, it should be understood to encompass an ESAT6 antigen consisting of the amino acid sequence shown as SEQ ID NO: 9, or a variant or fragment thereof, where reference is made to a CFP-10 antigen, it should be understood to encompass an CFP-IO antigen consisting of the amino acid sequence shown as SEQ ID NO: 10, or a variant or fragment thereof, where reference is made to a Rvl573 antigen, it should be understood to encompass an Rvl573 antigen consisting of the amino acid sequence shown as SEQ ID NO: 14, or a variant or fragment thereof, where reference is made to a Rvl580c antigen, it should be understood to encompass an Rvl580c antigen consisting of the amino acid sequence shown as SEQ ID NO: 15, or a variant or fragment thereof, where reference is made to a Rvl585c antigen, it should be understood to encompass an Rvl585c antigen consisting of the amino acid sequence shown as SEQ ID NO: 16, or a variant or fragment thereof

For example, with respect to Rv3616c, specific examples of particular fragments which may be used are provided as SEQ ID NOs 2-5. Likewise, with respect to CFP-IO, specific examples of particular fragments which may be used are provided as SEQ ID NOs 10-13. Variant polypeptide sequences in which one or more amino acid residues are modified may

also be used as the polypeptides/antigens in the invention. For example such variants may be useful in the preparation of vaccines or the raising of antibodies which may be used in kits of the invention. Modifications may involve insertion, addition, deletion and/or substitution of one or more amino acids. The modified amino acid residues in the amino acid sequences of the variant are preferably 30% or less, more preferably 20% or less, most preferably 10% or less, within the entire polypeptide. Such variants may be provided using the teaching of the present application and techniques known in the art. Preferably such variants involve the insertion, addition, deletion and/or substitution of 15 or fewer amino acids, more preferably of 10 or fewer, even more preferably of 5 or fewer, most preferably of 1 or 2 amino acids only.

Amino acid substitutions which do not essentially alter biological and immunological activities, have been described, e.g. by Neurath et al in "The Proteins" Academic Press New York (1979). Amino acid replacements between related amino acids or replacements which have occurred frequently in evolution are, inter alia, Ser/Ala, Ser/Gly, Asp/Gly, Asp/Asn, Ile/Val (see Dayhof, M.D., Atlas of protein sequence and structure, Nat. Biomed. Res. Found., Washington D.C, 1978, vol. 5, suppl. 3). Other amino acid substitutions may include but are not limited to Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr,

Ser/Asn, Ala/Val, Thr/Phe, Ala/Pro, Lys/Arg, Leu/Ile, Leu/Val and Ala/Glu.

In preferred embodiments , a variant or fragment retains the immune reactivity of the polypeptide having the amino acid sequence shown as any one of SEQ ID N0:l to SEQ ID NO: 16, of which it is a variant or fragment.

For the avoidance of any doubt, in the present application, where reference is made to the presence of two or more antigens selected from a list, it should be understood that reference is being made to two or more different antigens i.e. each having a different amino acid sequence.

Furthermore, for the avoidance of any doubt, in the present application, unless the context demands otherwise, where reference is made to the determination of the presence or absence of two or more polypeptides/antigens selected from a list, or the presence or absence of an immune response thereto, it should be understood that such a statement encompasses the determination of the presence or absence of two or more of said polypeptides/antigens selected from the list or the determination of the presence or absence of an immune response to two or more of said polypeptides/antigens or indeed a mixture thereof i.e. the determination of the presence or absence of one or more polypeptides/antigens selected from the list combined with the determination of an immune

response to one or more other polypeptides/antigens selected from the list.

Furthermore for the avoidance of any doubt, where reference is made to MPB70 or variants thereof and pepl (MPB70) together, pepl (MPB70) should not be considered as an MPB70 variant.

Antibody molecules

In the context of the present invention, an "antibody molecule" should be understood to refer to an immunoglobulin or part thereof or any polypeptide comprising a binding domain which is, or is homologous to, an antibody binding domain. Antibodies include but are not limited to polyclonal, monoclonal, monospecific, polyspecific antibodies and fragments thereof and chimeric antibodies comprising an immunoglobulin binding domain fused to another polypeptide.

Intact (whole) antibodies comprise an immunoglobulin molecule consisting of heavy chains and light chains, each of which carries a variable region designated VH and VL, respectively. The variable region consists of three complementarity determining regions (CDRs, also known as hypervariable regions) and four framework regions (FR) or scaffolds. The CDR forms a complementary steric structure with the antigen molecule and determines the specificity of the antibody.

Fragments of antibodies may retain the binding ability of the intact antibody and may be used in place of the intact antibody. Accordingly, for the purposes of the present invention, unless the context demands otherwise, the term "antibodies" should be understood to encompass antibody fragments. Examples of antibody fragments include Fab, Fab', F(ab')2, Fd, dAb, and Fv fragments, scFvs, bispecific scFvs, diabodies, linear antibodies (see US patent 5,641,870, Example 2 ; Zapata et al., Protein Eng 8 (10): 1057-1062 [1995]); single-chain antibody molecules; and multispecif ic antibodies formed from antibody fragments .

The Fab fragment consists of an entire L chain (VL and CL), together with VH and CHl. Fab' fragments differ from Fab fragments by having an additional few residues at the carboxy terminus of the CHl domain including one or more cysteines from the antibody hinge region. The F(ab')2 fragment comprises two disulfide linked Fab fragments. Fd fragments consist of the VH and CHl domains. Fv fragments consist of the VL and VH domains of a single antibody. Single-chain Fv (scFv) fragments are antibody fragments that comprise the VH and VL domains connected by a linker which enables the scFv to form an antigen binding site (see Pluckthun in The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenburg and Moore eds ., Springer-Verlag, New York, pp. 269-315 (1994) . Diabodies are small antibody fragments prepared by constructing scFv fragments

with short linkers (about 5-10 residues) between the VH and VL domains such that inter-chain but not intra-chain pairing of the V domains is achieved, resulting in a multivalent fragment, i.e. a fragment having two antigen-binding sites (see, for example, EP 404 097; WO 93/11161; and Hollinger *et al*., PNAS. USA, 90: 6444-6448 (1993)).

Further encompassed by fragments are individual CDRs. The CDRs may be carried on a framework structure comprising an antibody heavy or light chain sequence or part thereof. Preferably such CDRs are positioned in a location corresponding to the position of the CDR(s) of naturally occurring VH and VL domains. The positions of such CDRs may be determined as described in Rabat *et al,* Sequences of Proteins of Immunological Interest, US Dept of Health and Human Services, Public Health Service, Nat'l Inst, of Health, NIH Publication No. 91-3242, 1991 and online at www.kabatdatabase.com http ://immuno .bme .nwu .edu . Furthermore, modifications may alternatively or additionally be made to the Framework Regions of the variable regions. Such changes in the framework regions may improve stability and reduce immunogenicity of the antibody.

The antibody molecules for use in the present invention extend, for example, to any other antibody which specifically binds a polypeptide identified herein as inducing a strong immune response i.e. an antibody molecule which retains binding specificity

for a polypeptide consisting of amino acid sequence shown as any one of SEQ ID NOs :1 to 16.

Antibody molecules for use in the invention herein include "chimeric" antibodies in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain (s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (see US 4,816,567; and Morrison et *al.,* PNAS. USA, 81: 6851-6855 (1984)). Chimeric antibodies of interest herein include "primatized" antibodies comprising variable domain antigen-binding sequences derived from a non-human primate (e.g. Old World Monkey, Ape etc), and human constant region sequences.

Antibody molecules for use in the present invention may be produced in any suitable way, either naturally or synthetically. Such methods may include, for example, traditional hybridoma techniques (Kohler and Milstein (1975) Nature, 256 :495-499), recombinant DNA techniques (see e.g. US 4,816, 567), or phage display techniques using antibody libraries (see e.g. Clackson et *al.* (1991) Nature, 352: 624-628 and Marks et *al.* (1992) Bio/ Technology, 10: 779-783). Other antibody production

techniques are described in Antibodies: A Laboratory Manual, eds. Harlow et *al*., Cold Spring Harbor Laboratory, 1988.

Traditional hybridoma techniques typically involve the immunisation of a mouse or other animal with an antigen in order to elicit production of lymphocytes capable of binding the antigen. The lymphocytes are isolated and fused with a myeloma cell line to form hybridoma cells which are then cultured in conditions which inhibit the growth of the parental myeloma cells but allow growth of the antibody producing cells. The hybridoma may be amenable to genetic mutation, which may or may not alter the binding specificity of antibodies produced. Synthetic antibodies can be made using techniques known in the art (see, for example, Knappik et *al*, J. MoI. Biol. (2000) 296, 57-86 and Krebs et *al*, J. Immunol. Meth. (2001) 2154 67-84).

Modifications may be made in the VH, VL or CDRs of the antibody molecules, or indeed in the FRs using any suitable technique known in the art. For example, variable VH and/or VL domains may be produced by introducing a CDR, e.g. CDR3 into a VH or VL domain lacking such a CDR. Marks et *al.* (1992) Bio/ Technology, 10: 779-783 describe a shuffling technique in which a repertoire of VH variable domains lacking CDR3 is generated and is then combined with a CDR3 of a particular antibody to produce novel VH regions. Using analogous techniques, novel VH and VL domains comprising CDR

derived sequences of the present invention may be produced.

Alternative techniques of producing antibodies for use in the invention may involve random mutagenesis of gene(s) encoding the VH or VL domain using, for example, error prone PCR (see Gram *et al,* 1992, PNAS 89 3576-3580. Additionally or alternatively, CDRs may be targeted for mutagenesis e.g. using the molecular evolution approaches described by Barbas *et al* 1991 PNAS 3809-3813 and Scier 1996 J MoI Biol 263 551-567. Having produced such variants, antibodies and fragments they may be tested for binding to a Mycobacterium, for example *Mycobacterium bovis.*

The antibodies for use in the invention may comprise further modifications. For example the antibodies can be glycosylated, pegylated, or linked to albumin or a nonproteinaceous polymer.

The antibody molecules for use in the invention may be labelled. Labels which may be used include radiolabels, enzyme labels such as horseradish peroxidase or alkaline phosphatase, or biotin.

Nucleic Acid

Nucleic acid for use in the present invention may comprise DNA or RNA. It may be produced recombinantly, synthetically, or by any means

available to those in the art, including cloning using standard techniques.

The nucleic acid may be inserted into any appropriate vector. In one embodiment the vector is an expression vector and the nucleic acid is operably linked to a control sequence which is capable of providing expression of the nucleic acid in a host cell. A variety of vectors may be used. For example, suitable vectors may include viruses (e.g. vaccinia virus, adenovirus, etc.), baculovirus) ; yeast vectors, phage, chromosomes, artificial chromosomes, plasmids, or cosmid DNA.

The vectors may be used to introduce the nucleic acids into a host cell. A wide variety of host cells may be used for expression of the nucleic acid. Suitable host cells for use in the invention may be prokaryotic or eukaryotic. They include bacteria, e.g. E. coli, yeast, insect cells and mammalian cells. Mammalian cell lines which may be used include Chinese hamster ovary cells, baby hamster kidney cells, NSO mouse melanoma cells, monkey and human cell lines and derivatives thereof and many others.

A host cell strain that modulates the expression of, modifies, and/or specifically processes the gene product may be used. Such processing may involve glycosylation, ubiquitination, disulfide bond formation and general post-translational modification .

For further details relating to known techniques and protocols for manipulation of nucleic acid, for example, in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, see, for example, Current Protocols in Molecular Biology, 5th ed., Ausubel *et al.* eds ., John Wiley & Sons, 2005 and, Molecular Cloning: a Laboratory Manual: 3rd edition Sambrook *et al.,* Cold Spring Harbor Laboratory Press, 2001.

Diagnostic Methods, Assays and Kits

The invention may be used in the diagnosis of a variety of conditions and disorders associated with tuberculosis. These include infection with *Mycobacterium bovis, Mycobacterium avium* and human mycobacterium and para-tuberculosis diseases such as Johne's disease.

In using the methods of the invention to identify the infection with a mycobacterial strain, either as current or previous infection, the presence or absence of immunogenic polypeptides, or the presence or absence of an immune response to said polypeptides is determined from a biological sample. Any suitable biological sample may be used. For example, the biological sample may be a biological fluid, such as sputum, saliva, plasma, blood, urine or sperm, or a tissue, such as a biopsy of a tissue.

Diagnostic and assay means of detecting the presence of polypeptides or immune responses to said polypeptides are known in the art. For example, the presence of the polypeptides may be detected by use of antibodies specific to said polypeptides. Alternatively, using standard techniques in the art, the presence of nucleic acids encoding the polypeptide or indeed an antibody specific to said polypeptide may be used. Further, the presence of antibodies specific to said polypeptides may be used to determine the presence of an immune response to said polypeptide.

Techniques which may be employed include but are not limited to ELISA, Immunohistochemistry, Electron Microscopy, Latex agglutination, Immuno Blotting, immunochromatography, immunochips, lateral flow immunoassays and Dip Stick Immuno testing.

The ELISA test (enzyme linked immunoenzymatic assay) is frequently used for serological diagnosis. This method allows the identification and quantification of antigens or antibodies in biological fluids. The conventional ELISA consists in the detection of the complex antibody-antigen by a second antibody (against the antibody that reacts with the antigen) conjugated to an enzymatic activity (peroxidase, alkaline phosphatase and others) .

In the latex agglutination assay, the antigen preparation is affixed to latex beads. The biological sample is then incubated directly on a

slide with the latex particles. In a short time the reaction is examined for the presence of cross-linked, or agglutinated, latex particles indicating the presence of antibodies to polypeptides in the sample.

Immunochips may used to determine the presence of the specific Mycobacterium antigens. Generally, the specific antibodies to the antigens are immobilised on a transducer, e.g. electrodes, caloric meter, piezoelectric crystal, surface plasmon resonance transducer, surface acoustic resonance transducer or other light detecting device. The binding of antigens in the biological sample to the immobilised specific antibody is detected by a change in electric signal.

As described above, the presence of immunogenic antigens may be detected by detecting nucleic acids encoding the antigen or encoding antibodies raised against the antigen. Such techniques are well known in the art. For example, where large amounts of DNA are available, genomic DNA may be used directly. Alternatively, the region of interest is cloned into a suitable vector and grown in sufficient quantity for analysis. The nucleic acid may be amplified by conventional techniques, such as the polymerase chain reaction (PCR) (Saiki, et al. (1985) Science 239:487). Primers may be used to amplify sequences encoding the polypeptide of interest. Optionally, a detectable label, for example a fluorochrome, biotin or a radioactive label may be used in such an

amplification reaction. The label may be conjugated to one or both of the primers. Alternatively, the pool of nucleotides used in the amplification is labeled, so as to incorporate the label into the amplification product.

The sample nucleic acid, e.g. amplified or cloned may be analysed using any suitable method known in the art. For example, the nucleic acid may be sequenced by dideoxy or other methods, and the sequence of bases compared to the deleted sequence. Hybridization with the variant sequence may also be used to determine its presence, by Southern blots, dot blots, etc. The hybridization pattern of a control and variant sequence to an array of oligonucleotide probes immobilized on a solid support, as described in WO95/35505, may be used as a means of detecting the presence or absence of a sequence .

In one embodiment, the kit contains an antigen preparation prepared as described above and then fixed onto a solid support for use in a serological assay. The kit may also contain an explanatory note on how to proceed.

In one embodiment, the assay is in a dip stick or test strip format. Such assays typically employ a solid support medium, which is able to bind target substances such as proteins, for example protein antigens. In use, the test strip is exposed to a sample, for example serum, such that antibodies in

the serum may bind target substances such as antigens on the test strip. After washing, the dip stick/test strip is exposed to a labelled probe which binds the bound antibody of interest, with binding typically visualised by means of further incubation with a visualisable substrate, for example a chromogenic substrate.

In a further embodiment, the assay of the invention is provided as a lateral flow immunoassay. Such devices are well known in the art. Lateral flow immunoassay devices typically employ a microporous element along which a sample may flow laterally, the device having a capture region for binding an analyte of interest in the sample. Generally, the lateral flow device has an application zone, to which a sample is applied, a conjugate zone, containing a mobile reporter conjugate, which is typically a visually observable reporter e.g. colloidal gold conjugated to an antibody directed against the analyte and a capture region comprising a capture agent which binds the analyte. In use, the sample is applied to the application zone and flows along a strip to a first region, at which the reporter conjugate binds the analyte. Thereafter, movement of the reporter conjugate/analyte complex to the capture region results in capture of the complex at that region. Uncomplexed reporter conjugate does not bind at the capture region. By determining the amount of visually detectable signal at the capture region, the presence of analyte in a

sample may be determined. Variants of such kits are well known in the art.

In one embodiment of the invention, the methods, assays and kits of the invention comprise or use an assay in which the presence or absence of a number of antigens or an immune response thereto is analysed simultaneously, i.e. using a multiplex based assay. Any suitable multiplex assay may be used. For example, arrays of different tuberculosis antigens may be provided in microplate wells and a sample mixed with reporter antibodies in the wells. Other multiplex based methods may involve protein arrays placed on a matrix, with the response to individual proteins on the solid-phase array assayed. The use of such multiplex techniques enables the rapid identification of the presence or absence of a plurality of target antigens (or antibodies) during a single run of the analysis. A suitable multiplex system which may be used is that described by Whelan et al (Clinical and Vaccine Immunology, 2008, 1834-1838) .

The inventors have developed a multiplex technique in which a plurality of different antigens, for example up to 25, are spotted in a single well of a 96 well plate. The inventors have shown that high sensitivity of reaction with each of the antigens tested was possible in the multiplex reactant mixture despite the necessary requirement of a single buffer being used for each of the plurality of antigen- antibody reactions in a single well.

Using such a plate, up to 96 samples, each from a different animal, may be tested on a single plate with the each samples reactivity with each of the antigens measured simultaneously. Such a technique therefore enables the rapid analysis of infection with, for example, tuberculosis, in a large number of animals simultaneously.

Moreover, the inventors have surprisingly found that the advantages of such a system for the determination of tuberculosis infection is not just with respect to the convenience of being able to test many different antigens in many different samples simultaneously. The inventors have also demonstrated that the multiplex platform provided higher sensitivity and specificity than that obtained using conventional assays such as lateral flow assays and ELISA.

Accordingly, in a further aspect of the invention, there is provided a multiplex method of diagnosis of tuberculosis in an animal, the method comprising the steps :

providing a substrate on which is immobilised a plurality of different antigens, wherein said antigens comprise (i)an Rv3616 antigen, (ii) an MPB70 antigen, (iii) an MPB70 linear epitope, for example PEPl (MPB70) (iv) an MPB83 antigen, and (v) at least one of (a) a CFPlO antigen and (b) an ESAT 6 antigen,

bringing into contact with said substrate a biological sample from an animal,
identifying the presence or absence of binding of antibodies from said sample with said antigens,
wherein the determination of binding is indicative of the presence of tuberculosis infection in the animal .

In one embodiment, the substrate is a microplate. In one such embodiment, a plurality of different antigens are immobilised in a single well of a plate. In one embodiment of this aspect of the invention, the plurality of antigens comprises (i)an Rv3616 antigen, (ii) an MPB70 antigen, (iii) an MPB70 linear epitope, for example PEPl (MPB70) (iv) an MPB83 antigen, and (v) at least one of (a) a CFPlO antigen and (b) an ESAT 6 antigen or fragments or variants thereof.

In a further aspect of the invention, there is provided a multiplex method of diagnosis of tuberculosis in an animal, the method comprising the steps :
providing a substrate to which is attached a plurality of different antibody molecules, wherein said antibody molecules comprise i) an antibody molecule with binding specificity for an Rv3616 antigen, (ii) an antibody molecule with binding specificity for an MPB70 antigen, (iii) an antibody molecule with binding specificity for an MPB70 linear epitope, for example PEPl (MPB70) (iv) an antibody molecule with binding specificity for an

MPB83 antigen, and (v) at least one of (a) an antibody molecule with binding specificity for a CFPlO antigen and (b) an antibody molecule with binding specificity for an ESAT 6 antigen;

bringing into contact with said substrate a biological sample from an animal,

identifying the presence or absence of binding of antigens from said sample with said antibody molecules,

wherein the determination of binding is indicative of the presence of tuberculosis infection in the animal .

In one embodiment, the substrate is a microplate. In one such embodiment, a plurality of different antibody molecules are immobilised in a single well of a plate. In one embodiment of this aspect of the invention, the plurality of antibodies comprises i) an antibody molecule with binding specificity for an Rv3616 antigen, (ii) an antibody molecule with binding specificity for an MPB70 antigen, (iii) an antibody molecule with binding specificity for an MPB70 linear epitope, for example PEPl (MPB70) (iv) an antibody molecule with binding specificity for an MPB83 antigen, and (v) at least one of (a) an antibody molecule with binding specificity for a CFPlO antigen and (b) an antibody molecule with binding specificity for an ESAT 6 antigen..

The kit can be used to perform the methods of the invention described above.

Vaccines

As described above, the present invention also extends to vaccines for use in protecting against tuberculosis and tuberculosis-associated diseases.

One way of making a vaccine according of or for use in the invention is by biochemical purification of the immunogenic polypeptides from bacteria.

Alternatively, expression products of the genes encoding the polypeptides according to the invention may be used in vaccines. Such vaccines based upon the expression products of these genes can easily be made by admixing one or more proteins with a pharmaceutically acceptable carrier.

Alternatively, a vaccine according to the invention can comprise live recombinant carriers, capable of expressing the polypeptides according to the invention .

Vaccines described above all contribute to active vaccination, i.e. the host's immune system is triggered by one or more proteins according to the invention or immunogenic fragments thereof, to make antibodies against these proteins.

Alternatively, such antibodies can be raised in e.g. rabbits or can be obtained from antibody-producing cell lines. Such antibodies can then be administered to the host animal. This method of vaccination,

passive vaccination, is the vaccination of choice when an animal is already infected, and there is no time to allow the natural immune response to be triggered. It is also the preferred method for vaccinating immune-compromised animals.

Therefore, one other form of this embodiment of the invention relates to vaccines comprising antibodies against one or more of the immunogenic polypeptides used in the invention.

An alternative and efficient way of vaccination is direct vaccination with DNA encoding the relevant antigen. Direct vaccination with DNA encoding proteins has been successful for many different proteins. (As reviewed in e.g. Donnelly et al., The Immunologist 2:20-26(1993)). Therefore, still other forms of this embodiment of the invention relate to vaccines comprising nucleic acid sequences encoding a polypeptide as used in the invention or immunogenic fragments thereof, and to vaccines comprising DNA fragments that comprise such nucleic acid sequences.

Still other forms of this embodiment relate to vaccines comprising recombinant DNA molecules according to the invention.

DNA vaccines can easily be administered through intradermal application e.g. using a needle-less injector .

Vaccines according to the present invention may comprise a pharmaceutically acceptable carrier e.g. sterile water, a sterile physiological salt solution, or a buffer, and may also contain an adjuvant.

The invention will now be described further in the following non-limiting examples.

Example 1

The inventors have identified a panel of proteins capable of strain identification (Table 1).

Methodology Multiplex Assays

Serum samples

Serum samples used in the study were obtained from several sources. Blood samples were taken into serum tubes (Vacuette, serum clot activator tubes, Greiner-bio-one), transported at room temperature and then stored at 2-8 ^{0}C until processing. Following centrifugation (3000g, 30 minutes at 2-8^{0}c) the serum was removed, aliquoted and stored at -20^{0}C.

Two sets of sera were obtained, one from a negative sample bank including sera from herds of animals with a known history of being free of *Mbv.* A third group of sera were collected from animals that were proven to be positive for *Mbv* infection at the time

of slaughter based on subsequent histopathological/bacteriological examination.

The fourth set of serum samples was part of an infectivity study. The animals were non-vaccinated and challenged via the intra-tracheal route with a low dose of a virulent strain of *Mbv* (approximately 5,000 CFU). Sera were collected prior to challenge and then at 2, 5, 10 and 17 weeks post infection (PI) (data not shown). A single intradermal comparative cervical tuberculin test (SICCT) was carried out prior to challenge and also during week 15 PI. All animals in the study had lesions typical of a TB infection, and all animals at 17 weeks PI were culture positive for *Mbv.*

Preparation of antigens

The genes encoding different TB proteins were expressed in *E. coli* as N-terminal polyhistidine-tagged (6X HIS) fusion proteins by Fusion Antibodies Ltd. (Belfast) using the Fusion Expression Technology (FET) platform. Recombinant proteins were purified and polished to near homogeneity by using Fusion Antibodies Ltd. three-step chromatographic protocol. SDS-PAGE and western blot analysis were performed on all purified and polished recombinant TB proteins to confirm their level of purity was greater than 99%. Synthetic peptides were synthesised by Genosphere Biotechnologies (France) to a purity of >80% (RP-HPLC at 220nm). Amino acid residues were added to peptides to enhance the hydrophilicity of the peptides.

Lyophilised peptides were re-constituted to 2mg/ml in sterile phosphate buffer saline pH 7.4 (Sigma Aldrich, Dublin) and aliquots were prepared and stored at -20^{0}C. Antigen quantification was performed using the micro BCA™ protein assay kit (Pierce, Rockford, IL).

Multiplex Antigen printing

Antigens used in the study were printed in each well of a black polystyrene 96 well plate in a multiplex planar array format by Quansys Biosciences, (Logan, UT). Optimization of the antigen printing was carried out in conjunction with Enfer. Plates were printed by Quansys Biosciences, (Logan, UT) and testing of plates was carried out at Enfer Scientific (Naas, Ireland). Buffer mediated alterations in spot deposition and morphology were examined at Quansys Biosciences using an Alpha Innotech 8900 imager workstation and a Nikon Diaphot ELWD phase contrast inverted microscope. The effects of differing buffers used for spotting were screened for differential responses at Enfer. The buffer selected gave the optimal pixel intensity response with the optimal spot size/morphology on the plates. Antigen coating concentrations optimization was carried out at Enfer Scientific (Naas, Ireland) using a series of coating titrations printed by Quansys Biosciences in the optimized printing buffer. Panels of antigens were printed using the optimized printing protocol and buffers and were used for sample testing. All plates were shipped and stored at 2-8^{0}C. Plates were allowed to

warm to room temperature for 30 minutes prior to use .

Multiplex immunoassay method

The assay method was developed in-house at Enfer Scientific (Naas, Ireland) . Serum samples were diluted 1:250 into sample dilution buffer (Enfer Buffer A, Enfer Scientific, Naas, Ireland) and mixed. 30µl of sample was added per well. Sample dilution and plating was carried out using the automated pipettor Tecan Genesis RSP 150. The plates were incubated at room temperature with agitation (900 rpm) for 30minutes. The plates were washed with IX Enfer Wash Buffer (Enfer Scientific, Ireland) six times and aspirated. The detection antibody (polyclonal rabbit anti-bovine immunoglobulin -HRP, Dako, Denmark) was prepared to a dilution of 1:3000 in detection antibody dilution buffer (Enfer Buffer B, Enfer Scientific, Naas, Ireland) . After addition of 30µl of the detection antibody to test wells, the plates were incubated at room temperature for 15 minutes with agitation (900 rpm) . The plates were washed as above and 30µl of substrate (50:50 dilution of substrate and diluent) added per well (Chemilumenscent substrate and diluent, Quansys Biosciences, Logan, Utah) . Signals were captured over a 45 second exposure time using Quansys Biosciences Imaging system (Quansys Biosciences, Logan, Utah) . Images were saved as CR2 image files. Data were extracted from the captured images using the Quansys Q-View Software™ Version 2.0. The data were compiled and analysed in a

custom made macro in Microsoft Excel (Enfer Multiplex Macro, Version 1.0.1.0) .

Development of the multiplex chemiluminescent ELISA

Preliminary studies were conducted to optimize and obtain uniform binding of each antigen using a fluorescent tag. Following optimization of spotting, tests were performed to determine lot to lot variability in pixel intensity at Quansys Biosciences based on the fluorescent tag incorporated into the printing buffer. Plates were tested and evaluated by Enfer for signal variation using a panel of serum samples. Testing of the plates with the detection antibody, with and without sera from a panel of control sera from uninfected cattle, showed that background noise in the system in areas of the surface not coated with antigens was on an average 779.3 relative light units (RLU) . Optimisation of the printing concentration for all antigens was carried out on a series of plates printed based on a cohort of control sera. Optimal printing concentrations were determined and used in subsequent testing. Buffers, blocking agents and assay conditions were further optimised on the multiplex assay as well as optimisation of the exposure time for signal capture. The optimal exposure time was determined to be 45 seconds, this giving the best signal to noise ratios with a cohort of samples with varying signal intensities (data not shown) .

A total of 1489 TB negative and 522 TB positive animal sera were screened against the antigens listed in Table 1. The individual specificity and sensitivity of all antigens were calculated based on a range of cut offs before selecting individual thresholds for each individual antigen. The individual sensitivities of the antigens ranged from 5.4 - 95.0 % while individual specificities ranged from 69.1 - 99.1%. Calculation of results was based on a combinational approach to the data analysis on results from all antigens for each sample.

Example 2 Identification of Panel of Antigens for Diagnosis of TB in bovidae

The inventors analysed antigen expression from 447 cattle, which had been confirmed TB positive by histopathology and culture methods, using 1489 cattle, which had been confirmed TB negative using tuberculin skin tests, as the controls. The analysis was carried out using the multiplex platform described above in relation to Example 1 with the following changes: 50µl of diluted sample and diluted conjugate were added per well and 40µl of prepared substrate was added per well instead of the volumes recited in Example 1. Moreover, the sample incubation time was 90 minutes and the conjugate incubation time was 30 minutes.

The inventors identified two panels of five antigens, each panel of which when assayed together enable diagnosis of tuberculosis infection with very

high sensitivity and selectivity. The assaying for the presence of at least two of the five antigens ESAT-6+Rv3616c+pepl (MPB70) +MPB83+MPB70 gave a sensitivity of 93.7%, compared to 71.4% obtained with ESAT-6+Rv3616c+pepl (MPB70) +MPB83, 25.5% obtained using ESAT-6+Rv3616c +pepl (MPB70) . The specificity obtained with ESAT-6+Rv3616c+pepl (MPB70) +MPB83+MPB70 was 97.6%.

The assaying for the presence of at least two of the five antigens CFP-10+Rv3616c+pepl (MPB70) +MPB83+MPB70 gave a sensitivity of 97.8%. The specificity obtained with CFP-10+Rv3616c+pepl (MPB70) +MPB83+MPB70 was 97.6%.

The inventors then extended the study to another member of the bovidae family. The inventors analysed antigen expression from 62 goats, which had been confirmed TB positive by tuberculin skin tests and IFNg tests, using 31 goats, which had been confirmed TB negative using tuberculin skin tests, as the controls. The analysis was carried out using the multiplex platform described above in relation to Example 1 with the following changes: 50µl of diluted sample and diluted conjugate were added per well and 40µl of prepared substrate was added per well instead of the volumes recited in Example 1; moreover, the sample incubation time was 90 minutes and the conjugate incubation time was 30 minutes; samples were diluted to 1:1000, a different conjugate (rabbit anti-goat IgG whole molecule,

peroxidise) was used with appropriate buffers and the conjugate was diluted to 1:40,000.

The inventors identified a panel of five antigens which when assayed together enable diagnosis of tuberculosis infection with sensitivity and selectivity at levels hitherto not obtained with goats. The assaying for the presence of at least two of the five antigens CFP-IO + Rv3616c + pepl (MPB70) + MPB83 + MPB70 gave a sensitivity of 100% and a specificity of 100% i.e. even higher than the sensitivity and specificity obtained using the same antigens in cattle.

Example 3 Identification of Panel of Antigens for Diagnosis of TB in badgers

The inventors analysed antigen expression from 67 badgers, which had been confirmed TB positive by histopathology and culture methods, using 133 badgers, which had been confirmed TB negative using tuberculin skin tests, as the controls. The analysis was carried out using the multiplex platform described above in relation to Example 1 with the following changes: 50µl of diluted sample and diluted conjugate were added per well and 50µl of prepared substrate was added per well instead of the volumes recited in Example 1; moreover, the conjugate used was affinity purified mAb CF2, anti-badger IgG- HRP, which was used with appropriate buffers and which was diluted to 1:100,000; the samples were diluted to 1:450.

The inventors identified a panel of three antigens which when assayed together, enable diagnosis of tuberculosis infection with sensitivity and specificity at levels hitherto not obtained with badgers. The assaying for the presence of at least one of the three antigens Rv3616c+ MPB83+ MPB70 gave a sensitivity of 58.2%, compared to 52.2% obtained with Rv3616c+ MPB70 alone, and 31.3% obtained using MPB70 alone. The specificity obtained with Rv3616c+ MPB83+ MPB70 was 96.2%.

Example 4 Identification of Panel of Antigens for Diagnosis of TB in wild boar and cervids

The inventors analysed antigen expression from 5 wild boar, which had been confirmed TB positive, using 5 wild boar, which had been confirmed TB negative, as the controls. The analysis was carried out using the multiplex platform described above in relation to Example 1 with the following changes: 50µl of diluted sample and diluted conjugate were added per well and 40µl of prepared substrate was added per well instead of the volumes recited in Example 1; moreover, the sample incubation time was 90 minutes and the conjugate incubation time was 30 minutes; samples were diluted to 1:200; the conjugate used was rabbit anti-pig IgG whole molecule peroxidase conjugate with appropriate buffers and was diluted to 1:40,000

The inventors identified a panel of four antigens which when assayed together enable diagnosis of tuberculosis infection with sensitivity and selectivity at levels hitherto not obtained with wild boar. The assaying for the presence of at least two of the four antigens MPB70 + MPB83+ Rv3616c+CFP-10 gave a sensitivity of 100% and a specificity of 100%.

The inventors also analysed antigen expression in a further cohort of 45 wild boar confirmed positive for TB infection with *M.bovis,* using 102 wild boar, which had been confirmed TB negative, as the controls. The assaying for the presence of at least two of the four antigens MPB70 + MPB83+ Rv3616c+CFP-10 again demonstrated high levels of sensitivity and specificity using these antigens, the specificity being 97.1% and the sensitivity being 72.7%.

The inventors also analysed antigen expression in a further cohort of 10 wild boar confirmed positive for TB infection with *M.caprae,* using 102 wild boar, which had been confirmed TB negative, as the controls. The assaying for the presence of at least two of the four antigens MPB70 + MPB83+ Rv3616c+CFP-10 again demonstrated high levels of sensitivity and specificity using these antigens, the specificity being 97.1% and the sensitivity being 60%.

The inventors also analysed antigen expression from 20 cervids, which had been confirmed TB positive, using 20 cervids, which had been confirmed TB

negative, as the controls. The analysis was carried out using the multiplex platform described above in relation to Example 1 with the following changes: 50µl of diluted sample and diluted conjugate were added per well and 40µl of prepared substrate was added per well instead of the volumes recited in Example 1; moreover, the sample incubation time was 90 minutes and the conjugate incubation time was 30 minutes; the conjugate used was affinity purified antibody, peroxidase labelled, rabbit anti-deer IgG H+L which was used with appropriate buffers and was diluted to 1:100.

The panel of four antigens as employed with the identification of TB infection in wild boar was found when assayed together to enable diagnosis of tuberculosis infection with high sensitivity and selectivity with cervids . The assaying for the presence of at least one of the four antigens MPB70 + MPB83+ Rv3616c+CFP-10 gave a sensitivity of 70% and a specificity of 100%.

All documents referred to in this specification are herein incorporated by reference. Various modifications and variations to the described embodiments of the inventions will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments.

Indeed, various modifications of the described modes of carrying out the invention which are obvious to those skilled in the art are intended to be covered by the present invention.

Table 1

SEQ ID NO: 1 - Rv3616C (full length)

MSRAFIIDPT ISAIDGLYDL LGIGIPNQGG ILYSSLEYFE
KALEELAAAF PGDGWLGSAA DKYAGKNRNH VNFFQELADL
DRQLISLIHD QANAVQTTRD ILEGAKKGLE FVRPVAVDLT
YIPVVGHALS AAFQAPFCAG AMAVVGGALA YLVVKTLINA
TQLLKLLAKL AELVAAAIAD IISDVADI IK GTLGEVWEFI
TNALNGLKEL WDKLTGWVTG LFSRGWSNLE SFFAGVPGLT
GATSGLSQVT GLFGAAGLSA SSGLAHADSL ASSASLPALA
GIGGGSGFGG LPSLAQVHAA STRQALRPRA DGPVGAAAEQ
VGGQSQLVSA QGSQGMGGPV GMGGMHPSSG ASKGTTTKKY
SEGAAAGTED AERAPVEADA GGGQKVLVRN VV

SEQ ID NO: 2 - residues 300-370 of Rv3616c (Mtb40) :

ASTRQALRPRADGPVGAAAEQVGGQSQLVSAQGSQGMGGPVGMGGMHPSSGA
SKGTTTKKYSEGAAAGTED

SEQ ID NO: 3 - residues 35-94 of Rv3616c:

SLEYFEKALEELAAAFPGDGWLGSAADKYAGKNRNHVNFFQELADLDRQLIS
LIHDQANA

SEQ ID NO: 4 - residues 1-34 of Rv3616c:

MSRAFIIDPTISAIDGLYDLLGIGIPNQGGILYS

SEQ ID NO: 5 - residues 95-392 of Rv3616c:

VQTTRDILEGAKKGLEFVRPVAVDLTYIPVVGHALSAAFQAPFCAGAMAVVG
GALAYLVVKTLINATQLLKLLAKLAELVAAAIADIISDVADIIKGTLGEVWE
FITNALNGLKELWDKLTGWVTGLFSRGWSNLESFFAGVPGLTGATSGLSQVT
GLFGAAGLSASSGLAHADSLASSASLPALAGIGGGSGFGGLPSLAQVHAAST
RQALRPRADGPVGAAAEQVGGQSQLVSAQGSQGMGGPVGMGGMHPSSGASKG
TTTKKYSEGAAAGTEDAERAPVEADAGGGQKVLVRNVV

SEQ ID NO: 6 - MPB70

MKVKNTIAATSFAAAGLAALAVAVSPPAAAGDLVGPGCAEYAAANPTGPASV
QGMSQDPVAVAASNNPELTTLTAALSGQLNPQVNLVDTLNSGQYTVFAPTNA
AFSKLPASTIDELKTNSSLLTSILTYHVVAGQTSPANVVGTRQTLQGASVTV
TGQGNSLKVGNADVVCGGVSTANATVYMIDSVLMPPA

SEQ ID NO: 7 - PEP1(MPB70):
KGSGS SVQGMSQDPVAVAASNNPEL

SEQ ID NO: 8 - MPB83
MINVQAKPAAAASLAAIAIAFLAGCSSTKPVSQDTSPKPATSPAAPVTTAAM
ADPAADLIGRGCAQYAAQNPTGPGSVAGMAQDPVATAASNNPMLSTLTSALS
GKLNPDVNLVDTLNGGEYTVFAPTNAAFDKLPAATIDQLKTDAKLLSSILTY
HVIAGQASPSRIDGTHQTLQGADLTVIGARDDLMVNNAGLVCGGVHTANATV
YMIDTVLMPPAQ

SEQ ID NO: 9 - Rv3875 (ESAT6) :
TEQQWNFAGIEAAASAIQGNVTSIHSLLDEGKQSLTKLAAAWGGSGSEAYQG
VQQKWDATATELNNALQNLARTISEAGQAMASTEGNVTGMFA

SEQ ID NO: 10 - Rv3874 (CFP10) :
AEMKTDAATLAQEAGNFERISGDLKTQIDQVESTAGSLQGQWRGAAGTAAQA
AVVRFQEAANKQKQELDEISTNIRQAGVQYSRADEEQQQALSSQMGF

SEQ ID NO: 11 - PEP4 (CFP10)
KGSGS MAEMKTDAATLAQEAGN

SEQ ID NO: 12 - PEP9 (CFP10)
KGSGS NIRQAGVQYSRADEEQQQ

SEQ ID NO: 13 - PEP 17 (CFP10)

VVRFQEAANKQKQELDE I

SEQ ID NO: 14 - Rvl573:
MTTTPARFNHLVTVTDLETGDRAVCDRDQVAETIRAWFPDAPLEVREALVRL
QAALNRHEHTGELEAFLRISVEHADAAGGDECGPAILAGRSGPEQAAINRQL
GLAGDDEPDGDDTPPWSRMIGLGGGSPAEDER

SEQ ID NO: 15 - Rvl580c:
MAETPDHAELRRRIADMAFNADVGMATCKRCGDAVPYI ILPNLQTGEPVMGV
ADNKWKRANCPVDVGKPCPFLIAEGVADSTDDTIEVDQ

SEQ ID NO: 16 - Rvl585c:
MSRHHNIVIVCDHGRKGDGRIEHERCDLVAPI IWVDETQGWLPQAPAVATLL
DDDNQPRAVIGLPPNESRLRPEMRRDGWVRLHWEFACLRYGAAGVRTCEQRP
VRVRNGDLQTLCENVPRLLTGLAGNPDYAPGFAVQS DAVVVAMWLWRTLCE S
DTPNKLRATPTRGSC

Claims

1. An assay for the detection of the presence of Mycobacterium, in a biological sample, said method comprising the steps:

providing a biological sample from said animal; and determining the presence or absence of a group of antigens, or the presence or absence of an immune response to each member of said group of antigens, wherein said group of antigens comprises the group consisting of (i)an Rv3616 antigen, (ii) an MPB70 antigen, (iii) an MPB70 linear epitope, for example PEPl (MPB70) (iv) an MPB83 antigen, and (v) at least one of (a) a CFPlO antigen and (b) an ESAT 6 antigen;

wherein the identification of the presence of two or more of said antigens or of an immune response thereto is indicative of the presence of Mycobacterium in the biological sample.

2. The method according to claim 1 wherein the Mycobacterium is *Mycobacterium bovis.*

3. The method according to claim 1 or claim 2, wherein the identification of the presence of two or more of said antigens of said group of antigens, or of an immune response thereto, is indicative of the presence of tuberculosis infection in the animal.

4. The method according to any one of the preceding claims, further comprising determining the presence or absence of at least one of the antigens, or an immune response to at least one of the

antigens, of the group consisting of an Rvl573 antigen, an Rvl580c antigen or an Rvl585c antigen.

5. The method according to claim 4, wherein the identification of the presence of one or more of the antigens, or an immune response to at least one of the antigens, of the group consisting of an Rvl573 antigen, an Rvl580c antigen or an Rvl585c antigen is indicative of the presence of, or infection with, *Mycobacterium bovis.*

6. The method according to any one of claims 1 to 3, wherein the group of antigens consists of (i) an Rv3616 antigen, (ii) an MPB70 antigen, (iii) an MPB70 linear epitope, for example PEPl (MPB70) (iv) an MPB83 antigen, and (v) at least one of (a) a CFPlO antigen and (b) an ESAT 6 antigen.

7. The method according to any one of claims 1 to 3 or 6, wherein the group of antigens comprises (a) a CFPlO antigen but does not comprise (b) an ESAT-6 antigen .

8. The method according to any one of claims 1 to 7, wherein the animal is a bovid.

9. The method according to claim 8, wherein the animal is a goat or a cow.

10. An assay method for the detection of the presence of Mycobacterium, for example *Mycobacterium bovis,* in a biological sample from an animal, for

example a reservoir mammal, the method comprising the steps:

providing a biological sample from said animal; and

determining the presence or absence of a group of antigens, or the presence or absence of an immune response to each member of said group of antigens, wherein said group of antigens comprises an Rv3616 antigen, an MPB70 antigen, and an MPB83 antigen, wherein the identification of the presence of one or more of said antigens of said group of antigens, or of an immune response thereto, is indicative of the presence of tuberculosis infection in the animal.

11. The method according to claim 10, wherein the identification of the presence of one or more of said antigens of said group of antigens, or of an immune response thereto, is indicative of the presence of tuberculosis infection in the animal.

12. The method according to claim 10 or claim 11, wherein the method does not comprise determining the presence or absence of any mycobacterial antigen, or an immune response thereto, other than an Rv3616 antigen, an MPB70 antigen, and an MPB83 antigen.

13. The method according to any one of claims 10 to 12, wherein said mammal is a badger.

14. The method according to claim 10 or claim 11, wherein said method further comprises determining the presence or absence of an immune response to a CFP-10 antigen.

15. The method according to claim 14, wherein the method does not comprise determining the presence or absence of any mycobacterial antigen, or an immune response thereto, other than an Rv3616 antigen, an MPB70 antigen, an MPB83 antigen, and a CFP-IO antigen .

16. The method according to claim 15, wherein said mammal is a suidae mammal, for example wild boar, or a cervid.

17. The method according to any one of the preceding claims wherein the presence of said one or more antigens is determined by determining the presence of an antibody response to said antigens.

18. The method according to any one of claims 1 to 16 wherein the presence of said one or more antigens is determined by determining the presence of a nucleic acid encoding said antigen or by determining the presence of a nucleic acid encoding an antibody to said antigen.

19. A diagnostic kit for the diagnosis of the presence of tuberculosis in a subject, said kit comprising :
(i)an Rv3616 antigen, (ii) an MPB70 antigen, (iii) an MPB70 linear epitope, for example PEPl (MPB70) (iv) an MPB83 antigen, and (v) at least one of (a) a CFPlO antigen and (b) an ESAT 6 antigen.

20. The kit according to claim 19, wherein the kit comprises each of the antigens of the group consisting of (i) an Rv3616 antigen, (ii) an MPB70 antigen, (iii) an MPB70 linear epitope, for example PEPl (MPB70) (iv) an MPB83 antigen, and (v) at least one of (a) a CFPlO antigen and (b) an ESAT 6 antigen, but does not comprise another mycobacterial antigen .

21. The kit according to claim 20, wherein the kit comprises (a) a CFPlO antigen but does not comprise (b) an ESAT-6 antigen.

22. A diagnostic kit for the diagnosis of the presence of tuberculosis in a biological sample, said kit comprising:
(i) an antibody molecule with binding specificity for an Rv3616 antigen, (ii) an antibody molecule with binding specificity for an MPB70 antigen, (iii) an antibody molecule with binding specificity for an MPB70 linear epitope, for example PEPl (MPB70) (iv) an antibody molecule with binding specificity for an MPB83 antigen, and (v) at least one of (a) an antibody molecule with binding specificity for a CFPlO antigen and (b) an antibody molecule with binding specificity for an ESAT 6 antigen.

23. The diagnostic kit according to claim 22, wherein the kit does not comprise an antibody molecule with binding specificity for any mycobacterial antigen other than for a Rv3616 antigen, an MPB70 antigen, an MPB70 linear epitope,

for example PEPl (MPB70), an MPB83 antigen, a CFPlO antigen or an ESAT 6 antigen.

24. The kit according to claim 22 or claim 23, wherein the kit comprises (a) an antibody molecule with binding specificity for a CFPlO antigen, but does not comprise (b) an antibody molecule with binding specificity for an ESAT-6 antigen.

25. A diagnostic kit for the diagnosis of the presence of tuberculosis in a biological sample from a reservoir animal, said kit comprising an Rv3616 antigen, an MPB70 antigen, and a MPB83 antigen.

26. A diagnostic kit for the diagnosis of the presence of tuberculosis in a biological sample from a reservoir animal, said kit comprising: an antibody molecule with binding specificity for an Rv3616 antigen, an antibody molecule with binding specificity for an MPB70 antigen, and an antibody molecule with binding specificity for an MPB83 antigen .

27. The diagnostic kit according to claim 25 wherein the kit does not comprise a mycobacterial antigen other than an Rv3616 antigen, an MPB70 antigen, and a MPB83 antigen.

28. The diagnostic kit according to claim 26 wherein the kit does not comprise an antibody molecule with binding specificity for a

mycobacterial antigen other than for an Rv3616 antigen, an MPB70 antigen, or a MPB83 antigen.

29. The diagnostic kit according to any one of claims 25 to 28, wherein said animal is a badger.

30. The diagnostic kit according to claim 25 further comprising a CFPlO antigen.

31. The diagnostic kit according to claim 26 further comprising an antibody molecule with binding specificity for a CFPlO antigen.

32. The diagnostic kit according to claim 30 wherein the kit does not comprise a mycobacterial antigen other than an Rv3616 antigen, an MPB70 antigen, a MPB83 antigen, and a CFPlO antigen.

33. The diagnostic kit according to claim 31 wherein the kit does not comprise an antibody molecule with binding specificity for a mycobacterial antigen other than an Rv3616 antigen, an MPB70 antigen, a MPB83 antigen, and a CFPlO antigen .

34. The kit according to any one of claims 30 to 33, wherein said animal is a suidae mammal, for example wild boar, or a cervid.

35. A vaccine comprising an Rv3616 antigen, an MPB70 antigen, and an MPB83 antigen.

36. The vaccine according to claim 35 wherein the vaccine does not comprise another mycobacterial antigen .

37. The vaccine according to claim 35 further comprising a CFPlO antigen.

38. The vaccine according to claim 37 wherein the vaccine does not comprise another mycobacterial antigen.

39. A vaccine comprising (i)an Rv3616 antigen, (ii) an MPB70 antigen, (iii) an MPB70 linear epitope, for example PEPl (MPB70) (iv) an MPB83 antigen, and (v) at least one of (a) a CFPlO antigen and (b) an ESAT 6 antigen.

40. The vaccine according to claim 39, wherein the vaccine does not comprise an ESAT 6 antigen.

41. The vaccine according to claim 40, wherein the vaccine does not comprise another mycobacterial antigen .

42. The method according to claim 16, wherein the mammal is a suidae mammal, for example wild boar, and the identification of the presence of two of said antigens of said group of antigens, or of an immune response thereto, is indicative of the presence of tuberculosis infection in the suidae mammal .

43. The method according to claim 16, wherein the mammal is a cervid and the identification of the presence of one of said antigens of said group of antigens, or of an immune response thereto, is indicative of the presence of tuberculosis infection in the cervid.

44. The method according to claim 9, wherein the mammal is a goat and the identification of the presence of two of said antigens of said group of antigens, or of an immune response thereto, is indicative of the presence of tuberculosis infection in the goat.

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2010/050324

A CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/04 G01N33/569

According to International Patent Classification (IPC) or to both national classification and IPC

B FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K G0IN

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, EMBASE, **WPI Data**

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document with indication, where appropriate, of the relevant passages	Relevant to claim No
X,P	WO 2009/024822 A2 (FUSION ANTIBODIES LTD [GB]; ENFER TECHNOLOGY LTD [IE]; OLWILL SHANE A) 26 February 2009 (2009-02-26) the whole document	1-9, 17-24, 39-41,44
Y	WO 2006/117538 A2 (FUSION ANTIBODIES LTD [GB]; OLWILL SHANE A [GB]; BUICK RICHARD J [GB];) 9 November 2006 (2006-11-09) claim 1 pages 7-10 claim 13	1-9, 17-24, 39-41,44

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[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 May 2010	30/07/2010

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P B 5818 Patentlaan 2 NL - 2280 HV RijSWlJk Tel (+31-70) 340-2040, Fax (+31-70) 340-3016	Hohwy, Morten

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
Y	LIU ET AL: "A novel fusion protein-based indirect enzyme-linked immunosorbent assay for the detection of bovine tuberculosis" TUBERCULOSIS, ELSEVIER, GB LNKD- DOI:10.1016/J.TUBE.2006.07.007, vol. 87, no. 3, 11 April 2007 (2007-04-11), pages 212-217, XP022020620 ISSN: 1472-9792 abstract	1-9, 17-24, 39-41,44
Y	LYASHCHENKO K P ET AL: "PrimaTB STAT-PAK assay, a novel, rapid lateral-flow test for tuberculosis in nonhuman primates" CLINICAL AND VACCINE IMMUNOLOGY, AMERICAN SOCIETY FOR MICROBIOLOGY, WASHINGTON, DC, US LNKD- DOI:10.1128/CVI.00230-07, vol. 14, no. 9, 1 September 2007 (2007-09-01), pages 1158-1164, XP002511617 ISSN: 1556-6811 [retrieved on 2007-07-25] page 1161 page 1160, column 1; table 2 table 4	1-9, 17-24, 39-41,44
Y	WATERS W R ET AL: "Antibody responses in reindeer (Rangifer tarandus) infected with Mycobacterium bovis" CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, AMERICAN SOCIETY FOR MICROBIOLOGY, US LNKD- DOI:10.1128/CDLI.12.6.727-735.2005, vol. 12, no. 6, 1 June 2005 (2005-06-01), pages 727-735, XP002511618 ISSN: 1071-412X page 728, column 2; table 2	1-9, 17-24, 39-41,44
Y	WIKER H G ET AL: "Immunochemical characterization of the MPB70/80 and MPB83 proteins of Mycobacterium bovis." INFECTION AND IMMUNITY APR 1998 LNKD- PUBMED: 9529066, vol. 66, no. 4, April 1998 (1998-04), pages 1445-1452, XP002579405 ISSN: 0019-9567 figures 5,6,8	1-9, 17-24, 39-41,44

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB2010/050324

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect oi certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. IM Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

21, 24, 40, 41(completely); 1-9, 17-20, 22, 23, 39, 44(partial ly)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM POT/ISA/ 210

This International Searching Authority found multiple **(groups of) inventions in this international** application, **as follows:**

1. **claims:** 21, **24,** 40, **41(completely);** 1-9, 17-20, 22, 23, 39, **44(partially)**

A method for detecting mycobacteria comprising detecting antigens or an immune response to said antigens, a diagnostic kit comprising said antigens, a diagnostic kit comprising antibodies to said antigens, and a vaccine comprising said antigens, wherein the antigens are: an Rv3616 antigen, **an MPB70** antigen, **an MPB70 linear antigen (such as** PEPl), **an MPB83** antigen, **and a CFPlO antigen.**

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2. **claims:** 1-9, 17-20, 22, 23, 39, **44(all partially)**

A method for detecting mycobacteria comprising detecting antigens or an immune response to said antigens, **a diagnostic kit comprising said** antigens, **a diagnostic kit comprising antibodies to said** antigens, **and a vaccine comprising said** antigens, **wherein the antigens are: an Rv3616 antigen, an MPB70** antigen, **an MPB70 linear antigen (such as** PEPl), **an MPB83** antigen, **and an ESAT6 antigen.**

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3. **claims:** 10-16, 25-29, 35, 36, 42, **43(completely);** 17, **18(partially)**

A method for detecting mycobacteria comprising detecting antigens or an immune response to said antigens, **a diagnostic kit comprising said** antigens, **a diagnostic kit comprising antibodies to said antigens, and a vaccine comprising said** antigens, **wherein the antigens are: an Rv3616** antigen, **an MPB70** antigen, **and an MPB83 antigen.**

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4. **claims:** 30-34, 37, **38**

A diagnostic kit comprising antigens, **a diagnostic kit comprising antibodies to said** antigens, **and a vaccine comprising said** antigens, **wherein the antigens are: an Rv3616** antigen, **an MPB70** antigen, **an MPB83** antigen, **and a CFPlO antigen.**

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2010/050324

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2009024822 A2	26-02-2009	NONE	
WO 2006117538 A2	09-11-2006	CA 2606962 A1	09-11-2006
		EP 1904848 A2	02-04-2008
		US 2009053258 A1	26-02-2009