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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.

WO 2010/097625 A1

1

"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

2

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

5   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-

10   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.

15   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

20   recognize that there is regional variation in the infectious mycobacterial strains, for example *M. bovis* strains.

An antibody assay developed using strain specific

25   proteins could resolve both specificity and sensitivity issues.

A number of methods of discriminating between strains of tuberculosis have been suggested. US

30   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

4

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 al1996. 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

6

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.

5   **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

10   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

15   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

20   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

25   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

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

8

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

5   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
10   TQLLKLLAKL AELVAAAIAD IISDVADI IK GTLGEVWEFI
TNALNGLKEL WDKLTGWVTG LFSRGWSNLE SFFAGVPGLT
GATSGLSQVT GLFGAAGLSA SSGLAHADSL ASSASLPALA
GIGGGSGFGG LPSLAQVHAA STRQALRPRA DGPVGAAAEQ
VGGQSQLVSA QGSQGMGGPV GMGGMHPSSG ASKGTTTKKY
15   SEGAAAGTED AERAPVEADA GGGQKVLVRN VV

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

ASTRQALRPRADGPVGAAAEQVGGQSQLVSAQGSQGMGGPVGMGGMHPSSGA
20   SKGTTTKKYSEGAAAGTED

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

SLEYFEKALEELAAAFPGDGWLGSAADKYAGKNRNHVNFFQELADLDRQLIS
25   LIHDQANA

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

MSRAFIIDPTISAIDGLYDLLGIGIPNQGGILYS
30

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

VQTTRDILEGAKKGLEFVRPVAVDLTYIPVVGHALSAAFQAPFCAGAMAVVG
GALAYLVVKTLINATQLLKLLAKLAELVAAAIADIISDVADIIKGTLGEVWE
35   FITNALNGLKELWDKLTGWVTGLFSRGWSNLESFFAGVPGLTGATSGLSQVT
GLFGAAGLSASSGLAHADSLASSASLPALAGIGGGSGFGGLPSLAQVHAAST
RQALRPRADGPVGAAAEQVGGQSQLVSAQGSQGMGGPVGMGGMHPSSGASKG
TTTKKYSEGAAAGTEDAERAPVEADAGGGQKVLVRNVV

9

SEQ ID NO: 6 corresponds to MPB70

MKVKNTIAATSFAAAGLAALAVAVSPPAAAGDLVGPGCAEYAAANPTGPASV

QGMSQDPVAVAASNNPELTTLTAALSGQLNPQVNLVDTLNSGQYTVFAPTNA

AFSKLPASTIDELKTNSSLLTSILTYHVVAGQTSPANVVGTRQTLQGASVTV

TGQGNSLKVGNADVVCGGVSTANATVYMIDSVLMPPA

SEQ ID NO: 7 corresponds to PEP1(MPB7O):

<u>KGSGS</u> 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 (CFP1O):

AEMKTDAATLAQEAGNFERISGDLKTQIDQVESTAGSLQGQWRGAAGTAAQA

AVVRFQEAANKQKQELDEISTNIRQAGVQYSRADEEQQQALSSQMGF

SEQ ID NO: 11 corresponds to PEP4 (CFP1O)

<u>KGSGS</u> MAEMKTDAATLAQEAGN

SEQ ID NO: 12 corresponds to PEP9 (CFP1O)

<u>KGSGS</u> NIRQAGVQYSRADEEQQQ

SEQ ID NO: 13 corresponds to PEP 17 (CFP1O)

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 Rv1573:
MTTTPARFNHLVTVTDLETGDRAVCDRDQVAETIRAWFPDAPLEVREALVRL
QAALNRHEHTGELEAFLRISVEHADAAGGDECGPAILAGRSGPEQAAINRQL
GLAGDDEPDGDDTPPWSRMIGLGGGSPAEDER

SEQ ID NO: 15 corresponds to Rv1580c:
MAETPDHAELRRRIADMAFNADVGMATCKRCGDAVPYI ILPNLQTGEPVMGV
ADNKWKRANCPVDVGKPCPFLIAEGVADSTDDTIEVDQ

SEQ ID NO: 16 corresponds to Rv1585c:
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
PEP1 (MPB70) (iv) an MPB83 antigen, and (v) at least
one of (a) a CFP1O 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:

5   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

10   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;

15   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.

20   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

25   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.

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

13

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 CFP1O 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 CFP1O 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 .

17

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,
5   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
10   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
15   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
20   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 .

25   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
30   tuberculosis infection in the cervid.

18

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

5   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.

10

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

15   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

20   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

25   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) .

30   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

20

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

22

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

5   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
10   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 .
15
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
20   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.

25   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
30   PEPl (MPB70), MPB83 antigen, and CFPlO antigen
and/or ESAT 6 antigen.

23

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.

5   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

10   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

15   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

20   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

25   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

30   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
CFP1O 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 PEP1 (MPB70), an MPB83
antigen, a CFP1O 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 CFP1O 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
Rv1573 antigen, an antibody molecule with binding
specificity for an Rv1580c antigen, and an antibody
molecule with binding specificity for an Rv1585c
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*.

28

Detailed Description

Polypeptides , Variants and Fragments

5   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
10   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
15   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
20   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
25   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
30   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

5   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,

10   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

15   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.

20   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

25   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).

30   Other amino acid substitutions may include but are

not limited to Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr,

31

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 NO: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

32

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 pep1 (MPB70) together, pep1 (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
5   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
10   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
15   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,
20   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
25   improve stability and reduce immunogenicity of the
antibody.

The antibody molecules for use in the present
invention extend, for example, to any other antibody
30   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.

5   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

10   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

15   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).

20

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

25   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

30   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.

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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
5   sample.

Immunochips may used to determine the presence of
the specific Mycobacterium antigens. Generally, the
specific antibodies to the antigens are immobilised
10   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
15   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
20   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
25   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
30   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

5 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

10 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

15 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 .

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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

25 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

30 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

5   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

10   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

15   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.

20   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

25   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

30   determining the amount of visually detectable signal at the capture region, the presence of analyte in a

44

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.

45

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
5   antigens measured simultaneously. Such a technique
therefore enables the rapid analysis of infection
with, for example, tuberculosis, in a large number
of animals simultaneously.

10   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
15   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.
20

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 :
25   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 PEP1 (MPB70) (iv) an MPB83 antigen, and (v)
30   at least one of (a) a CFP1O 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,
5   wherein the determination of binding is indicative
of the presence of tuberculosis infection in the
animal .

In one embodiment, the substrate is a microplate.
10   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
15   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.

20   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
25   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
30   molecule with binding specificity for an MPB70
linear epitope, for example PEPl (MPB70) (iv) an
antibody molecule with binding specificity for an

47

MPB83 antigen, and (v) at least one of (a) an
antibody molecule with binding specificity for a
CFP10 antigen and (b) an antibody molecule with
binding specificity for an ESAT 6 antigen;

5   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,

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

In one embodiment, the substrate is a microplate.

15   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

20   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 PEP1 (MPB70) (iv)
an antibody molecule with binding specificity for an

25   MPB83 antigen, and (v) at least one of (a) an
antibody molecule with binding specificity for a
CFP10 antigen and (b) an antibody molecule with
binding specificity for an ESAT 6 antigen..

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

48

Vaccines

As described above, the present invention also
extends to vaccines for use in protecting against
5   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.

10

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
15   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
20   expressing the polypeptides according to the
invention .

Vaccines described above all contribute to active
vaccination, i.e. the host's immune system is
25   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.
30   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
5   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
10   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
15   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
20   encoding a polypeptide as used in the invention or
immunogenic fragments thereof, and to vaccines
comprising DNA fragments that comprise such nucleic
acid sequences.

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

DNA vaccines can easily be administered through
30   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

5   adjuvant.

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

10   **Example 1**

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

15   **Methodology Multiplex Assays**
     Serum samples

Serum samples used in the study were obtained from several sources. Blood samples were taken into

20   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

25   -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

30   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 -20^0c. Antigen quantification was

5   performed using the micro BCA™ protein assay kit (Pierce, Rockford, IL).

Multiplex Antigen printing

Antigens used in the study were printed in each well

10   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

15   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

20   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

25   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

30   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

53

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

54

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).

55

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

5 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

10 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

15

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

20 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

25 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.

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

56

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.

58

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

5   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%.

10   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

15   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%.

20   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

25   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%.

30   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

62

SEQ ID NO: 6 - MPB70

MKVKNTIAATSFAAAGLAALAVAVSPPAAAGDLVGPGCAEYAAANPTGPASV
QGMSQDPVAVAASNNPELTTLTAALSGQLNPQVNLVDTLNSGQYTVFAPTNA
AFSKLPASTIDELKTNSSLLTSILTYHVVAGQTSPANVVGTRQTLQGASVTV
TGQGNSLKVGNADVVCGGVSTANATVYMIDSVLMPPA

SEQ ID NO: 7 - PEP1(MPB7O):
<u>KGSGS</u> SVQGMSQDPVAVAASNNPEL

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

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

SEQ ID NO: 10 - Rv3874 (CFP1O):
AEMKTDAATLAQEAGNFERISGDLKTQIDQVESTAGSLQGQWRGAAGTAAQA
AVVRFQEAANKQKQELDEISTNIRQAGVQYSRADEEQQQALSSQMGF

SEQ ID NO: 11 - PEP4 (CFP1O)
<u>KGSGS</u> MAEMKTDAATLAQEAGN

SEQ ID NO: 12 - PEP9 (CFP1O)
<u>KGSGS</u> NIRQAGVQYSRADEEQQQ

SEQ ID NO: 13 - PEP 17 (CFP1O)

63

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

64

Claims

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

5   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

10   consisting of (i)an Rv3616 antigen, (ii) an MPB70
antigen, (iii) an MPB70 linear epitope, for example
PEP1 (MPB70) (iv) an MPB83 antigen, and (v) at least
one of (a) a CFP1O antigen and (b) an ESAT 6
antigen;

15   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.

20   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

25   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

30   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

5   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

10 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

15 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.

20 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.

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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,

30 wherein said method further comprises determining the presence or absence of an immune response to a CFP-10 antigen.

67

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 PEP1 (MPB70) (iv) an MPB83 antigen, and (v) at least one of (a) a CFP1O 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,
5   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.

10   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.

15   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
20   specificity for an MPB70 antigen, and an antibody
molecule with binding specificity for an MPB83
antigen .

27.   The diagnostic kit according to claim 25
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
30   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.

71

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

5   37.   The vaccine according to claim 35 further comprising a CFP1O antigen.

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

10   antigen.

39.   A vaccine comprising (i)an Rv3616 antigen, (ii) an MPB70 antigen, (iii) an MPB70 linear epitope, for example PEP1 (MPB70) (iv) an MPB83 antigen, and (v)

15   at least one of (a) a CFP1O antigen and (b) an ESAT 6 antigen.

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

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

25   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

30   presence of tuberculosis infection in the suidae mammal .

72

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
5   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
10   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.

15

	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

[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 Rijswijk Tel (+31-70) 340-2040, Fax (+31-70) 340-3016	Hohwy, Morten

Form PCT/ISA/210 (second sheet) (April 2005)

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 fusi on protein-based i ndi rect enzyme-l inked immunosorbent assay for the detection of bovine tubercul osi s" TUBERCULOSIS, ELSEVIER, GB LNKD-DOI : 10 . 1016/ J .TUBE .2006 .07 .007 , vol . 87, no. 3, 11 Apri l 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-fl ow test for tubercul osi s i n 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 i n reindeer (Rangifer tarandus) infected with Mycobacterium bovi s " 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, col umn 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 bovi s . " INFECTION AND IMMUNITY APR 1998 LNKD-PUBMED: 9529066, vol . 66, no. 4, Apri l 1998 (1998-04) , pages 1445-1452, XP002579405 ISSN : 0019-9567 figures 5,6,8 _____	1-9, 17-24, 39-41 ,44

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.
           ---

   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.
           ---

   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

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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