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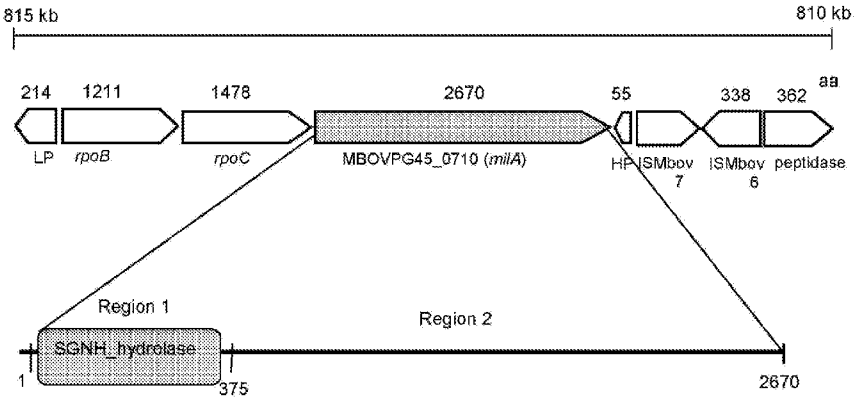

Figure 5

(57) Abstract: The present specification teaches a serological test for *Mycoplasma* and the development of prophylactic and therapeutic compositions to treat ruminant subjects infected with or potentially exposed to *Mycoplasma* species.

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SEROLOGICAL TEST

FILING DATA

[0001] This application is associated with and claims priority from Australian Provisional Patent Application No. 2013903549, filed on 16 September 2013, entitled "A serological test" and Australian Provisional Patent Application No. 2014902094, filed on 2 June 2014, entitled "A serological test - II, the entire contents of which, are incorporated herein by reference.

BACKGROUND

FIELD

[0002] The present specification teaches a serological test for *Mycoplasma* and the development of prophylactic and therapeutic compositions to treat ruminant subjects infected with or potentially exposed to *Mycoplasma* species.

DESCRIPTION OF PRIOR ART

[0003] Bibliographic details of the publications referred to by author in this specification are collected alphabetically at the end of the description.

[0004] Reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that this prior art forms part of the common general knowledge in any country.

[0005] *Mycoplasma bovis* is the most frequently isolated aetiological agent in naturally occurring outbreaks of bovine respiratory disease (BRD) in veal calves (Gagea *et al.* (2006) *J. Vet. Diagn. Invest. 18*:29-40; Arcangioli *et al.* (2008) *Vet. J. 177*:89-93), a

chronic disease characterized by pneumonia and arthritis. *Mycoplasma bovis* is also responsible for outbreaks of keratoconjunctivitis (Alberti *et al.* (2006) *J. Vet. Diagn,. Invest. 18*:41-51), mastitis (Hale *et al.* (1962) *Cornell Vet. 52*:193-200), meningitis (Stipovits *et al.* (1993) *Acta Vet. Hung. 41*:73-88), otitis media (Walz *et al.* (1997) *J. Vet. Diagn. Invest. 9*:250-254) and genital tract disease (LaFaunce and McEntee (1982) *Cornell Vet. 72*:160-167).

[0006] The significance of respiratory tract infection with *M. bovis* has become increasingly apparent in recent years because of greater recognition of its role in pneumonia in many parts of the world (Nicholas *et al.* (2002) *Vaccine 20*:3569-3575), and due to its increasing resistance to antimicrobial drugs (Ayling *et al.* (2000) *Vet. Rec. 146*:745-747). In the absence of an effective vaccine or antimicrobial therapy, an alternative strategy to controlling *M. bovis* infection in cattle is to separate infected from uninfected cattle (Pfuetzner (1996) *Rev. Sci. Tech. Off. Int. Epiz. 15*:1477). Currently *M. bovis* diagnostic methods, including culture (Sachse *et al.* (1993) *Rev. Sci. Tech. Off. Int. Epiz. 12*:571-580), polymerase chain reaction assays (González *et al.* (1995) *Vet. Microbiol. 47*:183-190; Ayling *et al.* (1997) *Vet. Rec. 141*:307-308) and antigen capture ELISAs (Heller *et al.* (1993) *Vet. Microbiol. 37*:127-133; Ball *et al.* (1994b) *IR. Vet. J. 47*:45), have not completely fulfilled the need for a rapid diagnostic test to detect infection, and there is a clear need for a sensitive and specific serological assay based on a species-specific immunologically reactive antigen.

[0007] Published genome sequences of several *M. bovis* strains have contributed to the identification of novel antigenic proteins. These include strain *Hubei-1* Li *et al.* (2011) PLoS *ONE 6*:e20999 and strain PG45 Wise *et al.* (2011) *Infect. Immun. 79*:982-983. . Most effort thus far has been concentrated on identifying and characterising the variable surface proteins (Vsps) [Behrens *et al.* (1994) *Infect. Immun. 62*:5075-5084; Poumarat *et al. FEMS Microbiol. Lett. 173*:103-110]. Apart from the Vsps, only a few antigenic proteins of *M. bovis* have been identified and characterized, including Hsp60 and P48 (Scherm *et al.* (2002) *Vet. Microbiol. 89*:141-150; Robino *et al.* (2005) *Vet. Microbiol. 109*:201-209). However, these are not ideal antigens for immunodiagnosis, because of the

phase and antigenic variation seen in Vsps, and the similarities between Hsp60 and P48 and their orthologs in the closely related species such as *M. agalactiae*. Therefore, there remains a need to identify a better *Mycoplasma* antigen for serological diagnosis.

SUMMARY

[0008] An immunogenic protein is identified useful in the identification of *Mycoplasma* spp and in the development of prophylactic and therapeutic compositions to manage *Mycoplasma* infection and exposure. The immunogenic protein is referred to herein as *Mycoplasma* immunogenic lipase A (MilA). Reference to "MilA" includes the MilA protein from *Mycoplasma bovis* and homologs of MilA from other *Mycoplasma* spp as well as antibody-binding fragments of MilA. Region 1 of MilA is defined by the consensus sequence set forth in SEQ ID NO:1. Examples of region 1 of MilA include MilA from *M. bovis* PG45 strain (type strain) [SEQ ID NO:2], and MilA homologs from *M. bovis* strain Hubei-1 (SEQ ID NO:3), *Mycoplasma agalactiae* strain PG2 (SEQ ID NO:4), *Mycoplasma columbinium* strain SF7 (SEQ ID NO:5), *Mycoplasma fermantans* strain JER (SEQ ID NO:6), *Mycoplasma pulmonis* (SEQ ID NO:7) and *Mycoplasma hyopneumoniae* (SEQ ID NO:8). Full length MilA is defined in SEQ ID NO:5 (*M bovis* PG45), SEQ ID NO:16 (*M. bovis* Hubei-1), SEQ ID NO:17 (*M. agalactiae* PG2), SEQ ID NO:18 (*M. columbinia* SF7), SEQ ID NO:19 (*M. fermantans* JER), SEQ ID NO:20 (*M. pulmonis*) and SEQ ID NO:21 (*M. hyopneumoniae*). The nucleotide sequence encoding MilA optimized for expression in *E. coli* is set forth in SEQ ID NO:13. The genomic nucleotide sequence from *M. bovis* PG45 is set forth in SEQ ID NO:14.

[0009] Enabled herein is a method for detecting current or prior exposure of a ruminant subject to a species of *Mycoplasma*, the method comprising contacting an antibody containing sample from the subject with an immobilized protein or derivative thereof comprising the amino acid sequence set forth in SEQ ID NO:1 or an amino acid sequence having at least 40% similarity to SEQ ID NO:1 after optimal alignment or an antibody-binding fragment of the protein, for a time and under conditions sufficient for an antibody specific for the protein, if present in the sample, to bind to the protein and then detecting for the presence of bound antibody wherein the presence of bound antibody is indicative of current or prior exposure to *Mycoplasma* spp.

[0010] Further enabled is a method for detecting current or prior exposure of a

ruminant subject to a species of *Mycoplasma*, the method comprising contacting an antibody containing sample from the subject with an immobilized protein or derivative thereof comprising the amino acid sequence set forth in SEQ ID NO:2 or an amino acid sequence having at least 40% similarity to SEQ ID NO:2 after optimal alignment or an antibody-binding fragment of the protein, for a time and under conditions sufficient for an antibody specific for the protein, if present in the sample, to bind to the protein and then detecting for the presence of bound antibody wherein the presence of bound antibody is indicative of current or prior exposure to *Mycoplasma* spp.

[0011] Taught herein is a method for detecting current or prior exposure of a ruminant subject to a species of *Mycoplasma*, the method comprising contacting an antibody containing sample from the subject with an immobilized protein or derivative thereof comprising the amino acid sequence set forth in SEQ ID NO:15 or an amino acid sequence having at least 40% similarity to SEQ ID NO:15 after optimal alignment or an antibody-binding fragment of the protein, for a time and under conditions sufficient for an antibody specific for the protein, if present in the sample, to bind to the protein and then detecting for the presence of bound antibody wherein the presence of bound antibody is indicative of current or prior exposure to *Mycoplasma* spp.

[0012] The present specification teaches a device for screening for current or prior exposure of a subject to a species of *Mycoplasma*, the device comprising an immobilized protein or derivative thereof comprising the amino acid sequence set forth in SEQ ID NO:1 or an amino acid sequence having at least 40% similarity to SEQ ID NO:1 after optimal alignment or an antibody-binding fragment of the protein, the device further comprising means to contact a sample from the subject with the immobilized protein.

[0013] Enabled herein is a device for screening for current or prior exposure of a subject to a species of *Mycoplasma*, the device comprising an immobilized protein or derivative thereof comprising the amino acid sequence set forth in SEQ ID NO:2 or an amino acid sequence having at least 40% similarity to SEQ ID NO:2 after optimal alignment or an antibody-binding fragment of the protein, the device further comprising

means to contact a sample from the subject with the immobilized protein.

[0014] Further enabled herein is a device for screening for current or prior exposure of a subject to a species of *Mycoplasma*, the device comprising an immobilized protein or derivative thereof comprising the amino acid sequence set forth in SEQ ID NO:15 or an amino acid sequence having at least 40% similarity to SEQ ID NO:15 after optimal alignment or an antibody-binding fragment of the protein, the device further comprising means to contact a sample from the subject with the immobilized protein.

[0015] Taught herein is a method for controlling infection by a species of *Mycoplasma* in a ruminant subject, the method comprising administering to the subject an antibody-inducing effective amount of a protein or derivative thereof comprising the amino acid sequence set forth in SEQ ID NO:1 or an amino acid sequence having at least 40% similarity to SEQ ID NO:1 after optimal alignment for a time and under conditions sufficient to generate antibodies to said protein.

[0016] Enabled herein is a method for controlling infection by a species of *Mycoplasma* or a condition caused or exacerbated by infection with *Mycoplasma* in a ruminant subject, the method comprising administering to the subject an antibody-inducing effective amount of a protein or derivative thereof comprising the amino acid sequence set forth in SEQ ID NO:2 or an amino acid sequence having at least 40% similarity to SEQ ID NO:2 after optimal alignment for a time and under conditions sufficient to generate antibodies to the protein. Infection by *Mycoplasma* spp can lead to bovine respiratory disease, keratoconjunctivits, mastitis, meningitis, otitis media, and genital tract disease.

[0017] Taught herein is a method for controlling infection by a species of *Mycoplasma* or a condition caused or exacerbated by infection with *Mycoplasma* in a ruminant subject, the method comprising administering to the subject an antibody-inducing effective amount of a protein or derivative thereof comprising the amino acid sequence set forth in SEQ ID NO:15 or an amino acid sequence having at least 40% similarity to SEQ ID NO:15 after optimal alignment for a time and under conditions sufficient to generate antibodies to the

protein. Infection by *Mycoplasma* spp can lead to bovine respiratory disease, keratoconjunctivits, mastitis, meningitis, otitis media, and genital tract disease.

[0018] The present specification is instructional on a composition comprising a protein or derivative thereof comprising the amino acid sequence set forth in SEQ ID NO:1 or an amino acid sequence having at least 40% similarity to SEQ ID NO:1 after optimal alignment and one or more pharmaceutically acceptable carriers, excipients and/or diluents.

[0019] Enabled herein is a composition comprising a protein or derivative thereof comprising the amino acid sequence set forth in SEQ ID NO:2 or an amino acid sequence having at least 40% similarity to SEQ ID NO:2 after optimal alignment and one or more pharmaceutically acceptable carriers, excipients and/or diluents.

[0020] Taught herein is a composition comprising a protein or derivative thereof comprising the amino acid sequence set forth in SEQ ID NO:15 or an amino acid sequence having at least 40% similarity to SEQ ID NO:15 after optimal alignment and one or more pharmaceutically acceptable carriers, excipients and/or diluents.

[0021] Enabled herein is the use of the above compositions in the manufacture of a medicament for the treatment or prophylaxis of *Mycoplasma* infection or a condition caused or exacerbated by *Mycoplasma* infection in a ruminant subject.

[0022] In an embodiment, the protein comprises an amino acid sequence selected from the listing consisting of SEQ ID NOs:2 through 8 or is an antibody-binding fragment thereof or comprises an amino acid sequence having at least 40% similarity to any one of SEQ ID NOs:2 through 8 after optimal alignment.

[0023] In an embodiment, the protein comprises an amino acid sequence selected from the listing consisting of SEQ ID NOs:15 through 21 or is an antibody-binding fragment thereof or comprises an amino acid sequence having at least 40% similarity to any one of

SEQ ID NOs:15 through 21 after optimal alignment.

[0024] Enabled herein is the method wherein the protein comprises the amino acid sequence set forth in SEQ ID NO:2 or is an antibody-binding fragment thereof.

[0025] Enabled herein is the method wherein the protein comprises the amino acid sequence set forth in SEQ ID NO:15 through 21 or is an antibody-binding fragment thereof.

[0026] In an embodiment, the ruminant animal is selected from the list consisting of cow, sheep, goat, giraffe, yak, camel, llama, antelope and macropod. In an embodiment, the *Mycoplasma* is *Mycoplasma bovis* and the ruminant animal is a cow.

[0027] Nucleotide and amino acid sequences are referred to by a sequence identifier number (SEQ ID NO). The SEQ ID NOs correspond numerically to the sequence identifiers <400>1 (SEQ ID NO:1), <400>2 (SEQ ID NO:2), etc. A summary of the sequence identifiers is provided in Table 1. A sequence listing is provided after the claims.

[0028] A summary of sequence identifiers used throughout the subject specification is provided in Table 1.

Table 1

Summary of sequence identifiers

SEQUENCE ID NO:	DESCRIPTION
1	Consensus amino acid sequence of region 1 of MilA from *M. bovis*
2	Amino acid sequence of region 1 of MilA from *M. bovis* (type strain)
3	Amino acid sequence of region 1 of MilA homolog from *M. bovis* strain Hubei-1
4	Amino acid sequence of region 1 of MilA homolog from *M. agalactiae* strain PG2
5	Amino acid sequence of region 1 of MilA homolog from *M. columbinium* strain SF7
6	Amino acid sequence of region 1 of MilA homolog from *M. fermantans* strain JER
7	Amino acid sequence of region 1 of MilA homolog from *M. pulmonis*
8	Amino acid sequence of region 1 of MilA homolog from *M. hyopneumoniae*
9	Restriction endonuclease cleavage sites: abF-*Bam*HI, CAACggatccATCAAAGACGTGA (binding site, 144 – 166)
10	Restriction endonuclease cleavage sites: abR-*Sal*I, TTCgtcgacGGATTTCGCCT (1931 – 1950)
11	Restriction endonuclease cleavage sites: cdF-*Bam*H1, CACTGggatccATCTCGAACATC (3128 – 3150)
12	Restriction endonuclease cleavage sites: cdR-*Sal*I, ACAgtcgacCCAGGTTCG (4955 – 4872)
13	Nucleotide sequence encoding *MilA-Aβ* fragment optimized for expression in *E. coli*
14	Genomic sequence of MilA from *M. bovis* PG45 (type strain)
15	Amino acid sequence of MilA from *M. bovis* PG45 (type strain)

SEQUENCE ID NO:	DESCRIPTION
16	Amino acid sequence of MilA homolog from *M. bovis* strain Hubei-1
17	Amino acid sequence of MilA homolog from *M. agalactiae* strain PG2
18	Amino acid sequence of MilA homolog from *M. columbinia* strain SF7
19	Amino acid sequence of MilA homolog from *M. fermantans* strain JER
20	Amino acid sequence of MilA homolog from *M. pulmonis*
21	Amino acid sequence of MilA homolog from *M. hyopneumoniae*
22	Nucleotide sequence encoding MilA-CD fragment optimized from expression in *E. coli*
23	Nucleotide sequence of pGEX fwd
24	Nucleotide sequence of pGEX rev
25	Nucleotide sequence of cloning site of pGEX
26	Amino acid sequence of cloning site of pGEX
27	Nucleotide sequence encoding *M. bovis* strain Hubei-1 MilA

[0029] A list of abbreviations used throughout the subject specification are provided in Table 2.

Table 2

Abbreviations

ABBREVIATION	DESCRIPTION
ABTS	2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
BRD	Bovine respiratory disease
DAB	3,3'-Diaminobenzidine
ELISA	Enzyme linked immunosorbent assay
HRP	Horse radish peroxidase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
MilA	Mycoplasma immunogenic lipase A
MilA	Gene encoding MilA
PBS	Phosphate buffered saline
PBS-T	PBS comprising 0.05% v/v Tween 20
Spp	Species
Vsp	Variable surface protein

BRIEF DESCRIPTION OF THE FIGURES

[0030] **Figure 1** is a diagrammatical representation of a 6 kbp region of MBOVPG45_710 (MilA) gene cloned as two 3 kbp fragments, AB and CD, and subcloned as four 1.5 kbp DNA fragments, A, B, C and D, with the expressed gene sequences bounded by restriction endonuclease cleavage sites. In addition, two 2 kbp DNA fragments were amplified using primers containing appropriate restriction endonuclease cleavage sites: abF-*Bam*HI, CAACggatccATCAAAGACGTGA (binding site, 144 – 166) [SEQ ID NO:9]; abR-*Sal*I, TTCgtcgacGGATTTCGCCT (1931 – 1950) [SEQ ID NO:10]; cdF-*Bam*H1, CACTGggatccATCTCGAACATC (3128 – 3150) [SEQ ID NO:11]; and cdR-*Sal*I, ACAgtcgacCCAGGTTCG (4955 – 4872) [SEQ ID NO:12].

[0031] **Figure 2** is a photographical representation of Western blots of whole cell proteins of *M. bovis* strain 3683 probed with sera from 6 calves (A-F) collected on: 1, day 0; 2, day 24. Uninfected, calves inoculated with Mycoplasma culture medium; Infected, calves inoculated with *M. bovis* strain 3683; Arrow, protein of interest seen in strip 2 in infected calves.

[0032] **Figure 3** is a photographical representation of SDS-PAGE and Western blot of MilA (226 kDa). Lanes: 1, Spectra (Trade Mark) Multicolor high range protein ladder (Fermentas); 2, whole cell proteins of *M. bovis* strain 3683 stained with Coomassie blue; 3, whole cell proteins of *M. agalactiae* strain PG2 stained with Coomassie blue; 4, Western blot of whole cell proteins of *M. bovis* strain 3683 probed with *M. bovis*-specific calf sera; 5, Western blot of whole cell proteins of *M. agalactiae* strain PG2 probed with *M. bovis*-specific calf sera.

[0033] **Figures 4(i) and (ii)** are photographical representations showing expression of recombinant proteins in *E. coli* JM 109 cells. (i) Whole cell proteins of induced clones separated in a 12% w/v polyacrylamide gel stained with Coomassie blue, (ii) transferred to a PVDF membrane and probed with pooled *M. bovis*-specific bovine sera. Lanes: 1, PageRuler prestained protein ladder (Thermo Scientific); 2, pGEX-4T-1-*milA*-AB induced

(125.5 kDa); 3, pGEX-4T-1-*milA*-CD induced (127 kDa); 4, pGEX-4T-1-*milA*-A induced (72.8 kDa); 5, pGEX-4T-1-*milA*-B induced (69 kDa); 6, pGEX-4T-1-*milA*-C induced (71.9 kDa); 7, pGEX-4T-1-*milA*-D induced (73.3 kDa); 8, pGEX-4T-1-*milA*-cd induced (94.3 kDa); 9, pGEX-4T-1-*milA*-ab induced (92.9 kDa).

[0034] **Figure 5** is a diagrammatical representation showing physical map of genes in the region surrounding MBOVPG45_0710 (*milA*). The predicted size of the gene product is shown as the number of amino acids (aa). LP, lipoprotein; *rpoB*, DNA-directed RNA polymerase subunit beta; *rpoC*, DNA-directed RNA polymerase subunit beta; MBOVPG45_0710 (*milA*), membrane protein; HP, hypothetical protein; ISMbov 7, transposase truncated protein; ISMbov 6, M42 family glutamyl aminopeptidase.

[0035] **Figure 6** is a diagrammatical representation showing multiple sequence alignment of region 1 of mycoplasma immunogenic lipase (MilA) with homologs in other mycoplasmas and the mycoplasma GDSL carboxyesterases.

[0036] Numbers on the right indicate the position of the adjacent amino acid. Identical amino acids are shaded black. Grey shading indicates at least one substitution with a very similar amino acid. Light grey shading indicates at least one substitution with a similar amino acid. Dashed lines indicate gaps in the amino acid sequence alignment. GDSL-like lipase conserved sequence blocks are underlined. Asterisks (*) indicate the active site residues and the arrowheads indicate the conserved amino acids of the SGNH_hydrolase family. The sequences included in the alignment are: MBOVPG45_0710 (MilA), *M. bovis* strain PG45; MMB_0654, *M. bovis* strain Hubei-1; MAGa6830, *M. agalactiae* PG2; MCSF7_01871, *M. columbinum* SF7; MFE_02570, *M. fermantans* JER; MYPU3130, *M. pulmonis*; and mhp677, *M. hyopneumoniae*.

[0037] **Figure 7** is a graphical representation showing release of resorufin by the lipase activity of GST-MilA-ab and GST-p65 over 30 min, with GST as the negative control.

[0038] **Figures 8(i) and (ii)** are graphical representations showing Log_{10} IgG antibody titers of individual calves on day (i) 17 and (ii) day 24 after infection in Experiment 1. The bar indicates the mean of each group.

[0039] **Figures 9(i) and (ii)** are graphical representations showing Log_{10} IgG antibody titers of individual calves on (i) day 17 and (ii) day 24 after infection in Experiment 2. The bar indicates the mean of each group.

[0040] **Figure 10** is a graphical representation of mean *M. bovis*-specific IgM antibody titers of each group in Experiment 3. Bars indicate standard deviation.

[0041] **Figure 11** is a graphical representation of mean *M. bovis*-specific IgM antibody titers of each group in Experiment 3. Bars indicate standard deviation.

[0042] **Figure 12** is a schematic diagram of the plasmid vector pGEX-4T-1 (reproduced from the GE Healthcare pGEX vector map).

[0043] **Figure 13** is a representation of physical maps of mycoplasma homologues of MBOVPG45_710 (*milA*) and adjacent genes. Homologous proteins are shaded the same colour. Numbers above the genes indicate the length of the predicted product in amino acids. MBOVPG45_710 (*milA*), membrane protein; MMB_0654, conserved hypothetical transmembrane protein; MAGa6830, conserved hypothetical protein; MAG_6100, conserved hypothetical protein; MMB_0318, conserved hypothetical protein; MFE_02570/MfeM64YM_0307, lipase/hypothetical protein; MCSF7_01871, lipase; HP, hypothetical protein; LP, lipoprotein; AP, aminopeptidase; *rpoB*, DNA-directed RNA polymerase subunit beta; *rpoC*, DNA-directed RNA polymerase subunit beta; *celM1*, endo-1,4-beta-glucanase; *topA*, DNA topoisomerase; *uvrA*, uvrABC system protein A; *vpmaY1*, variable surface lipoprotein Y; LicA, lichenan-specific IIA component.

[0044] **Figure 14** is a graphical representation of *M. bovis*-specific IgG ELISA results for sera from feedlot cattle.

DETAILED DESCRIPTION

[0045] Throughout this specification, unless the context requires otherwise, the word "comprise" or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or method step or group of elements or integers or method steps but not the exclusion of any element or integer or method step or group of elements or integers or method steps.

[0046] As used in the subject specification, the singular forms "a", "an" and "the" include plural aspects unless the context clearly dictates otherwise. Thus, for example, reference to "a *Mycoplasma* " includes a single *Mycoplasma* cell, as well as two or more *Mycoplasmas* cells; reference to "an antibody" includes a single antibody, as well as two or more antibodies; reference to "the disclosure" includes a single reference to "the disclosure" includes a single and multiple aspects taught by the disclosure; and so forth. Aspects taught and enabled herein are encompassed by the term "invention". All such aspects are enabled within the width of the present invention.

[0047] In an embodiment, the instant specification enables an isolated protein or an antibody-binding fragment thereof comprising an amino acid sequence set forth in SEQ ID NO:1 or an amino acid sequence having at least 40% similarity to SEQ ID NO:1 after optimal alignment.

[0048] Reference to "at least 40%" means 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 and 100% similarity to a recited sequence identifier.

[0049] In an embodiment, the protein or a derivative thereof is from *Mycoplasma bovis* type strain PG45 and comprises the amino acid sequence as set forth in SEQ ID NO:2 or an amino acid sequence having at least 40% similarity to SEQ ID NO:2 after optimal alignment.

[0050] In an embodiment, the protein or a derivative thereof is from *Mycoplasma bovis* type strain PG45 and comprises the amino acid sequence as set forth in SEQ ID NO:15 or an amino acid sequence having at least 40% similarity to SEQ ID NO:15 after optimal alignment.

[0051] In an embodiment, the protein or a derivative thereof is from *Mycoplasma bovis* strain Hubei-1 and comprises amino acid sequence as set forth in SEQ ID NO:3 or an amino acid sequence having at least 40% similarity to SEQ ID NO:3 after optimal alignment.

[0052] In an embodiment, the protein or a derivative thereof is from *Mycoplasma agalactiae* strain PG2 and comprises amino acid sequence as set forth in SEQ ID NO:4 or an amino acid sequence having at least 40% similarity to SEQ ID NO:4 after optimal alignment.

[0053] In an embodiment, the protein or a derivative thereof is from *Mycoplasma columbinium* strain SF7 and comprises amino acid sequence as set forth in SEQ ID NO:5 or an amino acid sequence having at least 40% similarity to SEQ ID NO:5 after optimal alignment.

[0054] In an embodiment, the protein or a derivative thereof is from *Mycoplasma fermantans* strain JER and comprises amino acid sequence as set forth in SEQ ID NO:6 or an amino acid sequence having at least 40% similarity to SEQ ID NO:6 after optimal alignment.

[0055] In an embodiment, the protein or a derivative thereof is from *Mycoplasma pulmonis* and comprises amino acid sequence as set forth in SEQ ID NO:7 or an amino acid sequence having at least 40% similarity to SEQ ID NO:7 after optimal alignment.

[0056] In an embodiment, the protein or a derivative thereof is from *Mycoplasma*

hyopneumoniae and comprises amino acid sequence as set forth in SEQ ID NO:8 or an amino acid sequence having at least 40% similarity to SEQ ID NO:8 after optimal alignment.

[0057] In an embodiment, the protein or a derivative thereof is from *Mycoplasma hyopneumoniae* and comprises amino acid sequence as set forth in SEQ ID NO:16 or an amino acid sequence having at least 40% similarity to SEQ ID NO:16 after optimal alignment.

[0058] In an embodiment, the protein or a derivative thereof is from *Mycoplasma hyopneumoniae* and comprises amino acid sequence as set forth in SEQ ID NO:17 or an amino acid sequence having at least 40% similarity to SEQ ID NO:17 after optimal alignment.

[0059] In an embodiment, the protein or a derivative thereof is from *Mycoplasma hyopneumoniae* and comprises amino acid sequence as set forth in SEQ ID NO:18 or an amino acid sequence having at least 40% similarity to SEQ ID NO:18 after optimal alignment.

[0060] In an embodiment, the protein or a derivative thereof is from *Mycoplasma hyopneumoniae* and comprises amino acid sequence as set forth in SEQ ID NO:19 or an amino acid sequence having at least 40% similarity to SEQ ID NO:19 after optimal alignment.

[0061] In an embodiment, the protein or a derivative thereof is from *Mycoplasma hyopneumoniae* and comprises amino acid sequence as set forth in SEQ ID NO:20 or an amino acid sequence having at least 40% similarity to SEQ ID NO:20 after optimal alignment.

[0062] In an embodiment, the protein or a derivative thereof is from *Mycoplasma hyopneumoniae* and comprises amino acid sequence as set forth in SEQ ID NO:21 or an

amino acid sequence having at least 40% similarity to SEQ ID NO:21 after optimal alignment.

[0063] The present specification teaches an isolated protein comprising an amino acid sequence selected from the list consisting of SEQ ID NOs:1 through 8or 15 through 21.

[0064] Enabled herein is an isolated protein comprising an amino acid sequence set forth in SEQ ID NO:1.

[0065] Enabled herein is an isolated protein comprising an amino acid sequence set forth in SEQ ID NO:2.

[0066] Enabled herein is an isolated protein comprising an amino acid sequence set forth in SEQ ID NO:3.

[0067] Enabled herein is an isolated protein comprising an amino acid sequence set forth in SEQ ID NO:4.

[0068] Enabled herein is an isolated protein comprising an amino acid sequence set forth in SEQ ID NO:5.

[0069] Enabled herein is an isolated protein comprising an amino acid sequence set forth in SEQ ID NO:6.

[0070] Enabled herein is an isolated protein comprising an amino acid sequence set forth in SEQ ID NO:7.

[0071] Enabled herein is an isolated protein comprising an amino acid sequence set forth in SEQ ID NO:8.

[0072] Enabled herein is an isolated protein comprising an amino acid sequence set

forth in SEQ ID NO:15.

[0073] Enabled herein is an isolated protein comprising an amino acid sequence set forth in SEQ ID NO:16.

[0074] Enabled herein is an isolated protein comprising an amino acid sequence set forth in SEQ ID NO:17.

[0075] Enabled herein is an isolated protein comprising an amino acid sequence set forth in SEQ ID NO:18.

[0076] Enabled herein is an isolated protein comprising an amino acid sequence set forth in SEQ ID NO:19.

[0077] Enabled herein is an isolated protein comprising an amino acid sequence set forth in SEQ ID NO:20.

[0078] Enabled herein is an isolated protein comprising an amino acid sequence set forth in SEQ ID NO:21.

[0079] The present specification teaches the use of a protein or a derivative thereof having the amino acid sequence set forth in SEQ ID NOs:1 through 8 or 15 through 21 or an amino acid sequence with at least 40% similarity to any of SEQ ID NOs:1 through 8 or 15 through 21 in the manufacture of a diagnostic agent to screen for current or prior exposure of a subject to a species of *Mycoplasma*. The protein or derivative may also be used to screen for potential bovine respiratory disease, keratoconjunctivitis, mastitis, meningitis, otitis media and genital tract disease.

[0080] Hence, the present specification contemplates a method for detecting current or prior exposure of a ruminant subject to a species of *Mycoplasma*, the method comprising contacting an antibody containing sample from the subject with an immobilized protein or

derivative thereof comprising the amino acid sequence set forth in SEQ ID NO:1 or an amino acid sequence having at least 40% similarity to SEQ ID NO:1 after optimal alignment or an antibody-binding fragment of the protein, for a time and under conditions sufficient for an antibody specific for the protein, if present in the sample, to bind to the protein and then detecting for the presence of bound antibody wherein the presence of bound antibody is indicative of current or prior exposure to *Mycoplasma* spp.

[0081] The present specification further enables a method for detecting current or prior exposure of a ruminant subject to a species of *Mycoplasma*, the method comprising contacting an antibody containing sample from the subject with an immobilized protein or derivative thereof comprising the amino acid sequence set forth in SEQ ID NO:2 or an amino acid sequence having at least 40% similarity to SEQ ID NO:2 after optimal alignment or an antibody-binding fragment of the protein, for a time and under conditions sufficient for an antibody specific for the protein, if present in the sample, to bind to the protein and then detecting for the presence of bound antibody wherein the presence of bound antibody is indicative of current or prior exposure to *Mycoplasma* spp.

[0082] The present specification further enables a method for detecting current or prior exposure of a ruminant subject to a species of *Mycoplasma*, the method comprising contacting an antibody containing sample from the subject with an immobilized protein or derivative thereof comprising the amino acid sequence set forth in SEQ ID NO:15 or an amino acid sequence having at least 40% similarity to SEQ ID NO:15 after optimal alignment or an antibody-binding fragment of the protein, for a time and under conditions sufficient for an antibody specific for the protein, if present in the sample, to bind to the protein and then detecting for the presence of bound antibody wherein the presence of bound antibody is indicative of current or prior exposure to *Mycoplasma* spp.

[0083] In an embodiment, the protein comprises an amino acid sequence selected from the listing consisting of SEQ ID NOs2: through 8 or 15 through 21 or is an antibody-binding fragment thereof.

[0084] In an embodiment, the protein comprises the amino acid sequence set forth in SEQ ID NO:2 or an amino acid sequence having at least 40% similarity to SEQ ID NO:2 after optimal alignment or is an antibody-binding fragment thereof.

[0085] In an embodiment, the protein comprises the amino acid sequence set forth in SEQ ID NO:15 or an amino acid sequence having at least 40% similarity to SEQ ID NO:15 after optimal alignment or is an antibody-binding fragment thereof.

[0086] In an embodiment, the protein comprises the amino acid sequence set forth in SEQ ID NO:2 or is an antibody-binding fragment thereof.

[0087] In an embodiment, the protein comprises the amino acid sequence set forth in SEQ ID NO:15 or is an antibody-binding fragment thereof.

[0088] In an embodiment, the protein comprises the amino acid sequence set forth in SEQ ID NO:16 through 21 or is an antibody-binding fragment thereof.

[0089] Reference to a "ruminant subject" includes a cow, sheep, goat, giraffe, yak, camel, llama, antelope and macropod.

[0090] In an embodiment, the species of *Mycoplasma* is *M. bovis* and the ruminant subject is a cow.

[0091] A "derivative" of the protein includes a fragment of the protein such as antibody-binding fragment. Fragments include MilA-AB, MilA-CD, MilA-A, MilA-B, MilA-C, MilA-D, MilA-cd and MilA-ab. A "derivative" also includes a protein having a single or multiple amino acid substitution, deletion and/or addition to SEQ ID NO:1 or any one of SEQ ID NOs:2 through 8 or SEQ ID NOs:15 through 21. A "derivative" further includes the incorporation of an unnatural amino acid or chemical alteration of an amino acid side chain.

[0092] Examples of side chain modifications contemplated herein include modifications of amino groups such as by reductive alkylation by reaction with an aldehyde followed by reduction with $NaBH_4$; amidination with methylacetimidate; acylation with acetic anhydride; carbamoylation of amino groups with cyanate; trinitrobenzylation of amino groups with 2, 4, 6-trinitrobenzene sulphonic acid (TNBS); acylation of amino groups with succinic anhydride and tetrahydrophthalic anhydride; and pyridoxylation of lysine with pyridoxal-5-phosphate followed by reduction with $NaBH_4$.

[0093] The guanidine group of arginine residues may be modified by the formation of heterocyclic condensation products with reagents such as 2,3-butanedione, phenylglyoxal and glyoxal.

[0094] The carboxyl group may be modified by carbodiimide activation *via* O-acylisourea formation followed by subsequent derivitization, for example, to a corresponding amide.

[0095] Sulphydryl groups may be modified by methods such as carboxymethylation with iodoacetic acid or iodoacetamide; performic acid oxidation to cysteic acid; formation of a mixed disulphides with other thiol compounds; reaction with maleimide, maleic anhydride or other substituted maleimide; formation of mercurial derivatives using 4-chloromercuribenzoate, 4-chloromercuriphenylsulphonic acid, phenylmercury chloride, 2-chloromercuri-4-nitrophenol and other mercurials; carbamoylation with cyanate at alkaline pH.

[0096] Tryptophan residues may be modified by, for example, oxidation with N-bromosuccinimide or alkylation of the indole ring with 2-hydroxy-5-nitrobenzyl bromide or sulphenyl halides. Tyrosine residues on the other hand, may be altered by nitration with tetranitromethane to form a 3-nitrotyrosine derivative.

[0097] Modification of the imidazole ring of a histidine residue may be accomplished by alkylation with iodoacetic acid derivatives or N-carbethoxylation with

diethylpyrocarbonate.

[0098] Examples of incorporating unnatural amino acids and derivatives during peptide synthesis include, but are not limited to, use of norleucine, 4-amino butyric acid, 4-amino-3-hydroxy-5-phenylpentanoic acid, 6-aminohexanoic acid, t-butylglycine, norvaline, phenylglycine, ornithine, sarcosine, 4-amino-3-hydroxy-6-methylheptanoic acid, 2-thienyl alanine and/or D-isomers of amino acids. A list of unnatural amino acids, contemplated herein is shown in Table 3.

Table 3

Codes for non-conventional amino acids

Non-conventional amino acid	Code	Non-conventional amino acid	Code
α-aminobutyric acid	Abu	L-N-methylalanine	Nmala
α-amino-α-methylbutyrate	Mgabu	L-N-methylarginine	Nmarg
aminocyclopropane-carboxylate	Cpro	L-N-methylasparagine	Nmasn
		L-N-methylaspartic acid	Nmasp
aminoisobutyric acid	Aib	L-N-methylcysteine	Nmcys
aminonorbornyl-carboxylate	Norb	L-N-methylglutamine	Nmgln
		L-N-methylglutamic acid	Nmglu
cyclohexylalanine	Chexa	L-Nmethylhistidine	Nmhis
cyclopentylalanine	Cpen	L-N-methylisolleucine	Nmile
D-alanine	Dal	L-N-methylleucine	Nmleu
D-arginine	Darg	L-N-methyllysine	Nmlys
D-aspartic acid	Dasp	L-N-methylmethionine	Nmmet
D-cysteine	Dcys	L-N-methylnorleucine	Nmnle
D-glutamine	Dgln	L-N-methylnorvaline	Nmnva
D-glutamic acid	Dglu	L-N-methylornithine	Nmorn
D-histidine	Dhis	L-N-methylphenylalanine	Nmphe
D-isoleucine	Dile	L-N-methylproline	Nmpro
D-leucine	Dleu	L-N-methylserine	Nmser
D-lysine	Dlys	L-N-methylthreonine	Nmthr
D-methionine	Dmet	L-N-methyltryptophan	Nmtrp
D-ornithine	Dorn	L-N-methyltyrosine	Nmtyr
D-phenylalanine	Dphe	L-N-methylvaline	Nmval
D-proline	Dpro	L-N-methylethylglycine	Nmetg
D-serine	Dser	L-N-methyl-t-butylglycine	Nmtbug
D-threonine	Dthr	L-norleucine	Nle
D-tryptophan	Dtrp	L-norvaline	Nva
D-tyrosine	Dtyr	α-methyl-aminoisobutyrate	Maib
D-valine	Dval	α-methyl-γ-aminobutyrate	Mgabu
D-α-methylalanine	Dmala	α-methylcyclohexylalanine	Mchexa
D-α-methylarginine	Dmarg	α-methylcylcopentylalanine	Mcpen
D-α-methylasparagine	Dmasn	α-methyl-α-napthylalanine	Manap
D-α-methylaspartate	Dmasp	α-methylpenicillamine	Mpen
D-α-methylcysteine	Dmcys	N-(4-aminobutyl)glycine	Nglu
D-α-methylglutamine	Dmgln	N-(2-aminoethyl)glycine	Naeg
D-α-methylhistidine	Dmhis	N-(3-aminopropyl)glycine	Norn
D-α-methylisoleucine	Dmile	N-amino-α-methylbutyrate	Nmaabu
D-α-methylleucine	Dmleu	α-napthylalanine	Anap

Non-conventional amino acid	Code	Non-conventional amino acid	Code
D-α-methyllysine	Dmlys	N-benzylglycine	Nphe
D-α-methylmethionine	Dmmet	N-(2-carbamylethyl)glycine	Ngln
D-α-methylornithine	Dmorn	N-(carbamylmethyl)glycine	Nasn
D-α-methylphenylalanine	Dmphe	N-(2-carboxyethyl)glycine	Nglu
D-α-methylproline	Dmpro	N-(carboxymethyl)glycine	Nasp
D-α-methylserine	Dmser	N-cyclobutylglycine	Ncbut
D-α-methylthreonine	Dmthr	N-cycloheptylglycine	Nchep
D-α-methyltryptophan	Dmtrp	N-cyclohexylglycine	Nchex
D-α-methyltyrosine	Dmty	N-cyclodecylglycine	Ncdec
D-α-methylvaline	Dmval	N-cylcododecylglycine	Ncdod
D-N-methylalanine	Dnmala	N-cyclooctylglycine	Ncoct
D-N-methylarginine	Dnmarg	N-cyclopropylglycine	Ncpro
D-N-methylasparagine	Dnmasn	N-cycloundecylglycine	Ncund
D-N-methylaspartate	Dnmasp	N-(2,2-diphenylethyl)glycine	Nbhm
D-N-methylcysteine	Dnmcys	N-(3,3-diphenylpropyl)glycine	Nbhe
D-N-methylglutamine	Dnmgln	N-(3-guanidinopropyl)glycine	Narg
D-N-methylglutamate	Dnmglu	N-(1-hydroxyethyl)glycine	Nthr
D-N-methylhistidine	Dnmhis	N-(hydroxyethyl))glycine	Nser
D-N-methylisoleucine	Dnmile	N-(imidazolylethyl))glycine	Nhis
D-N-methylleucine	Dnmleu	N-(3-indolylyethyl)glycine	Nhtrp
D-N-methyllysine	Dnmlys	N-methyl-γ-aminobutyrate	Nmgabu
N-methylcyclohexylalanine	Nmchexa	D-N-methylmethionine	Dnmmet
D-N-methylornithine	Dnmorn	N-methylcyclopentylalanine	Nmcpen
N-methylglycine	Nala	D-N-methylphenylalanine	Dnmphe
N-methylaminoisobutyrate	Nmaib	D-N-methylproline	Dnmpro
N-(1-methylpropyl)glycine	Nile	D-N-methylserine	Dnmser
N-(2-methylpropyl)glycine	Nleu	D-N-methylthreonine	Dnmthr
D-N-methyltryptophan	Dnmtrp	N-(1-methylethyl)glycine	Nval
D-N-methyltyrosine	Dnmtyr	N-methyla-napthylalanine	Nmanap
D-N-methylvaline	Dnmval	N-methylpenicillamine	Nmpen
γ-aminobutyric acid	Gabu	N-(*p*-hydroxyphenyl)glycine	Nhtyr
L-*t*-butylglycine	Tbug	N-(thiomethyl)glycine	Ncys
L-ethylglycine	Etg	penicillamine	Pen
L-homophenylalanine	Hphe	L-α-methylalanine	Mala
L-α-methylarginine	Marg	L-α-methylasparagine	Masn
L-α-methylaspartate	Masp	L-α-methyl-*t*-butylglycine	Mtbug
L-α-methylcysteine	Mcys	L-methylethylglycine	Metg
L-α-methylglutamine	Mgln	L-α-methylglutamate	Mglu
L-α-methylhistidine	Mhis	L-α-methylhomophenylalanine	Mhphe
L-α-methylisoleucine	Mile	N-(2-methylthioethyl)glycine	Nmet

Non-conventional amino acid	Code	Non-conventional amino acid	Code
L-α-methylleucine	Mleu	L-α-methyllysine	Mlys
L-α-methylmethionine	Mmet	L-α-methylnorleucine	Mnle
L-α-methylnorvaline	Mnva	L-α-methylornithine	Morn
L-α-methylphenylalanine	Mphe	L-α-methylproline	Mpro
L-α-methylserine	Mser	L-α-methylthreonine	Mthr
L-α-methyltryptophan	Mtrp	L-α-methyltyrosine	Mtyr
L-α-methylvaline	Mval	L-N-methylhomophenylalanine	Nmhphe
N-(N-(2,2-diphenylethyl) carbamylmethyl)glycine	Nnbhm	N-(N-(3,3-diphenylpropyl) carbamylmethyl)glycine	Nnbhe
1-carboxy-1-(2,2-diphenyl-ethylamino)cyclopropane	Nmbc		

[0099] Crosslinkers can be used, for example, to stabilize 3D conformations, using homo-bifunctional crosslinkers such as the bifunctional imido esters having $(CH_2)_n$ spacer groups with n = 1 to n = 6, glutaraldehyde, N-hydroxysuccinimide esters and hetero-bifunctional reagents which usually contain an amino-reactive moiety such as N-hydroxysuccinimide and another group specific-reactive moiety such as maleimido or dithio moiety (SH) or carbodiimide (COOH). In addition, peptides can be conformationally constrained by, for example, incorporation of C_α and N $_\alpha$-methylamino acids, introduction of double bonds between C_α and C_β atoms of amino acids and the formation of cyclic peptides or analogs by introducing covalent bonds such as forming an amide bond between the N and C termini, between two side chains or between a side chain and the N or C terminus.

[0100] Any immunoassay may be used to target the MilA protein or its antibody-binding fragment or its derivative form. Immunoassays are in effect binding assays. Examples of immunoassays include the various types of enzyme linked immunosorbent assays (ELSA), radioimmunoassays (RIA), Western blotting, dot blotting and FACS analyses.

[0101] In an embodiment, an ELISA is carried out using immobilized MilA protein,

its homolog, its derivative or an antibody-binding fragment (herein referred to as he "antigen"). In an embodiment, is immobilized onto a selected surface exhibiting protein affinity, such as a well in a polystyrene microtiter plate.

[0102] A sample putatively comprising antibodies to the naturally occurring MilA protein is then added to the wells. After binding and washing to remove non-specifically bound immune complexes, the antibody bound complex may be detected. Detection is generally achieved by the addition of a second antibody that is known to bind to the antibody and is linked to a detectable label. This type of ELISA is a simple "sandwich ELISA". Detection may also be achieved by the addition of the second antibody, followed by the addition of a third antibody that has binding affinity for the second antibody, with the third antibody being linked to a detectable label.

[0103] In an embodiment, the antibody-antigen complex is detected by a labeled anti-immunoglobulin antibody having an enzyme, chemiluminescence, radioisotope, impedance or optical signal generating moiety attached thereto. In an embodiment, the anti-immunoglobulin activity is an anti-IgG antibody.

[0104] The present specification further enables antibodies to MilA or its homolog or derivative. The antibodies may be monoclonal or polyclonal antibodies. The antibodies are useful for screening for MilA protein or its homolog or derivative and for purifying or capturing MilA protein or its homolog or derivative.

[0105] By "antibody" is meant a protein of the immunoglobulin family that is capable of combining, interacting or otherwise associating with an antigen (i.e. MilA protein or its homolog or derivative). An antibody is, therefore, an antigen-binding molecule. An "antibody" is an example of an immunointeractive molecule and includes a polyclonal or monoclonal antibody. Particularly useful immunointeractive molecules are monoclonal antibodies.

[0106] The term "antigen" is used herein in its broadest sense to refer to a MilA

substance, or its homologs or derivatives that are capable of reacting in and/or inducing an immune response.

[0107] By "antigen-binding molecule" is meant any molecule that has binding affinity for a target antigen. It will be understood that this term extends to immunoglobulins (e.g. polyclonal or monoclonal antibodies), immunoglobulin fragments and non-immunoglobulin derived protein frameworks that exhibit antigen-binding activity.

[0108] By "antigenic determinant" or "epitope" is meant that part of an antigenic molecule against which a particular immune response is directed and includes an antibody-binding fragment of a MilA antibody. Typically, in an animal, antigens present several or even many antigenic determinants simultaneously.

[0109] Antibodies described herein may be from any source. Examples of animal and avian sources and hosts include humans, primates, livestock animals (e.g. sheep, cows, horses, pigs, donkeys), laboratory test animals (e.g. mice, rabbits, guinea pigs, hamsters), companion animals (e.g. dogs, cats), poultry bird (e.g. chickens, ducks, geese, turkeys) and game birds (e.g. pheasants).

[0110] Immunization and subsequent production of monoclonal antibodies can be carried out using standard protocols as for example described by Köhler and Milstein (Kohler *et al.* (1975), *Nature 256*:495-499 and Kohler *et al.* (1976), *Eur. J. Immunol. 6(7)*:511-519, Coligan *et al.* (1991-1997) *Current Protocols in Immunology*, or Toyama *et al.* (1987), *Monoclonal Antibody, Experiment Manual*, published by Kodansha Scientific. Essentially, an animal is immunized with an antigen-containing (i.e. MilA or a fragment or homolog thereof) by standard methods to produce antibody-producing cells, particularly antibody-producing somatic cells (e.g. B lymphocytes). These cells can then be removed from the immunized animal for immortalization. The antigen may need to first be associated with a carrier.

[0111] By "carrier" is meant any substance of typically high molecular weight to

which a non- or poorly immunogenic substance (e.g. a hapten) is naturally or artificially linked to enhance its immunogenicity.

[0112] Immortalization of antibody-producing cells may be carried out using methods, which are well-known in the art. For example, the immortalization may be achieved by the transformation method using Epstein-Barr virus (EBV) (Kozbor *et al.* (1986), *Methods in Enzymology 121*:140). In an embodiment, antibody-producing cells are immortalized using the cell fusion method (described in [Coligan *et al.. (*1991-1997) *supra*]), which is widely employed for the production of monoclonal antibodies. In this method, somatic antibody-producing cells with the potential to produce antibodies, particularly B cells, are fused with a myeloma cell line. These somatic cells may be derived from the lymph nodes, spleens and peripheral blood of primed animals, preferably rodent animals such as mice and rats. In an embodiment murine spleen cells are used. It would be possible, however, to use rat, rabbit, sheep or goat cells, or cells from other animal species instead.

[0113] Specialized myeloma cell lines have been developed from lymphocytic tumours for use in hybridoma-producing fusion procedures (Kohler *et al.* (1976) *supra*, Kozbor *et al. (*1986) *supra*, and Volk *et al.* (1982) *J. Virol. 42(1)*:220-227). These cell lines have been developed for at least three reasons. The first is to facilitate the selection of fused hybridomas from unfused and similarly indefinitely self-propagating myeloma cells. Usually, this is accomplished by using myelomas with enzyme deficiencies that render them incapable of growing in certain selective media that support the growth of hybridomas. The second reason arises from the inherent ability of lymphocytic tumor cells to produce their own antibodies. To eliminate the production of tumor cell antibodies by the hybridomas, myeloma cell lines incapable of producing endogenous light or heavy immunoglobulin chains are used. A third reason for selection of these cell lines is for their suitability and efficiency for fusion.

[0114] Many myeloma cell lines may be used for the production of fused cell hybrids, including, e.g. P3X63-Ag8, P3X63-AG8.653, P3/NS1-Ag4-1 (NS-1), Sp2/0-Ag14 and S194/5.XXO.Bu.1. The P3X63-Ag8 and NS-1 cell lines have been described by Köhler

and Milstein (Kohler *et al.* 1976 *supra*). Shulman *et al.* (1978), *Nature 276*:269-270, developed the Sp2/0-Ag14 myeloma line. The S194/5.XXO.Bu.1 line was reported by Trowbridge (1982), *J. Exp. Med. 148(1)*:220-227.

[0115] Methods for generating hybrids of antibody-producing spleen or lymph node cells and myeloma cells usually involve mixing somatic cells with myeloma cells in a 10:1 proportion (although the proportion may vary from about 20:1 to about 1:1), respectively, in the presence of an agent or agents (chemical, viral or electrical) that promotes the fusion of cell membranes. Fusion methods have been described (Kohler *et al.* (1975) *supra*, Kohler *et al.* (1976) *supra*, Gefter *et al.* (1977), *Somatic Cell Genet. 3*:231-236 and Volk *et al.* (1982) *supra*). The fusion-promoting agents used by those investigators were Sendai virus and polyethylene glycol (PEG).

[0116] Because fusion procedures produce viable hybrids at very low frequency (e.g. when spleens are used as a source of somatic cells, only one hybrid is obtained for roughly every $1x10^5$ spleen cells), it is preferable to have a means of selecting the fused cell hybrids from the remaining unfused cells, particularly the unfused myeloma cells. A means of detecting the desired antibody-producing hybridomas among other resulting fused cell hybrids is also necessary. Generally, the selection of fused cell hybrids is accomplished by culturing the cells in media that support the growth of hybridomas but prevent the growth of the unfused myeloma cells, which normally would go on dividing indefinitely. The-somatic cells used in the fusion do not maintain long-term viability in *in vitro* culture and hence do not pose a problem. In the example of the present invention, myeloma cells lacking hypoxanthine phosphoribosyl transferase (HPRT-negative) were used. Selection against these cells is made in hypoxanthine/aminopterin/thymidine (HAT) medium, a medium in which the fused cell hybrids survive due to the HPRT-positive genotype of the spleen cells. The use of myeloma cells with different genetic deficiencies (drug sensitivities, etc.) that can be selected against in media supporting the growth of genotypically competent hybrids is also possible.

[0117] Several weeks are required to selectively culture the fused cell hybrids. Early

in this time period, it is necessary to identify those hybrids which produce the desired antibody, so that they may subsequently be cloned and propagated. Generally, around 10% of the hybrids obtained produce the desired antibody, although a range of from about 1 to about 30% is not uncommon. The detection of antibody-producing hybrids can be achieved by any one of several standard assay methods, including enzyme-linked immunoassay and radioimmunoassay techniques as, for example, described in US Patent No. 6,056,957.

[0118] Once the desired fused cell hybrids have been selected and cloned into individual antibody-producing cell lines, each cell line may be propagated in either of two standard ways. A suspension of the hybridoma cells can be injected into a histocompatible animal. The injected animal will then develop tumors that secrete the specific monoclonal antibody produced by the fused cell hybrid. The body fluids of the animal, such as serum or ascites fluid, can be tapped to provide monoclonal antibodies in high concentration. Alternatively, the individual cell lines may be propagated *in vitro* in laboratory culture vessels. The culture medium containing high concentrations of a single specific monoclonal antibody can be harvested by decantation, filtration or centrifugation, and subsequently purified.

[0119] The cell lines are tested for their specificity to detect the antigen of interest by any suitable immunodetection means. For example, cell lines can be aliquoted into a number of wells and incubated and the supernatant from each well is analyzed by enzyme-linked immunosorbent assay (ELISA), indirect fluorescent antibody technique, or the like. The cell line(s) producing a monoclonal antibody capable of recognizing the target antigen but which does not recognize non-target epitopes are identified and then directly cultured *in vitro* or injected into a histocompatible animal to form tumors and to produce, collect and purify the required antibodies.

[0120] The present specification is further directed to a composition comprising a protein comprising an amino acid sequence set forth in SEQ ID NO:1 or a derivative thereof or a protein having at lest 40% sequence similarity to SEQ ID NO:1 in the manufacture of a medicament for the treatment or prophylaxis of infection by *Mycoplasma*

species.

[0121] Also enabled herein is a composition comprising a protein comprising an amino acid sequence set forth in SEQ ID NO:2 or a derivative thereof or a protein having at lest 40% sequence similarity to SEQ ID NO:2 in the manufacture of a medicament for the treatment or prophylaxis of infection by *Mycoplasma* species.

[0122] Also enabled herein is a composition comprising a protein comprising an amino acid sequence set forth in SEQ ID NO:15 or a derivative thereof or a protein having at lest 40% sequence similarity to SEQ ID NO:15 in the manufacture of a medicament for the treatment or prophylaxis of infection by *Mycoplasma* species.

[0123] In an embodiment, the protein comprises the amino acid sequence set forth by SEQ ID NO:2 or an amino acid sequence having at least 40% similarity thereto or is a derivative of the protein.

[0124] In an embodiment, the protein comprises the amino acid sequence set forth by SEQ ID NO:15 or an amino acid sequence having at least 40% similarity thereto or is a derivative of the protein.

[0125] In an embodiment, the protein comprises the amino acid sequence set forth by SEQ ID NO:3 or an amino acid sequence having at least 40% similarity thereto or is a derivative of the protein.

[0126] In an embodiment, the protein comprises the amino acid sequence set forth by SEQ ID NO:4 or an amino acid sequence having at least 40% similarity thereto or is a derivative of the protein.

[0127] In an embodiment, the protein comprises the amino acid sequence set forth by SEQ ID NO:5 or an amino acid sequence having at least 40% similarity thereto or is a derivative of the protein.

[0128] In an embodiment, the protein comprises the amino acid sequence set forth by SEQ ID NO:6 or an amino acid sequence having at least 40% similarity thereto or is a derivative of the protein.

[0129] In an embodiment, the protein comprises the amino acid sequence set forth by SEQ ID NO:7 or an amino acid sequence having at least 40% similarity thereto or is a derivative of the protein.

[0130] In an embodiment, the protein comprises the amino acid sequence set forth by SEQ ID NO:8 or an amino acid sequence having at least 40% similarity thereto or is a derivative of the protein.

[0131] In an embodiment, the protein comprises the amino acid sequence set forth by SEQ ID NO:16 or an amino acid sequence having at least 40% similarity thereto or is a derivative of the protein.

[0132] In an embodiment, the protein comprises the amino acid sequence set forth by SEQ ID NO:17 or an amino acid sequence having at least 40% similarity thereto or is a derivative of the protein.

[0133] In an embodiment, the protein comprises the amino acid sequence set forth by SEQ ID NO:18 or an amino acid sequence having at least 40% similarity thereto or is a derivative of the protein.

[0134] In an embodiment, the protein comprises the amino acid sequence set forth by SEQ ID NO:19 or an amino acid sequence having at least 40% similarity thereto or is a derivative of the protein.

[0135] In an embodiment, the protein comprises the amino acid sequence set forth by SEQ ID NO:20 or an amino acid sequence having at least 40% similarity thereto or is a

derivative of the protein.

[0136] In an embodiment, the protein comprises the amino acid sequence set forth by SEQ ID NO:21 or an amino acid sequence having at least 40% similarity thereto or is a derivative of the protein.

[0137] Generally, the protein is formulated with a pharmaceutical carrier, excipient or diluent, which is non toxic to a ruminant subject.

[0138] The carrier may take a wide variety of forms depending on the form of preparation desired for administration, e.g., oral or parenteral (including intravenous). In preparing the compositions for oral dosage form, any of the usual pharmaceutical media may be employed, such as, for example, water, glycols, oils, alcohols, flavouring agents, preservatives, coloring agents and the like in the case of oral liquid preparations, such as, for example, suspensions, elixirs and solutions; or carriers such as starches, sugars, microcrystalline cellulose, diluents, granulating agents, lubricants, binders, disintegrating agents and the like in the case of oral solid preparations such as, for example, powders, capsules and tablets, with the solid oral preparations being preferred over the liquid preparations. Because of their ease of administration, tablets and capsules represent the most advantageous oral dosage unit form in which case solid pharmaceutical carriers are obviously employed. If desired, tablets may be coated by standard aqueous or nonaqueous techniques.

[0139] Compositions disclosed herein suitable for oral administration may be presented as discrete units such as capsules, cachets or tablets each containing a predetermined amount of the active ingredient, as a powder or granules or as a solution or a suspension in an aqueous liquid, a non-aqueous liquid, an oil-in-water emulsion or a water-in-oil liquid emulsion. Such compositions may be prepared by any of the methods of pharmacy but all methods include the step of bringing into association the active ingredient with the carrier which constitutes one or more necessary ingredients. In general, the compositions are prepared by uniformly and intimately admixing the active ingredient with

liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product into the desired presentation. For example, a tablet may be prepared by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine, the active ingredient in a free-flowing form such as powder or granules, optionally mixed with a binder, lubricant, inert diluent, surface active or dispersing agent. Molded tablets may be made by molding in a suitable machine, a mixture of the powdered compound moistened with an inert liquid diluent.

[0140] The components and/or extracts enabled herein may be administered orally, parenterally (including subcutaneous injections, intravenous, intramuscular, intrasternal injection or infusion techniques), by inhalation spray, or rectally, in dosage unit formulations containing conventional non-toxic pharmaceutically acceptable carriers, excipient and diluents. The composition may also further comprise an adjuvant.

[0141] When administered by nasal aerosol or inhalation, these compositions are prepared according to techniques well-known in the art of pharmaceutical formulation and may be prepared as solutions in saline, employing benzyl alcohol or other suitable preservatives, absorption promoters to enhance bioavailability, fluorocarbons, and/or other solubilizing or dispersing agents known in the art.

[0142] The protein may also be administered in intravenous (both bolus and infusion), intraperitoneal, subcutaneous, topical with or without occlusion, or intramuscular form, all using forms well known to those of ordinary skill in the pharmaceutical arts. When administered by injection, the injectable solutions or suspensions may be formulated according to known art, using suitable non-toxic, parenterally-acceptable diluents or solvents, such as mannitol, 1,3-butanediol, water, Ringer's solution or isotonic sodium chloride solution, or suitable dispersing or wetting and suspending agents, such as sterile, bland, fixed oils, including synthetic mono- or diglycerides, and fatty acids, including oleic acid.

[0143] When rectally administered in the form of suppositories, these compositions may be prepared by mixing the protein with a suitable non-irritating excipient, such as cocoa butter, synthetic glyceride esters or polyethylene glycols, which are solid at ordinary temperatures, but liquefy and/or dissolve in the rectal cavity to release the protein.

[0144] The effective dosage of the protein employed in therapy may vary depending on the particular compound employed, the mode of administration, the condition being treated and the severity of the condition being treated. Thus, the dosage regimen utilizing the proteins of the present disclosure is selected in accordance with a variety of factors including type, species, age, weight, sex and medical condition of the ruminant subject; the severity of the *Mycoplasma* infection to be treated; the route of administration; the renal and hepatic function of the subject; and the particular peptide employed. A veterinarian of ordinary skill can readily determine and prescribe the effective amount of the drug required to prevent, counter or arrest the progress of the condition. Optimal precision in achieving concentration of protein within the range that yields efficacy without toxicity requires a regimen based on the kinetics of the protein's availability to target sites. This involves a consideration of the distribution, equilibrium, and elimination of the protein.

[0145] Another aspect enabled herein is a method for controlling infection by a species of *Mycoplasma* in a subject, the method comprising administering to the subject an antibody-inducing effective amount of a protein or derivative thereof comprising the amino acid sequence set forth in SEQ ID NO:1 or an amino acid sequence having at least 40% similarity to SEQ ID NO:1 after optimal alignment for a time and under conditions sufficient to generate antibodies to the protein.

[0146] Another aspect enabled herein is a method for controlling infection by a species of *Mycoplasma* in a subject, the method comprising administering to the subject an antibody-inducing effective amount of a protein or derivative thereof comprising the amino acid sequence set forth in SEQ ID NO:2 or an amino acid sequence having at least 40% similarity to SEQ ID NO:2 after optimal alignment for a time and under conditions sufficient to generate antibodies to the protein.

[0147] Another aspect enabled herein is a method for controlling infection by a species of *Mycoplasma* in a subject, the method comprising administering to the subject an antibody-inducing effective amount of a protein or derivative thereof comprising the amino acid sequence set forth in SEQ ID NO:15 or an amino acid sequence having at least 40% similarity to SEQ ID NO:15 after optimal alignment for a time and under conditions sufficient to generate antibodies to the protein.

[0148] In an embodiment, the protein comprises the amino acid sequence set forth in SEQ ID NO:2 or a protein having at least 40% similarity to SEQ ID NO:2 or is a derivative thereof.

[0149] In an embodiment, the protein comprises the amino acid sequence set forth in SEQ ID NO:15 or a protein having at least 40% similarity to SEQ ID NO:15 or is a derivative thereof.

[0150] In an embodiment, the protein comprises the amino acid sequence set forth in SEQ ID NO:3 or a protein having at least 40% similarity to SEQ ID NO:3 or is a derivative thereof.

[0151] In an embodiment, the protein comprises the amino acid sequence set forth in SEQ ID NO:4 or a protein having at least 40% similarity to SEQ ID NO:4 or is a derivative thereof.

[0152] In an embodiment, the protein comprises the amino acid sequence set forth in SEQ ID NO:5 or a protein having at least 40% similarity to SEQ ID NO:5 or is a derivative thereof.

[0153] In an embodiment, the protein comprises the amino acid sequence set forth in SEQ ID NO:6 or a protein having at least 40% similarity to SEQ ID NO:6 or is a derivative thereof.

[0154] In an embodiment, the protein comprises the amino acid sequence set forth in SEQ ID NO:7 or a protein having at least 40% similarity to SEQ ID NO:7 or is a derivative thereof.

[0155] In an embodiment, the protein comprises the amino acid sequence set forth in SEQ ID NO:8 or a protein having at least 40% similarity to SEQ ID NO:8 or is a derivative thereof.

[0156] In an embodiment, the protein comprises the amino acid sequence set forth in SEQ ID NO:16 or a protein having at least 40% similarity to SEQ ID NO:16 or is a derivative thereof.

[0157] In an embodiment, the protein comprises the amino acid sequence set forth in SEQ ID NO:17 or a protein having at least 40% similarity to SEQ ID NO:17 or is a derivative thereof.

[0158] In an embodiment, the protein comprises the amino acid sequence set forth in SEQ ID NO:18 or a protein having at least 40% similarity to SEQ ID NO:18 or is a derivative thereof.

[0159] In an embodiment, the protein comprises the amino acid sequence set forth in SEQ ID NO:19 or a protein having at least 40% similarity to SEQ ID NO:19 or is a derivative thereof.

[0160] In an embodiment, the protein comprises the amino acid sequence set forth in SEQ ID NO:20 or a protein having at least 40% similarity to SEQ ID NO:20 or is a derivative thereof.

[0161] In an embodiment, the protein comprises the amino acid sequence set forth in SEQ ID NO:21 or a protein having at least 40% similarity to SEQ ID NO:21 or is a

derivative thereof.

[0162] These aspects include conditions caused or exacerbated by *Mycoplasma* infection such as bovine respiratory disease, keratoconjunctivitis, mastitis, meningitis, otitis media and genital tract disease.

[0163] The formulation of protein and its subsequent administration (dosing) is within the skill of those in the art. Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the subject. Persons or ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be estimated based on EC_{50}s found to be effective in *in vitro* and *in vivo* animal models. In general, dosage is from 0.01μg to 100g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20 years. Persons of ordinary skill in the art can easily estimate repetition rats for dosing based on measured residence times and concentrations of the drug in bodily fluids or tissues. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence o the disease state, wherein the oligonucleotide is administrated in maintenance doses, ranging from 0.01μg to 100 g per kg of body weight, once or more daily, to once every 20 years.

[0164] In another embodiment, a ruminant subject is administered an antibody to MilA or its homolog or derivative. This is regarded as passive immunotherapy. Antibodies to MilA or its homolog or derivative are generated as previously described herein.

EXAMPLES

[0165] Embodiments contemplated herein are now described by the following non-limiting Examples.

Materials and Methods

Bacterial strains and plasmids

[0166] *Mycoplasma bovis* wild-type strains 3683, 149040, PG45 and the temperature sensitive (ts) mutant strains A, B and C were used. *M. bovis* wild-type strains (1/500 dilutions) were incubated for 17 h at 37˚C in MB medium, and 1/100 dilutions of the ts mutants were incubated for 24 h at 33˚C in MB medium. pGEX-4T-1 plasmid (Figure 12) was used for cloning different regions of *MilA* and *E. coli* JM109 cells were used to express recombinant GST-MilA proteins.

Serum samples from experimentally infected calves

[0167] *Experiment 1*- Sixteen, one-month-old Friesian-cross calves were weighed and allocated into two groups. Group 1 consisted of 4 animals exposed only to mycoplasma culture medium, while Group 2 consisted 12 calves exposed to an overnight culture of *M. bovis* strain 3683 twice on day 1 and 3, with each calf receiving an estimated dose of $10^{5.2}$ CCU, using an aerosol exposure method described previously (Czaja *et al.* (2002) *Vet. Rec. 150*:9-11; Wawegama *et al.* (2012) *Vet. Microbiol. 158*:220-224). The calves in Groups 1 and 2 calves were held in separate facilities. Blood samples were taken from all the calves on days 0, 10, 17 and 24 after infection.

[0168] *Experiment 2* - Thirty five, one-month-old Friesian-cross calves were grouped according to their weight and randomly grouped into 2 groups. Group 1, the uninfected group, consisted of 5 calves exposed to an aerosol of mycoplasma culture medium, and Group 2 consisted of 30 calves exposed to an aerosol of *M. bovis* strain 3683. Infection and sampling were performed as described for Experiment 1.

[0169] *Experiment 3* - Group 3 cattle totaled 31 and were provided with vaccine A.

SDS-PAGE and Western blots analysis

[0170] The whole cell proteins of *M. bovis* strain 3683 were separated by sodium dodecyl sulfate polyacrylamide in 10% w/v polyacrylamide gels and Western blotting performed as described by Markham *et al.* (1998) *Infect. Immun. 66*:2845-2853. Cells from a 10 ml culture were collected following centrifugation at 16,000 x g for 5 min, and washed 3 times with PBS. After the final centrifugation the supernatant was discarded and the cell pellet was resuspended in a volume of distilled water $1/50^{th}$ that of the original broth using a 25 gauge needle and a 1 ml syringe. The cell lysate was then mixed with 2 x SDS-PAGE lysis buffer (10% w/v SDS, 100% v/v glycerol, 0.5 M Tris-HCl, pH 6.8, 10% v/v β-mercaptoethanol, 10% w/v bromophenol blue) and heated at 100°C for 5 min.

Preparation and running of SDS-PAGE gels

[0171] The total proteins were separated in 10% w/v or 12% w/v polyacrylamide separating gels by electrophoresis at 180 V for 1 h in the BioRad mini-vertical PAGE system containing Laemmli's buffer (0.1% w/v SDS, 0.025 M Tris, 0.19 M glycine). Broad range prestained (Fermentas) or unstained protein markers (New England Biolabs, USA), or biotinylated protein markers (Cell Signaling Technology, USA) were used as molecular mass standards. The gels were stained with Coomassie brilliant blue R-250 (BioRad, USA).

Western blotting

[0172] After SDS-PAGE the proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, USA) using the BioRad mini-vertical PAGE and blotting system containing chilled Western transfer buffer (Laemmli's buffer containing 20% v/v methanol) for 1 h at a constant voltage of 100 V. After transfer, the PVDF membrane was blocked overnight with 5% w/v skim milk (Diploma, Australia) in distilled water at 4°C. The membrane was then washed three times (5 min each) with PBS-T [phosphate buffered saline (140 mM NaCl, 2.7 mM KCl, 10 mM $Na_2H_2PO_4$, 2 mM KH_2PO_4) containing 0.1% v/v Tween 20 (Asia Pacific Specialty Chemicals, Australia)].

The membrane was then probed with a pool of *M. bovis*-specific calf sera, diluted to 1/100 in 1% w/v skim milk in PBS-T, and incubated for 1 h at room temperature with gentle rocking. Alternatively, anti-*M. bovis* rabbit antibody diluted to 1/20,000 in PBS-T/1% w/v skim milk, was used. The membrane was then washed three times with PBS-T as before. The membrane was then probed with horseradish peroxidase (HRP)-conjugated sheep anti-bovine IgG antibody (Bethyl Laboratories, Inc, USA), diluted to 1/5000 in 1% w/v skim milk in PBS-T, or HRP-conjugated swine anti-rabbit IgG antibody (Dako, Denmark), diluted to 1/5000 in 1% w/v skim milk in PBS-T, for 1 h at room temperature. After incubation the membrane was washed three times with PBS-T (5 min each) and the bound enzyme was detected using chemiluminescence (ECL, Western blotting detection reagent and analysis system, Amersham Biosciences, UK), or with *SIGMAFAST (Trade Mark)* 3,3' diaminobenzidine (DAB) according to the manufacturer's instructions.

Triton X-114 partitioning

[0173] *Mycoplasma bovis* cells from a 25 ml culture were pelleted by centrifugation at 16,000 x g for 20 min at 4°C in a Sorvall RC-5B centrifuge (Sorvall, USA). The cell pellet was washed three times with PBS and resuspended in 0.25 ml PBS using a 25 gauge needle and syringe. The cells were lysed with a 0.25 ml volume of 1% v/v Triton X-114 (Sigma, USA) and the mixture incubated on ice for 60 min, with mixing every 15 min. The cell suspension was centrifuged at 13,000 x g for 30 min at 4°C, and the supernatant was carefully loaded onto a 1 ml 6% w/v sucrose cushion containing 0.06% v/v Triton X-114 and incubated for 9 min at 37°C in a water bath in order to achieve phase separation. The sample was centrifuged at 400 x g for 7 min at 37°C and the supernatant (hydrophilic protein fraction) was carefully aspirated, leaving on oily pellet predominantly containing the hydrophobic fraction. The oily pellet was resuspended in 0.5 ml of ice-cold PBS and placed on a 6% w/v sucrose cushion and phase separation carried out again as described above. Finally, the oily pellet was resuspended in 0.5 ml ice-cold PBS. The hydrophobic and hydrophilic fractions were precipitated using the methanol/chloroform method. To 1 volume of protein suspension 4 volumes of ice-cold methanol, 1 volume of ice-cold chloroform and 3 volumes of distilled water were added, the solutions vigorously mixed and then centrifuged for 1 min at 16,000 x g. The supernatant was discarded and the

protein pellet resuspended in 4 volumes of methanol, vortexed and centrifuged for 5 min at 16,000 x g at RT. The supernatant was removed, the pellet dried under a vacuum and then dissolved in 8 M urea. The proteins were then analyzed by SDS-PAGE or two dimensional electrophoresis (2-DE).

Mass spectrometric analysis

[0174] For mass spectrometric identification of proteins, the hydrophobic protein fraction of *M. bovis* strain 3683 was separated in a 7.5% w/v polyacrylamide gel and stained with colloidal Coomassie blue (Life Technologies) according to the manufacturer's instructions. The major bands between 180 kDa and 250 kDa were excised and analyzed at the Adelaide Proteomics Centre, The University of Adelaide, using liquid chromatography electrospray ionization ion – trap (LC-eSI-IT) mass spectrometry.

Two dimensional gel electrophoresis (2-DE)

Sample preparation

[0175] Sample preparation for two dimensional gel electrophoresis (2-DE) was carried out as described by Görg (2004). A 20 ml overnight culture of *M. bovis* was pelleted by centrifugation at 16,000 x g for 20 min at 4°C and the cell pellet washed three times with PBS, and resuspended using a 25 gauge needle in 0.5 ml of sample preparation solution [8 M urea, 4% w/v CHAPS (GE Healthcare, USA), 2% v/v IPG buffer pH 3-11 (GE Healthcare, USA) and 40 mM DL-dithiothreitol (DTT) (Sigma-Aldrich)]. The sample was then sonicated 4 times (15 sec each with 5 min intervals on ice) and centrifuged at 42,000 x g for 30 min at 15°C. The supernatant was stored in 0.1 ml aliquots at -80°C. The protein content of the samples was quantified using the 2D Quant kit (GE Healthcare, Sweden) according to the manufacturer's instructions. A 100 µg quantity of each sample was cleaned using the 2D clean-up kit (GE Healthcare, USA) according to the manufacturer's instructions and resuspended in urea rehydration solution [8 M urea, 2% w/v CHAPS (GE Healthcare, USA)]. Prior to electrophoresis, 0.5% v/v IPG buffer (pH 3-11) and 2.8 mg DTT/ml were added.

First dimension isoelectri focusing (IEF)

[0176] For the first dimension isoelectric focusing, 60 µg of the protein in 125 µl of rehydration solution was pipetted into a 7 cm IEF strip holder, and a dry 7 cm strip gel (pH 3-11) placed gel side down into the holder and overlaid with mineral oil (GE Healthcare). The strip holder was placed on an Ettan IPGphorII isoelectric focusing system (Amersham-Bioscience) and the gel was re-swelled for 12 h at 30 V, then at 60 V for 6 h, and the isoelectric focusing performed for 5.5 h, at 200 V for 1 h, at 500 V for 1 h, at 1000 V for 1 h, at a gradient of 1000 V to 8000 V for 1 h, and at 8000 V for 1.5 h. Once the IEF was complete, the strip was removed from the strip holder and equilibrated in SDS equilibration buffer (6 M urea, 75 mM Tris-HCl [pH 8.8], 87% v/v glycerol, 2% w/v SDS, 0.002% w/v bromophenol blue with 10 mg DTT/ml) for 15 min, followed by another 15 min with 25 mg iodoacetamide/ml (Amersham, USA) substituted for the DTT.

Second dimension electrophoresis

[0177] The IEF strip gel was placed on top of a 12.5% w/v polyacrylamide SDS-PAGE gel and sealed with agarose sealing solution (0.5% w/v agarose, 0.002% w/v bromophenol blue in Laemmli's buffer) and the proteins separated according to their mass by electrophoresis. Once electrophoresis was completed the proteins were either transferred onto a PVDF membrane or stained with either colloidal Coomassie blue or silver (Plusone Silver Staining Kit-Protein, GE Healthcare, USA) according to the manufacturer's instructions.

Expression of recombinant Mycoplasma immunogenic lipase A (MilA) protein

[0178] A 6 kb region of the *MilA* gene was codon-optimized for expression in *E. coli* and the TGA tryptophan codons altered to TGG to confirm with *E. coli* codon usage. The synthetic gene sequence was designed to contain a number of restriction endonuclease cleavage sites to aid manipulation in pGEX-4T-1 (*Bam*HI, *Sal*I, *Xho*I and *Bgl*II) [Figure 1]. The modified DNA sequences were then manufactured by Genscript (USA) and cloned in pUC57.

[0179] The gene sequence optimized for expression in *E. coli* is set forth in SEQ ID

Nos:13 and 22. The *MilA*-AB (SEQ ID NO:13) and *milA*-CD (SEQ ID NO:22) fragments were excised from pUC57 using *Bam*HI, *Bgl*I and *Sal*I, purified by agarose gel electrophoresis and extraction with a QIAEX II kit (Qiagen, Germany) according to the manufacturer's instructions, and ligated in to pGEX-4T-1 (GE Healthcare) that had been digested with *Bam*HI and *Sal*I. The *MilA*-A, *MilA*-B, *MilA*-C and *MilA*-D DNA fragments were excised from pGEX-4T-1-*MilA*-AB or pGEX-4T-1-*MilA*-CD with appropriate restriction endonucleases (Figure 1), purified and ligated to similarly digested pGEX-4T-1. In order to develop the *MilA*-ab and *MilA*-cd DNA fragments, PCR was performed using 2 ng of pGEX-4T-1-*milA*-AB or pGEX-4T-1-*MilA*-CD DNA as the template and 0.4 µM of the appropriate primers (Figure 1) in a reaction volume of 50 µl containing 2 mM $MgSO_4$, 100 µM of each deoxynucleoside triphosphate and 1.5 U of Platinum *Taq* thermo-polymerase (Life Technologies). Cycling conditions included an initial denaturation step at 94°C for 4 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min and 68°C for 2 min. The 1.8 kb products were excised, purified and ligated into pGEX-4T-1. The ligated plasmids were used to transform *E. coli* JM109 cells by electrophoration (Noormohammadi *et al.* (1999) *Microbiology 145*:2087-2094). The transformants were selected on LB agar containing ampicillin (50 µg/ml) and selected clones were incubated for 3 h at 30°C in the presence of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) [Invitrogen]. Expression of GST fusion proteins from the recombinant plasmids was assessed by SDS-PAGE and Western blotting of whole cell lysates. Fusion protein GST-MilA-ab was purified by affinity chromatography using glutathione-Sepharose 4B beads (GE Healthcare) and elution with free glutathione (Crabb *et al.* (1995) *Arch. Virol. 140*:245-258).

Constructing the recombinant protein

Preparing plasmid DNA

[0180] The vector pGEX-4T-1 (Figure 12) was used to transform *E. coli* JM109 cells by electroporation, according to the manufacturer's instructions (Gene Pulser, Bio Rad). The transformants were selected on LB agar containing ampicillin (50 µg/ml), and a single transformant was selected and grown overnight in LB broth supplemented with ampicillin

(50 μg/ml). The plasmid DNA was purified from the *E. coli* cells using the Qiagen Plasmid Midi Kit (Qiagen) according to the manufacturer's instructions.

[0181] Purification of the plasmids pUC57/*milA*-AB and pUC57/*milA*-CD was performed in the same manner.

Generation of pGEX-4T-1-milA-AB and pGEX-4T-1-milA-CD

[0182] The pUC57-*milA*-AB and pUC57-*milA*-CD plasmids were digested with appropriate restriction endonucleases (RE), *Bam*HI, *Bgl*I and *Sal*I, and pGEX-4T-1 was digested with *Bam*HI and *Sal*I according to the manufacturer's instructions (New England BioLabs). The digested DNA was separated in a 1% w/v agarose gel and the required DNA bands (3 kb for the *milA*-AB and *milA*-CD fragments and 5 kb for pGEX-4T-1) were excised and the DNA extracted using the QIAEX II kit (Qiagen, Germany) according to the manufacturer's instructions. The amount of DNA was quantified by measuring the absorbance at 260 nm using a NanoDrop spectrophotometer (Biolab, Australia). The *milA*-AB and *milA*-CD DNA fragments were ligated to similarly digested pGEX-4T-1 with T4 DNA ligase (Promega) in ligation buffer (Promega) at 4°C overnight. The ligation mixture was then used to transform *E. coli* JM109 cells by electroporation as above. Transformants were selected on LB agar containing ampicillin (50 μg/ml) and resistant transformants screened by PCR to confirm insertion of the *milA*-AB or *milA*-CD DNA fragment in the pGEX-4T-1 vector.

Screening clones

[0183] Ampicillin resistant colonies were selected and resuspended in 25 μl of dH_2O, and 1 μl of this was used as template in a screening PCR that contained 1.25 U Gotaq (Registered Trade Mark) Flexi DNA polymerase (Promega), 5 x Green Gotaq (Registered Trade Mark) Flexi buffer, 1.5 mM $MgCl_2$, 200 μM of each dNTP and 1 μM of each of the pGEX-4T-1 forward and reverse primers (pGEXfwd [GGGCTGGCAAGCCACGTTTGGTG (SEQ ID NO:23)] and pGEXrev [CCGGGAGCTGCATGTGTCAGA GG (SEQ ID NO:24)]). The reactions were incubated at 95°C for 4 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 7.5

min, finishing with 5 min at 72°C, in an iCycler (BioRad). PCR products were visualized by agarose gel electrophoresis and the image captured using the ChemiDoc imaging system (BioRad).

[0184] Those clones containing a 3 kb product as confirmed by PCR were selected and cultured overnight in LB broth supplemented with 50 µg ampicillin/ml. The plasmid DNA was purified using the Wizard Plus SV Minipreps kit (Promega), digested with *Bam*HI and *Sal*I and screened for the presence of the 3 kb insert and the 4.9 kb pGEX-4T-1 vector by electrophoresis in a 0.8% w/v agarose gel. The clones with the expected RE digestion pattern were selected for expression.

Screening clones for protein expression

[0185] A single transformant was used to set up an overnight culture in 5 ml LB broth containing 100 µg ampicillin/ml. The following day a 1/100 dilution of the overnight culture was used to inoculate 3 ml LB broth containing 100 µg ampicillin/ml and this culture incubated at 37°C with shaking until it reached an OD_{600} of 0.6-0.8 (around 2.5 h). A 1 ml sample of the culture was centrifuged at 16,000 x g for 5 min and the cells resuspended in 100 µl of PBS and stored at -20°C to be used as an uninduced sample. To the remaining culture 1 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) (Invitrogen) was added and the culture incubated at 30°C with shaking for 3 h. A 500 µl sample of the culture was centrifuged at 16,000 x g for 5 min and the cell pellet resuspended in 100 µl of PBS, stored at -20°C and used as the induced sample.

[0186] Whole cell proteins of the induced and uninduced samples were separated in a 10% polyacrylamide gel by SDS-PAGE and either stained with Coomassie blue or transferred to a PVDF membrane as described in Section 5.2.3 and probed with pooled *M. bovis*-specific calf sera and then with HRP-conjugated sheep anti-bovine IgG antibody.

Constructing the milA-A, milA-B, milA-C and milA-D plasmids

[0187] The pGEX-4T-1-*milA*-AB and pGEX-4T-1-*milA*-CD plasmids were digested with *Bam*HI and *Bgl*II to release the *milA*-B and *milA*-D regions and with *Xho*I to release

the *milA*-A and *milA*-C regions. Each of these four DNA fragments was separately ligated into similarly digested pGEX-4T-1 and ampicillin resistant clones were screened by PCR. Clones were screened by RE digestion using the appropriate RE combinations (*Bam*HI and *Bgl*II for *milA*-B and *milA*-D, *Xho*I for *milA*-A and *milA*-C), and positive clones were then screened for protein expression.

Constructing pGEX-4T-1-milA-ab and pGEX-4T-1-milA-cd plasmids using specific primers

[0188] Primers were designed using Geneious Pro 5.1.6 to amplify a 1.8 kb gene product from the original gene sequence of region *milA*-AB, extending from base 144 to base 1950, encompassing the region between regions *milA*-A and *milA*-B that were excluded in these constructs. A *Bam*HI cleavage site was added to the 5' end of the forward primer (abF-*Bam*HI) and a *Sal*I cleavage site was added to the 3' end of the reverse primer (abR-*Sal*I) (Table 7). Forward and reverse primers (cdF-*Bam*HI, cdR-*Sal*I) were similarly designed to amplify the region between bases 3128 and 4872 (Table 13).

[0189] PCR was performed with 2 ng of pGEX-4T-1-*milA*-AB plasmid DNA as the template with primers abF-*Bam*HI and abR-*Sal*I in a reaction volume of 50 µl containing 2 mM $MgSO_4$, 100 µM of each deoxynucleoside triphosphate, 0.4 µM of each primer and 1.5 U of Platinum *Taq* thermo-polymerase (Life Technologies). Cycling conditions included an initial denaturation step at 94°C for 4 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min and 68°C for 2 min. The final cycle included an elongation step at 68°C for 5 min. PCR products were resolved by electrophoresis in a 1% w/v agarose gel containing SyberSafe (Trade Mark) DNA gel stain (Molecular Probes) at 80 V for 1.5 h and visualized under UV illumination using the ChemiDoc imaging system (BioRad). The 1.8 kb product was excised and the DNA extracted and digested with *Bam*HI and *Sal*I as. The digested DNA was purified using the Wizard Plus SV Miniprep kit (Promega) according to the manufacturer's instructions. A similar PCR was performed to produce the 1.84 kb cd DNA fragment using the cdF-*Bam*HI and cdR-*Sal*I primers (Table 13), and the product digested and purified as above. The *milA*-ab and *milA*-cd fragments were cloned into pGEX-4T-1 and used to transform *E. coli* JM109 cells. Colonies expressing the GST-

MilA-ab and GST-MilA-cd proteins were screened as described above.

Large scale fusion protein production

[0190] A single colony was used to inoculate 5 ml of LB broth containing 100 µg ampicillin/ml and this culture incubated overnight at 37°C. The 5 ml overnight culture was inoculated into 500 ml LB containing 100 µg ampicillin/ml and incubated at 37°C with shaking until it reached an OD_{600} of 0.6-0.8 (around 2.5 h). A 1 ml sample of the culture was centrifuged at 16,000 x g for 5 min, resuspended in 100 µl of PBS and stored at -20°C for use as the uninduced sample. To the remaining culture, 1 mM of IPTG was added and the culture incubated at 30°C with shaking for approximately 3 h. After induction, the cells were pelleted by centrifuging the culture at 8,500 x g for 10 min, the pellet resuspended in 25 ml of ice cold PBS, 1 mg lysozyme/ml (aMResco, USA) added and the mixture incubated for 10 min on ice. To the mixture, 1 mM phenylmethanesulfonyl fluoride (PMSF) (Sigma) was added, and this mixture vortexed and then incubated overnight at 4°C. The cell suspension was sonicated 10 times for 10 sec each on ice and 1% v/v Triton X-100 (Plusone-Pharmacia Biotech) added and the mixture vortexed and incubated for 30 min on ice. The mixture was then centrifuged at 6,500 x g for 10 min at 4°C and the pellet and supernatant were stored separately. To prepare the glutathione affinity column, 1.33 ml of glutathione-sepharose 4B beads (GE Healthcare) were centrifuged at 500 x g for 5 min and the supernatant removed. The beads were washed with PBS, centrifuged as above and the pellet of beads carefully placed in a Poly-Prep chromatography column (BioRad) and left to settle at 4°C for 10 min. The column was equilibrated by passing 5 ml of ice cold PBS through it. The cell supernatant was filtered through a 0.45 µm nitrocellulose filter (Millipore) and then placed on the column and allowed to pass through. The column was then washed 3 times with 10 ml ice cold PBS and 10 ml of elution buffer (50 mM Tris-HCl [pH 8], 10 mM reduced glutathione [GE Healthcare]) was added to the column and left on it overnight at 4°C. After 18 h of incubation, the protein was eluted from the column in 1 ml aliquots. The eluted fractions were tested by electrophoresing a 2 µl sample of each aliquot through a 10% w/v polyacrylamide gel by SDS-PAGE and then staining the proteins with Coomassie blue.

Purification and estimation of protein concentration

[0191] The aliquots containing significant amounts of the recombinant proteins were dialysed (10 kDa cutoff [BioRad]) against 4 changes of 100 ml volumes of PBS at 4°C. The concentration of protein was determined using the BioRad protein assay (BioRad) according to the manufacturer's instructions. The protein was then stored at -80°C in 1 ml aliquots.

Sequencing of the pGEX-4T-1 gene constructs expression milA gene fragments

[0192] Plasmid DNA of clones expressing the MilA-AB, MilA-CD, MilA-A, MilA-B, MilA-C, MilA-D, MilA-ab and MilA-cd proteins was extracted using the Wizard Plus SV Minipreps kit (Promega). Approximately 600 ng of plasmid DNA of each construct was used as the template in 20 µl sequencing reactions containing the BDT sequencing terminator mix, BDT dilution buffer and 5 µM of the primer (pGEXFwd or pGEXRev). The reaction was incubated at 96°C for 1 min, followed by 30 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 4 min. The reaction products were purified by adding 125 mM EDTA, 2.5 M sodium acetate and 100% v/v ethanol to a total volume of 74.4 µl, and the mixture vortexed, incubated on ice for 15 min and centrifuged for 20 min at 16,000 x g at RT. The supernatant was carefully removed and the pellet was washed in 70% v/v ethanol, centrifuged as above for 5 min, the supernatant discarded and the pellet dried by incubation in a heating block at 95°C for 1 min. The sequencing products were analysed by capillary sequencing and the sequences aligned to the predicted sequence using Geneious Pro 5.1.6.

Characterization of MilA protein

[0193] The protein sequences of the different recombinant proteins were aligned to the predicted gene sequences using both Pfam (Sanger Institute) and BlastP (NCBI [National Center for Biotechnology Information]).

[0194] For the specific detection of lipase activity, two fold dilutions (2 µM, 1 µM, 0.5 µM, 0.25 µM and 0.125 µM) of recombinant GST-MilA-ab were incubated at room temperature with 100 µg of 1,2-O-dilauryl-rac-glycero-3-glutaric acid resorufin methyl ester (Sigma), as described by Schmidt *et al.* (2004) *J. Bacteriol 186*:5790-5798. Similar

concentrations of GST-P65 and GST were used as positive and negative controls, respectively. The release of resorufin was detected using a spectrophotometer (BioTek) with absorbance measured at 572 nm each minute for 30 min.

Optimized indirect IgG ELISA using GST-MilA-ab

[0195] The ELISA method used was adapted from Duffy *et al.* (1999) *J. Clin. Microbiol. 37*:1024-1029. Each well contained 1.2 µg of GST-MilA-ab and blocking was performed with 5% v/v sheep serum in phosphate buffer saline. Dilutions of positive control, negative control and test sera were made in 2.5% v/v sheep serum in PBS-T (PBS containing 0.05% v/v Tween 20) by preparing two fold dilutions of the positive control calf serum pool starting from 1/75, to 1/9600, as well as a single dilution of the negative control calf serum pool (1/300) and of test sera (1/300). HRP-conjugated sheep anti-bovine IgG heavy and light chain antibody (Bethyl) was diluted at 1/2000 in 2.5% sheep serum in PBS-T and used as the conjugate. After the final wash, 100 µl of 0.3 g/L 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) [ABTS Peroxidase Substrate, KPL] was added to each well and the plate incubated for 7 min at room temperature. The reaction was stopped by addition of 100 µl of 1% w/v SDS to each well, and the absorbance of each well measured at 405 nm using a Hybrid Multimode microplate reader (BioTeK).

Testing the sensitivity of indirect IgG ELISA

[0196] Calf sera collected at different time points during Experiments 1 and 2 were tested using the IgG ELISA protocol described as above. The antibody titer for each serum sample was calculated by plotting the OD values on the standard curve generated using the dilutions of the positive control serum pool on each plate using the program DeltaSoft 3 (Biometallic, Inc). All statistical analyses were performed using the Mann-Whitney test in SPSS version 20 (IBM). The cut-off value for the IgG ELISA was calculated using the test results of all the uninfected animals in both experiments, including all the samples from Group 1 and the samples from day 0 from Group 2 (total of 83). The average antibody titer of these uninfected animals was calculated and considered as the cut-off value.

[0197] Test results from both experiments were pooled to calculate the sensitivity and

specificity of the IgG ELISA. The sensitivity was calculated separately for each time point and the sensitivity on day 24 after infection was considered to be the overall sensitivity of the IgG ELISA. The specificity was calculated using one randomly selected sample from each of the uninfected calves (Group 1-uninfected, Group 2-before infection, Group 3-vaccine A).

EXAMPLE 1

Identification of Mycoplasma immunogenic lipase A (MilA)

[0198] In Western blots a single protein with a molecular weight greater than 170 kDa was recognized in serum antibodies from all the infected calves in Experiment 1 at day 24 after infection. Sera from the uninfected calves and sera collected on day 0 from infected calves did not contain antibody recognising this protein (Figure 2). A similar sized protein (226 kDa) was detected in whole cell proteins of *M. bovis* strain 3683 and *M. agalactiae* strain PG2, but antibodies in infected calf sera only bound to the protein in *M. bovis* (Figure 3). Mass spectrometric analysis identified the coding sequence as that of MBOVPG45_710, which is predicted to be a 302.9 kDa membrane protein in *M. bovis* PG45 (accession number 313678759). Only the amino terminal 60% of the coding sequence was identified, with peptides derived from the remaining 40% not detected, explaining the lower molecular weight estimated for the protein from SDS-polyacrylamide gels.

EXAMPLE 2

Expression of recombinant MilA protein

[0199] The *MilA*-AB, *MilA*-CD, *MilA*-A, *MilA*-B, *MilA*-C, *MilA*-D, *MilA*-ab and *MilA*-cd regions were successfully inserted into pGEX-4T-1 and introduced into *E. coli* JM109 cells. Recombinant GST-MilA-CD, GST-MilA-B, GST-MilA-C, GST-MilA-D and GST-MilA-cd were highly expressed, but did not react with *M. bovis*-specific calf sera. GST-MilA-AB and GST-MilA-A reacted weakly, but GST-MilA-ab reacted strongly with *M. bovis*-specific calf sera (Figure 4). DNA sequencing established that all the cloned inserts had the expected sequences.

EXAMPLE 3

Construction of pGEX-4T-1 expression plasmids for regions of MBOVPG45710 (milA)

[0200] The coding sequences of *milA* were synthesized as two 3 kb fragments, *milA*-AB and *milA*-CD, with *Bam*HI and *Sal*I cleavage sites located at the amino and carboxyl terminal ends, respectively. The two 3 kb fragments were able to be reduced in size by inclusion of *Bgl*II and *Xho*I sites at their mid-points, producing four 1.5 kb fragments, *milA*-A, *milA*-B, *milA*-C and *milA*-D. The primers abF and abR, and cdF and cdR, were designed to amplify the 1.8 kbp *milA*-ab and 1.84 kbp *milA*-cd products, respectively. PCR and RE digestion screening also confirmed that the plasmids pGEX-4T-1-*milA*-AB, pGEX-4T-1-*milA*-CD, pGEX-4T-1-*milA*-A, pGEX-4T-1-*milA*-B, pGEX-4T-1-*milA*-C and pGEX-4T-1-*milA*-D, which were cloned in *E. coli* JM109 cells, contained the expected inserts. PCR and RE digestion screening also confirmed that pGEX-4T-1-*milA*-ab and pGEX-4T-1-*milA*-cd, which were cloned in *E. coli* JM109 cells, contained the expected inserts.

EXAMPLE 4

Expression of recombinant proteins

[0201] The predicted molecular weight (MW) of the recombinant proteins GST-MilA-AB, GST-MilA-CD, GST-MilA-A, GST-MilA-B, GST-MilA-C and GST-MilA-D were 135.5 kDa, 137 kDa, 82.8 kDa, 79 kDa, 72 kDa and 73.3 kDa, respectively. The actual MWs of the recombinant proteins are summarized. The GST-MilA-CD, GST-MilA-B, GST-MilA-C and GST-MilA-D recombinant proteins were highly expressed, but did not react with *M. bovis*-specific calf sera. The GST-MilA-AB and GST-MilA-A proteins reacted weakly with *M. bovis*-specific calf sera. All the GST fusion proteins bound anti-GST antibody strongly. The recombinant proteins GST-MilA-ab and GST-MilA-cd were predicted to have MWs of 92.9 kDa and 94.3 kDa, respectively. Only GST-MilA-ab reacted with *M. bovis*-specific calf sera, while both GST-MilA-ab and GST-MilA-cd bound to anti-GST antibody.

[0202] DNA sequencing and analysis established that the cloned *milA*-AB, *milA*-CD, *milA*-A, *milA*-B, *milA*-C, *milA*-D, *milA*-ab and *milA*-cd inserts had the expected sequences.

EXAMPLE 5

Characterization of MilA

[0203] The gene encoding MBOVPG45_710 (*milA*) is predicted to code for a 302 kDa protein of 2700 aa in the *M. bovis* reference strain PG45. Further analysis revealed that it contained a membrane spanning region at its amino terminal end, with the remainder of the protein predicted be located extracellularly. Homologs were found in *M. bovis* strain Hubei-1, *M. agalactiae* strains 5632 and PG2, *M. columbinum* SF7 and *M. fermentans* strains JER and M64 (Table 4). Searches of the Pfam database identified an SGNH_hydrolase region at the amino terminal end (aa 1–375) that belonged to a family of GDSL-like lipases (Figure 5). The SGNH_hydrolase region is characterized by the conserved catalytic site amino acids Ser, Gly, Asp and His. The active site catalytic triad, Ser-His-Asp is in block 1 (Ser 99) and block V (His 362 and Asp 360). Alignment of region 1 of MilA (aa 1-375) with other mycoplasma lipases demonstrated that in MilA, the Ser, Gly, Asp and His residues in blocks I, II, III and V were conserved (Figure 6). In block I, the conserved amino acid sequence is GDSI, rather than the GDSL motif of the GDSL family lipases, as found in *M. hyoneumoniae* mhp677 {Schmidt, 2004 #1241}. In block II, Gly is conserved, while in block III, the sequence AXND is conserved in both *M. bovis* PG45 and *M. bovis* Hubei-1, although in most of the hydrolase family this sequence is GXND, as it is in *M. fermentans*, *M. agalactiae* and *M. columbinum*. In Block V all mycoplasma MilA homologs have the sequence DIHP, instead of the more typical DXXHP sequence. Analysis of genome sequences adjacent to *MilA* revealed the presence of *rpoC*, *rpoB* and a lipoprotein gene upstream, and a hypothetical protein, transposase and aminopeptidase genes downstream (Figure 5). Physical maps of MilA homologs are shown in Figure 13. The *MilA* gene is located downstream of *rpoC* and *rpoB* in all genomes, the exception being the second copy of the gene in *M. bovis* strain Hubei-1. In most species the genes downstream of the *MilA* homolog differ from each other.

[0204] The recombinant GST-MilA-ab protein catalyzed release of resorufin from 1,2-O-dilauryl-rac-glycero-3-glutaric acid resorufin methyl ester over a 30 min period. Within the 30 min period the OD_{572} of GST-MilA-ab changed from 0.18 to 0.3, while GST-P65 changed from 0.17 to 0.2 only indicating the release of resorufin by GST-MilA-ab was greater than that released by GST-P65. The OD_{572} of GST was constant at 0.135 as it was unable to catalyze any release of resorufin (Figure 7).

EXAMPLE 6

Sensitivity of IgG ELISA in experimental studies

Experiment 1

[0205] No calves in the uninfected group had detectable changes in antibody titers against GST-MilA-ab, throughout the experiment. The calves in the infected group did not have significant increase in antibody titers until 3 weeks after infection (Table 5). The mean IgG titer of uninfected calves was 23.4 ± 12, so a cutoff of 47.4 (mean + 2 x SD) was used to define positive test results. On day 10 after inoculation none of the infected calves were positive, but by day 17 two animals were positive, and by day 24 eleven animals were positive.

Experiment 2

[0206] As in Experiment 1, infected calves had high IgG titers by day 24 (Figure 9 and Table 6). In the uninfected group 2 calves were above the cutoff value of 47.4 at day 10, but only one remained above the cutoff on days 17 and 24. In the infected group four calves were positive on day 17 and twenty eight were positive on day 24.

[0207] The sensitivity of the IgG ELISA was 0% and 14.3% on days 10 and 17, respectively. The overall sensitivity of IgG ELISA was 92.8% and specificity was 98.7%.

Experiment 3

[0208] There was no increase in the antibody titer against the recombinant *M. bovis* MilA-ab protein in calves in Group 3 (Vaccine A) until 2 weeks after challenge, after

which it continued increasing until the calves were euthanized (day 45) [Table 7]. The titers were significantly higher than those of animals in the negative and challenged only groups on day 17, but by day 24 were only significantly different from those of calves in the negative control group, as in the calves in Group 2 IgG titers had increased by 2 weeks after challenge.

[0209] The results were similar to those in Experiment 2 when a cut off value of 65 was assigned to the assay. On day 0, before vaccination or challenge, 9 animals were positive, but by day 7 the same animals were negative, except for two in Group 3. Ten days after challenge, 4 animals in Group 3 were positive, while 3 and 19 animals on day 17, and 27 and 31 animals on day 24 were positive in Groups 2 and 3, respectively (Table 8 and Figure 10).

[0210] The sensitivity of the IgG ELISA for wild-type and vaccinated calves at each time point is shown in Table 9. The overall sensitivity of the IgG ELISA was 91 % and specificity was 98.4 %.

Optimization of the IgM ELISA

[0211] A 1/50 test serum dilution was selected, together with a HRP-conjugate dilution of 1/2000, as this offered the best discrimination between infected and uninfected animals. The development time for the ABTS substrate was increased to 20 min and the dilution series of the primary antibody for the standard curve for the test plate was 1/5, 1/10, 1/20, 1/40, 1/80, 1/160, 1/320 and 1/640.

Sensitivity of the IgM ELISA in experimental studies

[0212] Based on the results shown in Table 10, on the day of challenge there was no significant difference between the IgM titers of the three groups. On day 10 after challenge the calves in Group 3 had begun to develop significantly higher IgM titers than the calves in the other two groups, while by day 17 calves in Group 2 were also developing increased titers compared to the calves in Group 1. The calves in both Groups 2 and 3 continued to show an increase in IgM antibody titers up to day 45 (Table 10).

[0213] The mean titer for the uninfected calves in the IgM ELISA was 99, and the cut off value was calculated to be 287 (99 + 188). On day 0, three animals were positive, but all were considered negative one week later. At day 14 after vaccination, 2 animals in Group 3 were positive, and the number of positive animals increased to 3, 5, 9 and 11 at each time point. Only one animal in Group 2 was positive before challenge, but the number increased to 11 and 18 on days 17 and 24 after challenge (Table 11 and Figure 11).

[0214] The sensitivity of the IgM ELISA for wild-type and vaccinated calves at each time point is shown in Table 12. The overall sensitivity of the IgM ELISA was 47.5 % and specificity was 98.8 %.

[0215] The data show that the test can be used to evaluate the immune status of vaccinated cows.

EXAMPLE 7

IgG titers in feedlot cattle

Materials and Methods

Cattle Sera

Experimental study

[0216] Sera from ninety Frisian-cross claves aged one month were collected from two similar experiments. Experiments 1 and 2 have been described above. Briefly, experiment 1 had 24 calves, which were allocated into three groups. Group 1 consisted of 4 calves exposed to aerosol of mycoplasma culture medium, Group 2 consisted of 12 calves exposed to *Mycoplasma bovis* strain 3683, while Group 3 consisted of 8 calves exposed to *M. bovis* temperature sensitive vaccine strain A on days 1 and 3 and challenged together with calves from Group 2 with *M. bovis*strain 3683 three weeks after vaccination. Aerosol exposure was carried out as described before(Wawegama *et al.* (2012) *supra*). Sera were collected from all the calves on days 0, 7, 14, 18, 28, 35 and 42 and all the calves were euthanized and necropsied on day 42 for lung lesions as described previously (Wawegama *et al.* (2013) *supra*). In Experiment 2, sixty six one-month old Frisian cross calves were allocated into three groups. Group 1 contained 5 calves exposed to aerosol of mycoplasma culture medium, Group 2 contained 30 calves exposed to *M. bovis*strain 3683 and Group 3 contained 31 calves vaccinated and challenged as described in experiment 1. Aerosol infection and sampling was performed as described for Experiment 1.

[0217] All the calves were tested negative for BVDV and *M. bovis*prior to the commencement of these experiments. The three groups were separately housed and observed for clinical signs throughout the experiments. At necropsy Group 2 calves showed varied degree of lobular pulmonary consolidation and Group 3 had mild lobular lesions but none in Group 1 calves. Culture and PCR confirmed presence of*M. bovis* only from swabs from trachea, bronchi and lesions in Group 2 and 3 calf lungs and no specific pathogen from Group 1.

Dairy cattle herd

[0218] Fifty two serum samples were collected from adult cattle in a dairy herd in New South Wales, Australia. The herd have been tested negative for BVDV and *M. bovis* using PCR assay and had no record of respiratory infection for the last few years and there have been no heifer introduction to the herd from other farms, therefore, considered as a closed herd for *M. bovis* incidence.

Feedlot cattle

[0219] The sera supplied from University of Queensland, Australia were collected at two time points (1) introduction to a feedlot; and (2) six weeks later. A total of 7,448 cattle were tested. The sera were frozen at -20 °C until analyzed. All the sera were analysed blindly.

In-house IgG ELISA

[0220] The recombinant protein based *M. bovis*-specific IgG ELISA (see above) was used to test sera collected from all the three resources. Antibody units (AU) were calculated for each sample using the standard curve on each ELISA plate and DeltaSoft 3 (Biometallic, inc.).

Data analysis

[0221] Statistical analysis and graphs were performed using Graphpad prism 5. The results of the two experimental studies were combined together and descriptive statistics were computed for the 3 groups at each time point. Differences between AU on each day and between each group were analysed using Mann-Whitney tests.

[0222] The cut-off value for the IgG ELISA was calculated by considering all the uninfected calves in both experiments, including all the sera from Group 1, sera from day 0 to day 18 from Group 2 and sera from day 0 from Group 3. The sensitivity was calculated at day 42 and the specificity was calculated for a randomly selected time point.

[0223] The closed herd was considered as negative for *M. bovis* and descriptive statistics were computed and cut off value was calculated.

[0224] The feedlot cattle sera collected at introduction (Day 0) were considered as negative for *M. bovis* as their original herd disease status is unknown and the cut-off value was computed. The cattle with AU higher than the calculated cut-off value were grouped into 3 band scores >300AU, 300-250 AU and 250-200AU and the cattle come under each band and any other cow in contact with them within 2 weeks before the introduction to the feedlot were removed from the negative sample and descriptive statistics and cut-off value were calculated. Once the cut-off is selected *M. bovis*sero-prevalence was calculated using the AU values of all the cattle sera.

Results

Experimental study

[0225] The results of Group 1 and Group 2 of both experiments are discussed above. The new mean AU calculated after addition of Group 3 antibody titres are summarized in Table 14. The calves in Group 3 had significantly higher AU than animals in Group 1 and 2 on day 17 after challenge but by day 24 the animals in Group 2 had developed AU similar to those calves of Group 3. The mean AU of uninfected calves was 25 ± 20, therefore, a cut-off of 65 (mean + 2 x SD) was computed. In combined experiments results 24 days after challenge 36 animals out of 42 in Group 2 and 38 out of 39 in Group 3 were sero positive for *M. bovis* (Table 14).

[0226] The sensitivity at day 42 (24 days after challenge) was calculated as 91 % and specificity as 98.4%.

Dairy cattle herd

[0227] The mean antibody titer for the dairy herd was calculated as 36 ± 14.13, therefore, the cut-off was calculated as 64.3 AU. According to the cut-off all the cows except 2 were sero-negative for *M. bovis* and the 2 positive cows had antibody units less

than 1.5 times the cut-off. Specificity of the IgG ELISA using the dairy herd sera was calculated as 96%.

Feedlot cattle

[0228] The descriptive statistics of the *M. bovis*-specific antibody units of the cattle before introduction to the feedlot herd and after are summarised in Table 15. The mean was calculated as 56.5 ±70.2 with a cut-off of 196.8. After removal of cattle with antibody units higher than 300 and their contact cattle data the mean had dropped to 46.55 and cut off was calculated as 123.5 AU. With the removal of cattle with AU between 300-250 and 250-200 have sightly changed the cut-off from 117.3 to 111.4 AU (Table 15). The cut-off 111.4 was selected as the steady cut-off point for the feedlot cattle as the two serum populations day 0 and day 42 cross over close to 111.4 (Figure 14). Out of 7448 cattle, 90% were sero-negetive for *M. bovis* before introduction to the feedlot herds and 73.5 % of them have sero-converted after 6 weeks in the feedlot, either due to infection, exposure or subclinical infection with *M. bovis*.

[0229] Hence, the *M. bovis*-specific IgG ELISA is able to detect *M. bovis* infection in calves as well as adult cattle and can be used as a sero-diagnostic assay to screen for carriers for *M. bovis*.

EXAMPLE 8

Further characterization of MilA

[0230] MilA is a 226 kDa protein identified from *M. bovis* strain 3683 by Western blotting. MilA is immunoreactive based on *M. bovis*-specific calf sera. MilA was estimated to be 226 kDa, but the gene encoding it, MBOVPG45_0710 (*MilA*), which was identified by mass spectrometri analysis of tryptic peptides derived from it, was predicted to encode a 302 kDa protein, suggesting that MilA may be cleaved into 2 peptides, as is seen with the ciliary adhesin of *M. hyopneumoniae*.

[0231] A search of the Pfam database with MilA revealed that it belonged to the GDSL-like lipase family. The SGNH_hydrolase superfamily is characterized by conserved single residues of serine, glycine, aspargine and histidine in each of the four blocks I, II, III and V (Mølgaard *et al.* (2000) *Structure 8*:373-383). Although most carboxylesterase genes encode the conserved GxSxG motif (Wei *et al.* (1995) *Nat. Struct. Mol. Biol. 2*:218-223), Upton and Buckley (1995) *Sci. 20*:178-179 identified this new family of hydrolases (lipases) to contain a GxSxS (GDSLS) motif. In MilA, the conserved amino acid serine is present in block I in the motif GDSI, not GDSL as in the amino-terminal homolog mhp677 / P65 of *M. hyopneumoniae*. In block III, MilA and the *M. columbinum* and *M. fermentans* homologs (Rechnitzer *et al.* (2011) *Microbiology 157*:760-773; Shu *et al.* (2011) *J. Bacteriol. 193*:4302-4303) contain the conserved AND motif, but not the glycine in the conserved GXND motif found in other hydrolases (Lo *et al.* (2003) *J. Mol. Biol. 330*:539-551). Block V contains the conserved motif DXXHP, but in all the Mycoplasma proteins, including mhp677 in *M. hyopneumoniae* and MilA, the conserved motif was DXHP. Therefore, the Mycoplasma lipases/hydrolases comprise a new lipolytic enzyme family.

[0232] Under experimental conditions an IgG ELISA based on a fragment of MilA is a fast, convenient, sensitive and reproducible method to detect cattle infected with *M. bovis*. The concentration of recombinant MilA antigen needed (1.2 µg/well) was quite low, highlighting its sensitivity for use as an ELISA antigen. The dilution used to test sera (1/300) was higher than that used in other ELISAs (Bereiter *et al.* (1990) *Vet. Microbiol.*

25:177-192), allowing more freedom to adjust the test serum dilution and thus assess very low concentrations of antibody against *M. bovis*. An increase in *M. bovis*-specific IgG titers could be detected 3 weeks after experimental infection and the ELISA was found to have a high sensitivity, making it a suitable diagnostic tool for field situations.

[0233] Use of a single, highly immunoreactive protein is highly likely to result in a more specific ELISA. The most immunoreactive antigens in the *M. bovis* cell membrane are the Vsps (variable surface proteins), but while the Vsps may have diagnostic utility (Brank *et al.* (1999) *Clin. diagn. Lab. Immunol. 6*:861-867), they are subject to phase variation and antigenic variation, which may result in variations in the antibody response against them in individual infected cattle and compromise the sensitivity of the assay (Poumarat *et al.* (1999) *supra*; Buchenau *et al.* (2010) *Res. Vet. Sci. 89*:223-229). Therefore, use of a protein such as MilA that is not subject to phase or antigenic variation, but is still highly immunoreactive, is proposed to result in a useful diagnostic tool.

[0234] Those skilled in the art will appreciate that the disclosure described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the disclosure contemplates all such variations and modifications. The disclosure also enables all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of the steps or features or compositions or compounds.

Table 4

Homologs of milA in other mycoplasma species

Organism	Gene name	Predicted gene function	Query coverage (% of residues)	Amino acid sequence similarity
M. bovis PG45	MBOVPG45_0710 (*milA*)	Membrane protein	100%	100%
M. bovis Hubei-1	MMB_0654	Conserved hypothetical protein	99%	92%
M. agalactiae str 5632	MAGa6830	Conserved hypothetical protein	100%	89%
M. agalactiae PG2	MAG_6100	Conserved hypothetical protein	100%	89%
M. bovis Hubei-1	MMB_0318	Conserved hypothetical protein	99%	75%
M. columbinum SF7	MCSF7_01871	Lipase	99%	48%
M. fermentans JER	MFE_02570	Lipase	97%	49%
M. fermentans M64	MfeM64YM_0307	Hypothetical protein	97%	49%

Table 5

Anti-M. bovis IgG titers of calf sera from Experiment 1

	Group	Number of calves	Geometric mean of anti-*M. bovis* IgG titers		
			Days after challenge		
			10	17	24
1	Uninfected	4	1.38[a]	1.4[a]	1.35[a]
2	Infected	12	1.26[a]	1.35[a]	2.15[b]

Values marked with the same superscript letters are not significantly different ($P < 0.05$)

Table 6

Anti-M. bovis IgG titers of calf sera from Experiment 2

	Group	No. calves	Geometric mean of anti-*M. bovis* IgG titers		
			Days after challenge		
			10	17	24
1	Uninfected	5	1.35[a]	1.23[a]	1.25[a]
2	Infected	30	1.12[a]	1.3[a]	2.3[b]

Values marked with the same superscript letters are not significantly different (P <0.05)

Table 7

Anti-M. bovis IgG titres of calf sera from Experiment 3

	Group	No. calves	Geometric mean anti-*M. bovis* IgG titres		
			Days after challenge		
			10	17	24
1	Uninfected	5	1.35[a]	1.23[a]	1.25[a]
2	Wild-type	30	1.12[a]	1.3[a]	2.3[d]
3	Vaccine A	31	1.4[b]	1.97[c]	2.5[d]

Values marked with the same superscript letters are not significantly different (p < 0.05)

Table 8

Number of calves positive in each group in Experiment 3 using a cut off value of 65

Group	No. calves	Number of animals positive on day:						
		0	7	14	18	28	35	42
Uninfected	5	2	0	0	0	0	0	0
Wild-type	30	3	0	0	0	0	3	27
Vaccine A	31	4	2	0	3	4	19	31

Days after challenge are highlighted.

Table 9

Sensitivity of IgG ELISA

Group	Total no. calves	Sensitivity on day:					
		7	14	18	28	35	42
Wild-type	42	-	-	-	0	12%	85.7%
Vaccine A	39	5%	0	7.7%	15%	59%	97%
Total	81	-	-	-	-	-	91%

Days after challenge are highlighted.

Table 10

Anti-M. bovis IgM titres of calf sera from Experiment 3

	Group	No. calves	Geometric mean anti-*M. bovis* IgM titres			
			Days after challenge			
			0	10	17	24
1	Uninfected	5	2.02^{a}	1.9^{a}	1.78^{a}	1.89^{a}
2	Wild-type	30	1.89^{a}	1.99^{a}	2.3^{b}	2.35^{b}
3	Vaccine A	31	1.9^{a}	2.15^{a}	2.35^{b}	2.5^{b}

Values marked with the same superscript letters are not significantly different ($p < 0.05$)

Table 11

Number of calves positive in each group in Experiment 3 using a cut off value 287

Group	No. calves	No. positive on day:						
		0	7	14	18	28	35	42
Uninfected	5	0	0	0	0	0	0	0
Wild-type	30	1	0	0	0	1	11	18
Vaccine A	31	2	0	2	3	5	9	11

Days after challenge are highlighted.

Table 12

Sensitivity of IgM ELISA

Group	Total no. calves	Sensitivity on day:					
		7	14	18	28	35	42
Wild-type	30	-	-	-	3%	36.7%	60%
Vaccine A	31	0	6.5%	9.7%	16%	29%	35.5%
Total	61	-	-	-	-	-	47.5%

Days after challenge are highlighted.

Table 13

Primers used to clone regions of the MBOVPG450710* (milA) *gene for expression

Name	Binding site	Sequence (5'-3')	SEQ ID NO:
abF-*Bam*HI	144-166	CAACggatccATCAAAGACGTGA	9
abR-*Sal*I	1931-1950	TTCgtcgacGGATTTCGCCT	10
cdF-*Bam*HI	3128-3150	CACTGggatccATCTCGAACATC	11
cdR-*Sal*I	4955-4872	ACAgtcgacCCAGGTTCG	12

Lower case letters – restriction endonuclease cleavage sites included to aid cloning of PCR products

Table 14

Number of calves positive in each group in experimental studies using a cut-off of 65 antibody units (AU)

Group	No. calves	Number of calves positive on day:						
		0	7	14	18	28	35	42
Uninfected	9	2	0	0	0	0	0	0
Challenged only	42	3	0	0	0	0	5	36
Vaccinated and challenged	39	5	2	0	3	6	22	35

Days after challenge are shaded

Table 15

Descriptive statistics for deedlot cattle* M. bovis*-specific antibody units on Day 0

	No. of animals	Mean AU	SD	Cut-off value	Sero-negative %
Total data	7448	56.5	70.2	196.8	96.5
AU >300 removed	5395	46.6	38.4	123.5	95.4
AU 300-250 removed	5373	45.6	35.8	117.3	95.1
AU 250-200 removed	5343	44.6	33.4	111.4	94.7

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CLAIMS:

1. A method for detecting current or prior exposure of a ruminant subject to a species of *Mycoplasma*, said method comprising contacting an antibody containing sample from the subject with an immobilized protein or derivative thereof comprising the amino acid sequence set forth in SEQ ID NO:1 or an amino acid sequence having at least 40% similarity to SEQ ID NO:1 after optimal alignment or an antibody-binding fragment of said protein, for a time and under conditions sufficient for an antibody specific for said protein, if present in the sample, to bind to said protein and then detecting for the presence of bound antibody wherein the presence of bound antibody is indicative of current or prior exposure to *Mycoplasma* spp.

2. The method of Claim 1 wherein the protein comprises an amino acid sequence selected from the listing consisting of SEQ ID NOs2: through 8 or SEQ ID Nos:15 through 21 or is an antibody-binding fragment thereof.

3. The method of Claim 2 wherein the protein comprises the amino acid sequence set forth in SEQ ID NO:2 or SEQ ID NO:15 or an amino acid sequence having at least 40% similarity to SEQ ID NO:2 or SEQ ID NO:15 after optimal alignment or is an antibody-binding fragment thereof.

4. The method of Claim 3 wherein the protein comprises the amino acid sequence set forth in SEQ ID NO:2 or SEQ ID NO:15 or is an antibody-binding fragment thereof.

5. The method of Claim 4 wherein the species of *Mycoplasma* is *Mycoplasma bovis*.

6. The method of Claim 1 wherein bound antibody is detected by an anti-immunoglobulin antibody labeled with a reporter molecule capable of providing an identifiable signal.

7. The method of Claim 6 wherein the anti-immunoglobulin antibody is an anti-IgG antibody.

8. The method of Claim 6 wherein the reporter molecule is selected from the list consisting of an enzyme, chemiluminescence molecule, radioactive isotope, or a molecule providing an impedance or optical signal.

9. The method of Claim 8 wherein the reporter molecule is an enzyme.

10. The method of Claim 8 wherein the chemiluminescence molecule is a fluorescent molecule.

11. The method of any one of Claims 1 to 10 wherein the sample is selected from the list consisting of serum, whole blood, milk, respiratory fluid or a nasal swab.

12. The method of Claim 1 wherein the ruminant subject is selected from a cow, sheep, goat, giraffe, yak, camel, llama, antelope and macropod.

13. The method of Claim 1 wherein the subject is a cow.

14. A device for screening for current or prior exposure of a ruminant subject to a species of *Mycoplasma*, the device comprising an immobilized protein or derivative thereof comprising the amino acid sequence set forth in SEQ ID NO:1 or an amino acid sequence having at least 40% similarity to SEQ ID NO:1 after optimal alignment or an antibody-binding fragment of said protein, the device further comprising means to contact a sample from the subject with the immobilized protein.

15. The device of Claim 14 wherein the protein comprises an amino acid sequence selected from the listing consisting of SEQ ID NOs: 2 through 8 or SEQ ID NOs:15 through 21 or is an antibody-binding fragment thereof.

16. The device of Claim 15 wherein the protein comprises the amino acid sequence set forth in SEQ ID NO:2 or SEQ ID NO:15 or an amino acid sequence having at least 40% similarity to SEQ ID NO:2 or SEQ ID NO:15 after optimal alignment or is an antibody-binding fragment thereof.

17. The device of Claim 16 wherein the protein comprises the amino acid sequence set forth in SEQ ID NO:2 or SEQ ID NO:15 or is an antibody-binding fragment thereof.

18. The device of Claim 14 wherein the ruminant subject is selected from a cow, sheep, goat, giraffe, yak, camel, llama, antelope and macropod.

19. The device of claim 18 wherein the ruminant subject is a cow.

20. The device of Claim 19 wherein the species of *Mycoplasma* is *Mycoplasma bovis*.

21. A method for controlling infection by a species of *Mycoplasma* in a ruminant subject, said method comprising administering to said subject an antibody-inducing effective amount of a protein or derivative thereof comprising the amino acid sequence set forth in SEQ ID NO:1 or an amino acid sequence having at least 40% similarity to SEQ ID NO:1 after optimal alignment for a time and under conditions sufficient to generate antibodies to said protein.

22. The method of Claim 21 wherein the protein comprises an amino acid sequence selected from the listing consisting of SEQ ID Nos: 2 through 8 or SEQ ID NOs:15 through 21 or is an antibody-binding fragment thereof.

23. The method of Claim 22 wherein the protein comprises the amino acid sequence set forth in SEQ ID NO:2 or SEQ ID NO:15 or an amino acid sequence having at least 40% similarity to SEQ ID NO:2 or SEQ ID NO:15 after optimal alignment or is an antibody-binding fragment thereof.

24. The method of Claim 23 wherein the protein comprises the amino acid sequence set forth in SEQ ID NO:2 or SEQ ID NO:15 or is an antibody-binding fragment thereof.

25. The method of Claim 21 wherein the ruminant subject is selected from a cow, sheep, goat, giraffe, yak, camel, llama, antelope and macropod.

26. The method of Claim 25 wherein the ruminant subject is a cow.

27. The method of Claim 26 wherein the species of *Mycoplasma* is *Mycoplasma bovis*.

28. A composition comprising a protein or derivative thereof comprising the amino acid sequence set forth in SEQ ID NO:1 or an amino acid sequence having at least 40% similarity to SEQ ID NO:1 after optimal alignment and one or more pharmaceutically acceptable carriers, excipients and/or diluents.

29. The composition of Claim 28 wherein the protein comprises an amino acid sequence selected from the listing consisting of SEQ ID NOs2: through 8 or SEQ ID NOs:15 through 21 or is an antibody-binding fragment thereof.

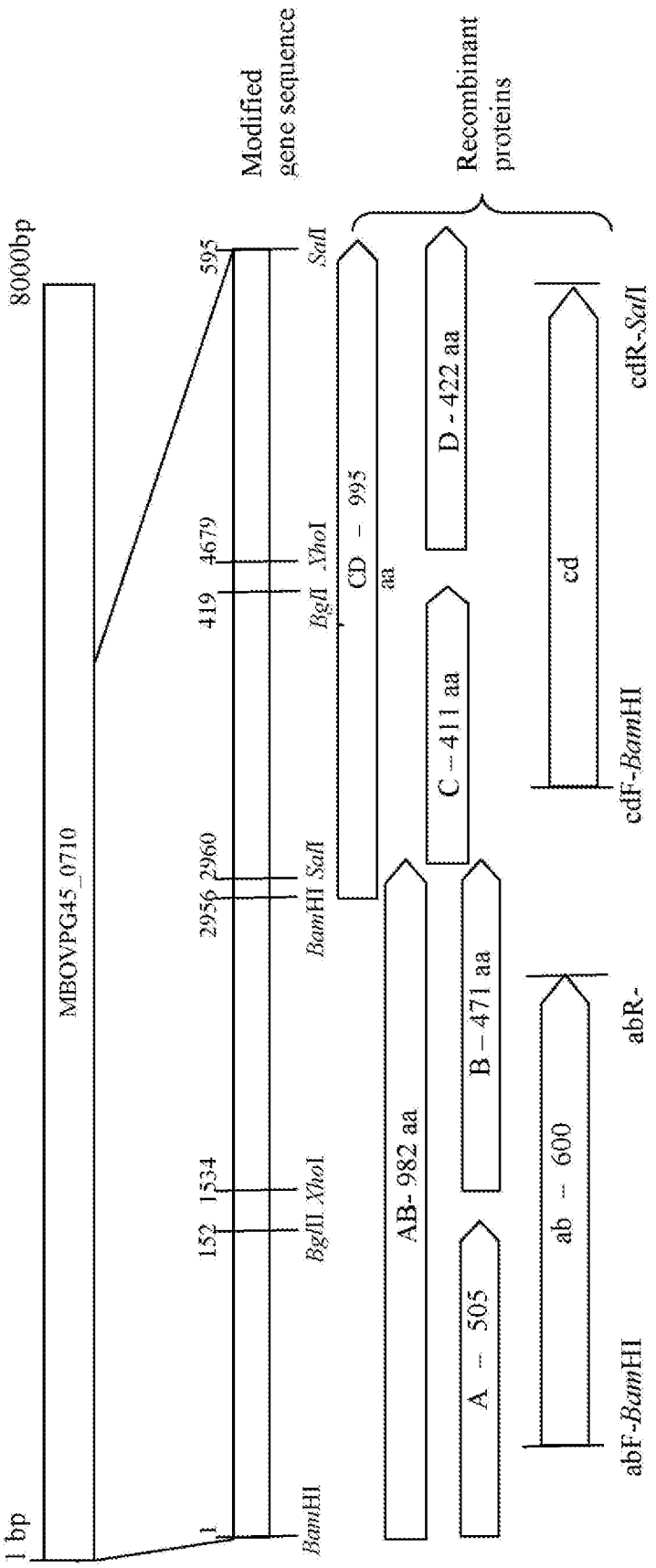
30. The composition of Claim 29 wherein the protein comprises the amino acid sequence set forth in SEQ ID NO:2 or SEQ ID NO:15 or an amino acid sequence having at least 40% similarity to SEQ ID NO:2 or SEQ ID NO:15 after optimal alignment or is an antibody-binding fragment thereof.

31. The composition of Claim 30 wherein the protein comprises the amino acid sequence set forth in SEQ ID NO:2 or SEQ ID NO:15 or is an antibody-binding fragment thereof.

32. Use of the composition of any one of Claims 28 to 31 in the manufacture of a medicament for the treatment or prophylaxis of *Mycoplasma* infection in a ruminant subject.

33. Use of Claim 32 wherein the ruminant subject is selected from a cow, sheep, goat, giraffe, yak, camel, llama, antelope and macropod.

34. Use of Claim 33 wherein the subject is a cow.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BglII
XhoI
BamHI
SalI
BglII
XhoI
SalI
AB- 982 aa
A – 505
B – 471 aa
ab – 600
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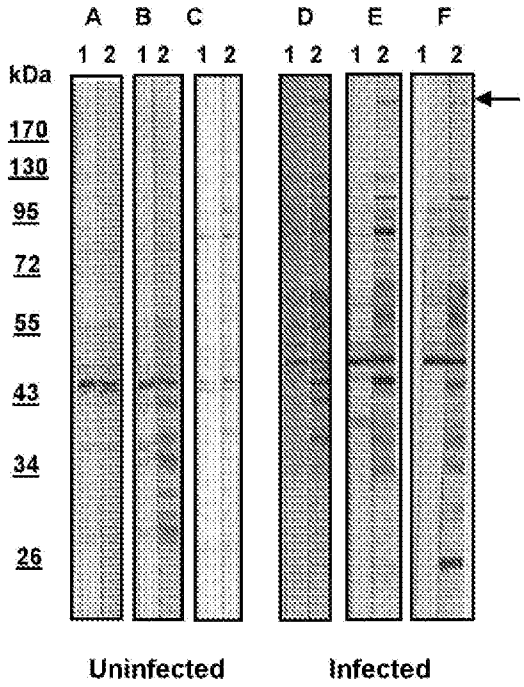
abR-
cdF-BamHI
cdR-SalI

Figure 1


A B C D E F
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kDa
170
130
95
72
55
43
34
26
Uninfected
Infected

Figure 2

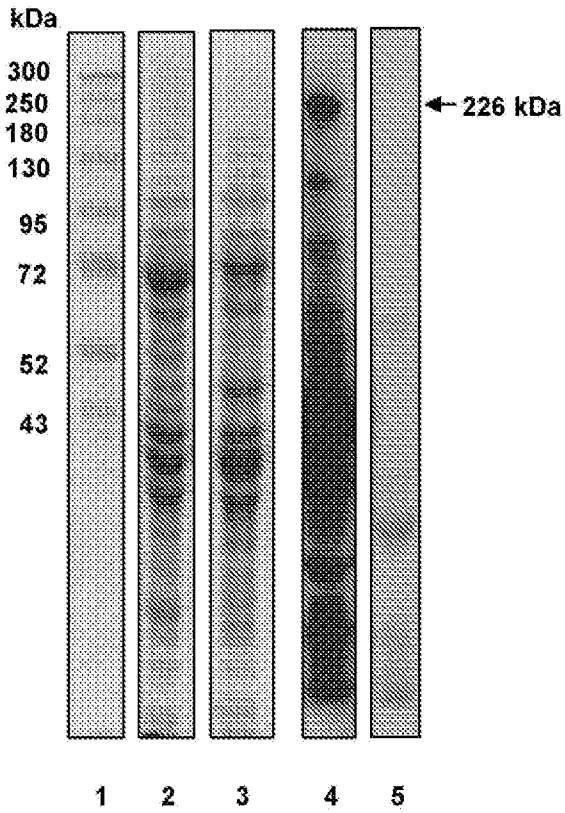
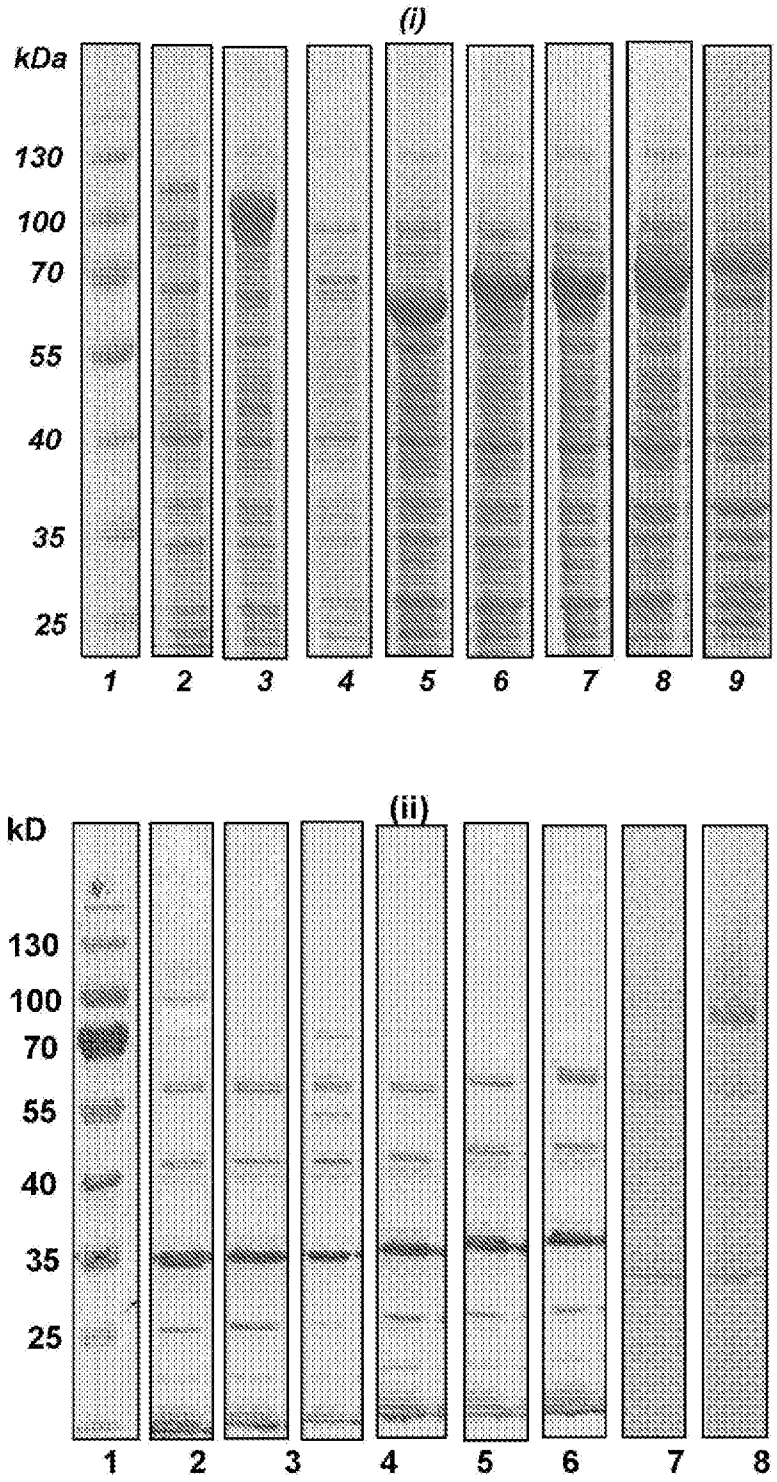
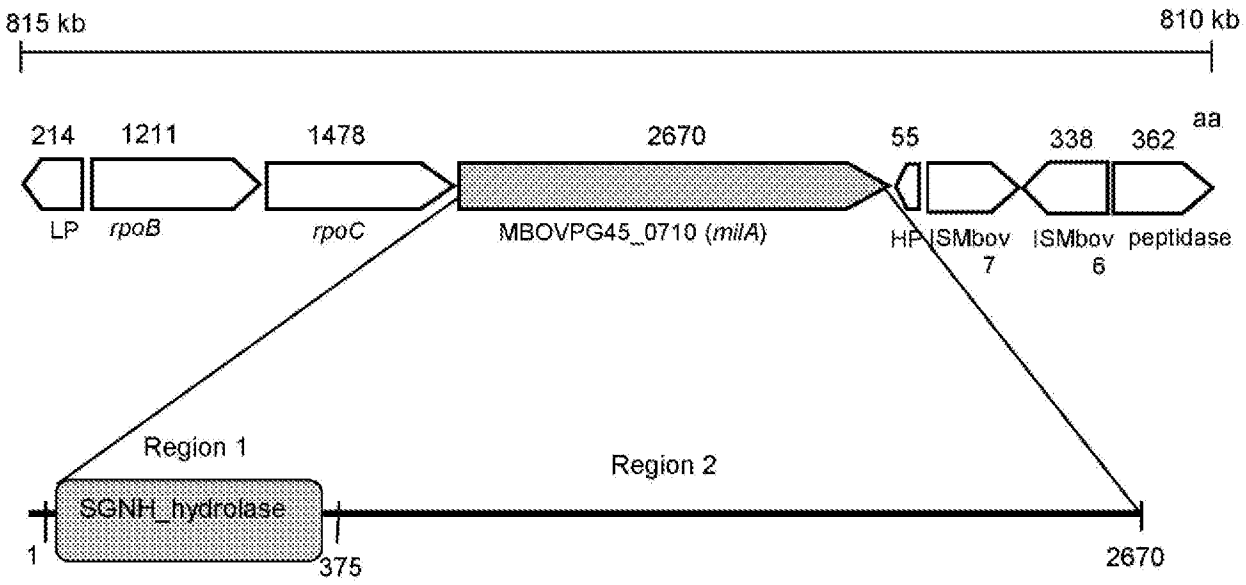


Figure 3



Figures 4(i) and (ii)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810 kb
214
1211
1478
2670
55
338
362
aa
LP
rpoB
rpoC
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ISMbov 7
ISMbov 6
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Region 1
Region 2
SGNH_hydrolase
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2670

Figure 5

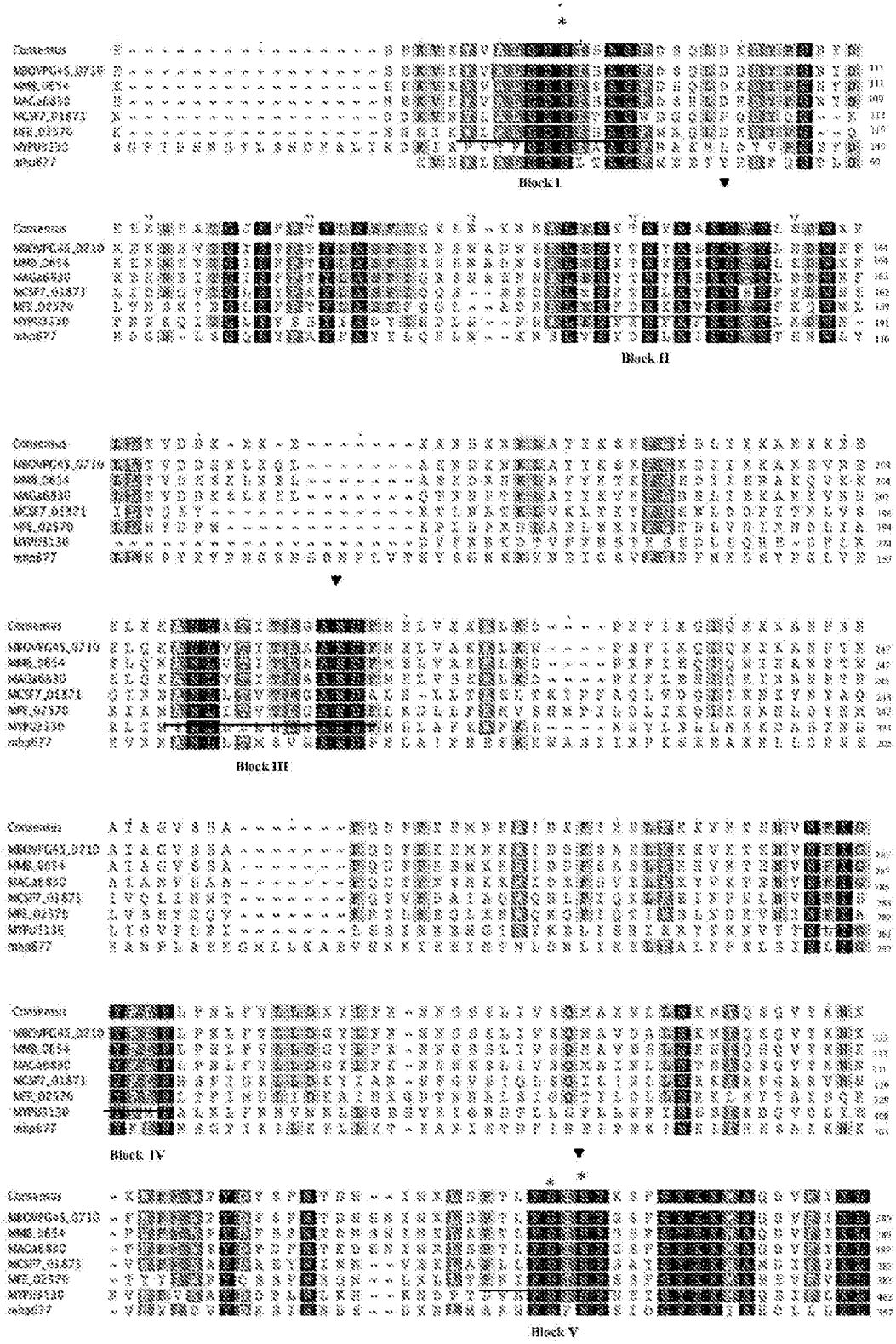
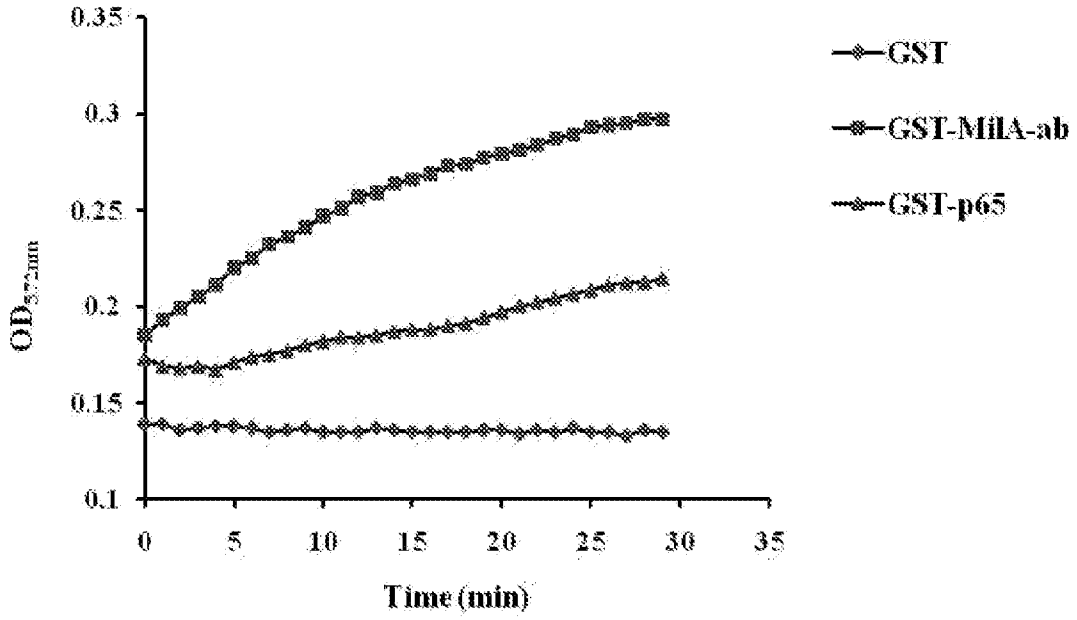


Figure 6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0.15
0.1
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15
20
25
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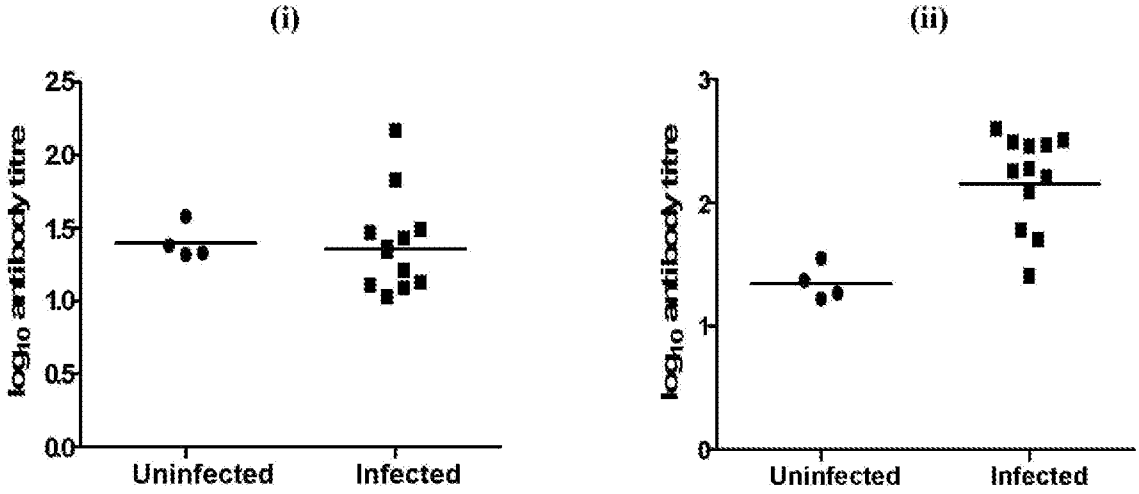
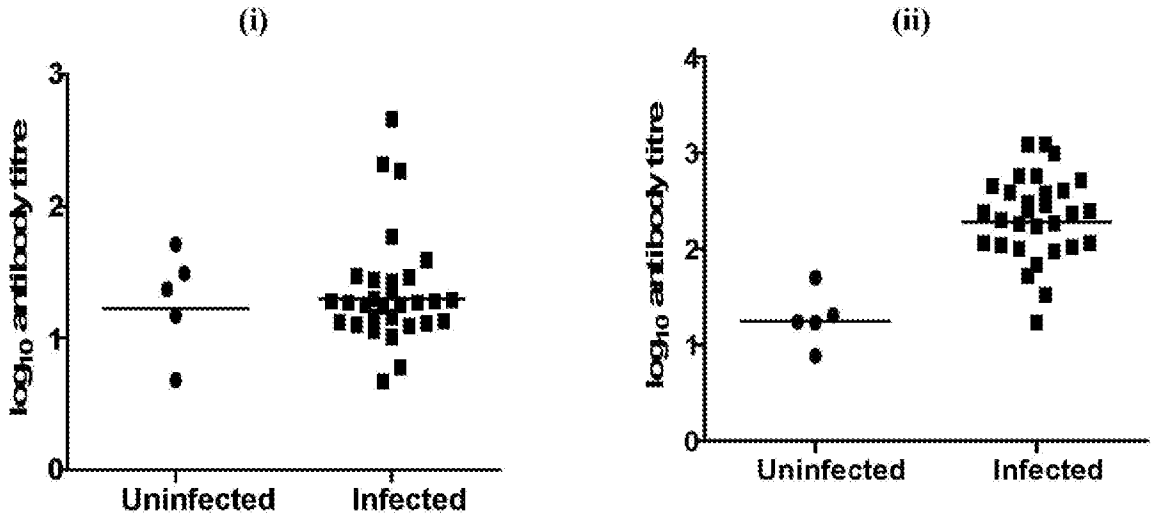
Time (min)
GST
GST-MiIA-ab
GST-p65

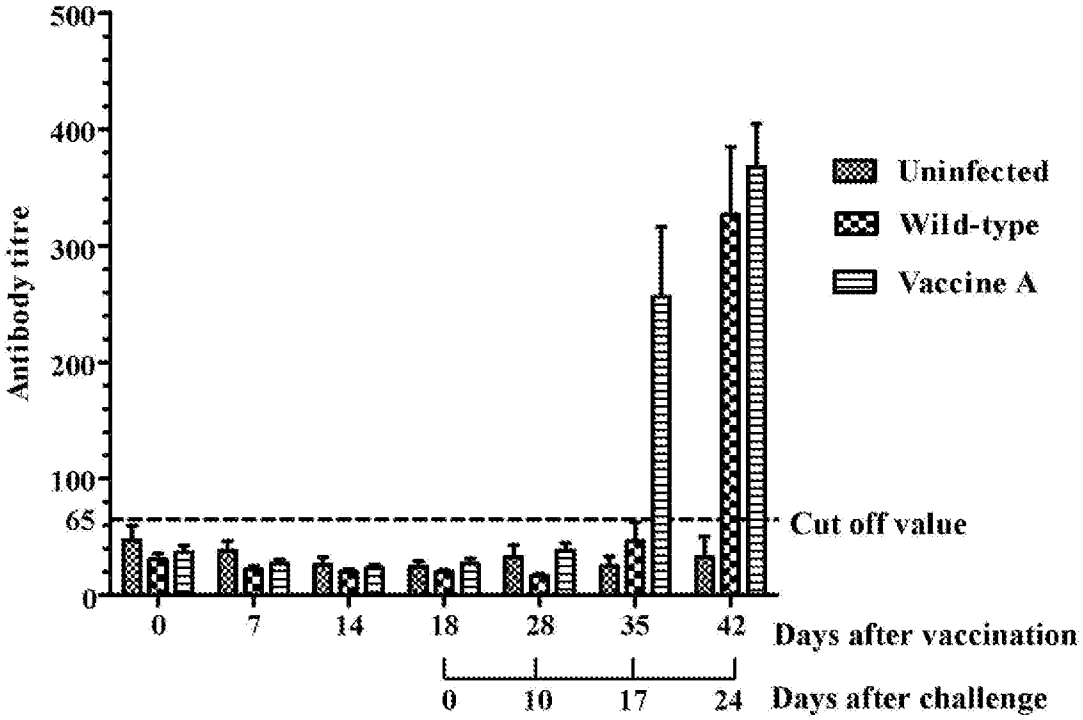
Figure 7



Figures 8(i) and (ii)



Figures 9(i) and (ii)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300
200
100
65
0
Antibody titre
Uninfected
Wild-type
Vaccine A
Cut off value
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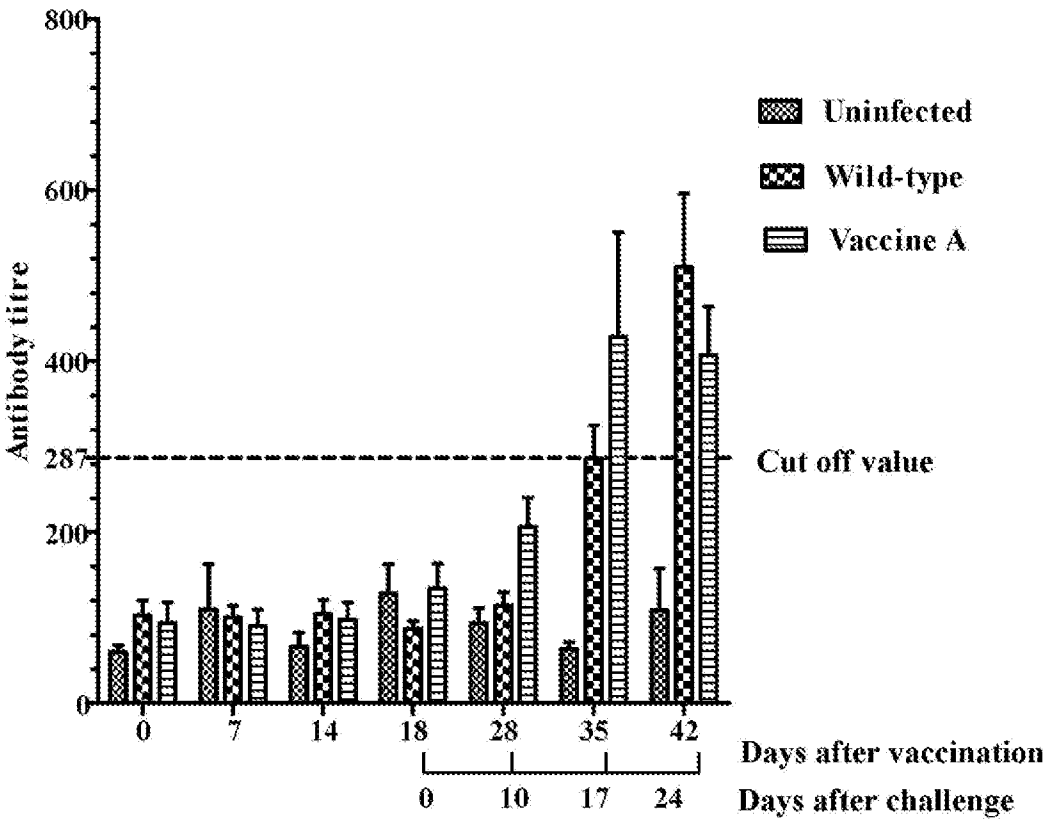
7
14
18
28
35
42
Days after vaccination
0
10
17
24
Days after challenge

Figure 10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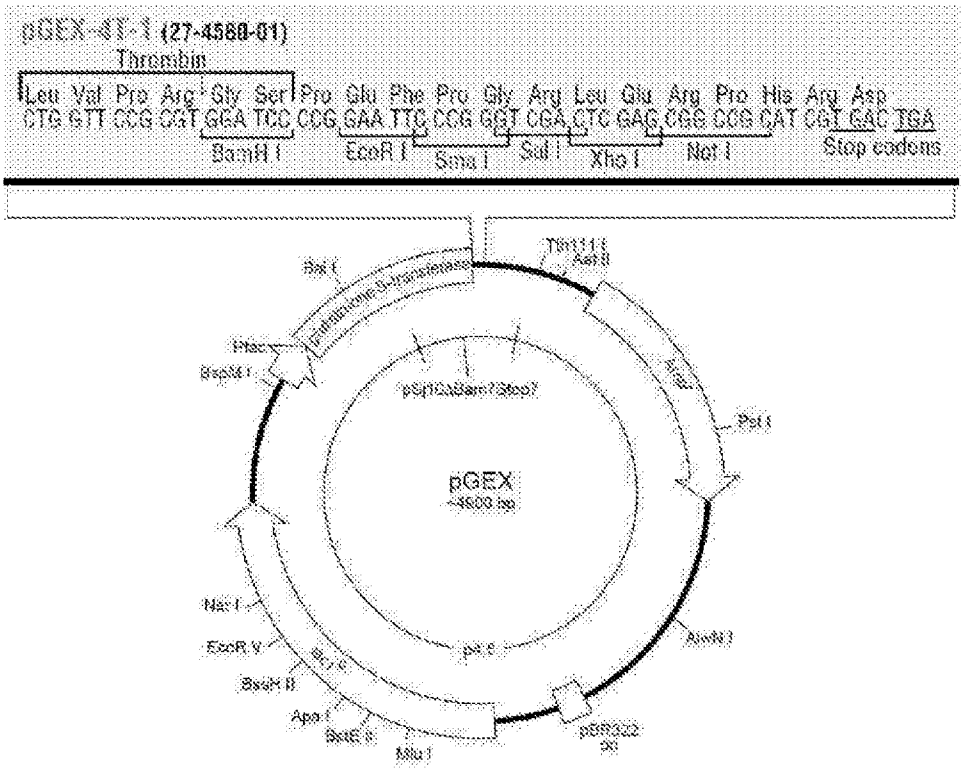
600
400
287
200
0
Antibody titre
Uninfected
Wild-type
Vaccine A
Cut off value
0
7
14
18
28
35
42
Days after vaccination
0
10
17
24
Days after challenge

Figure 11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Thrombin
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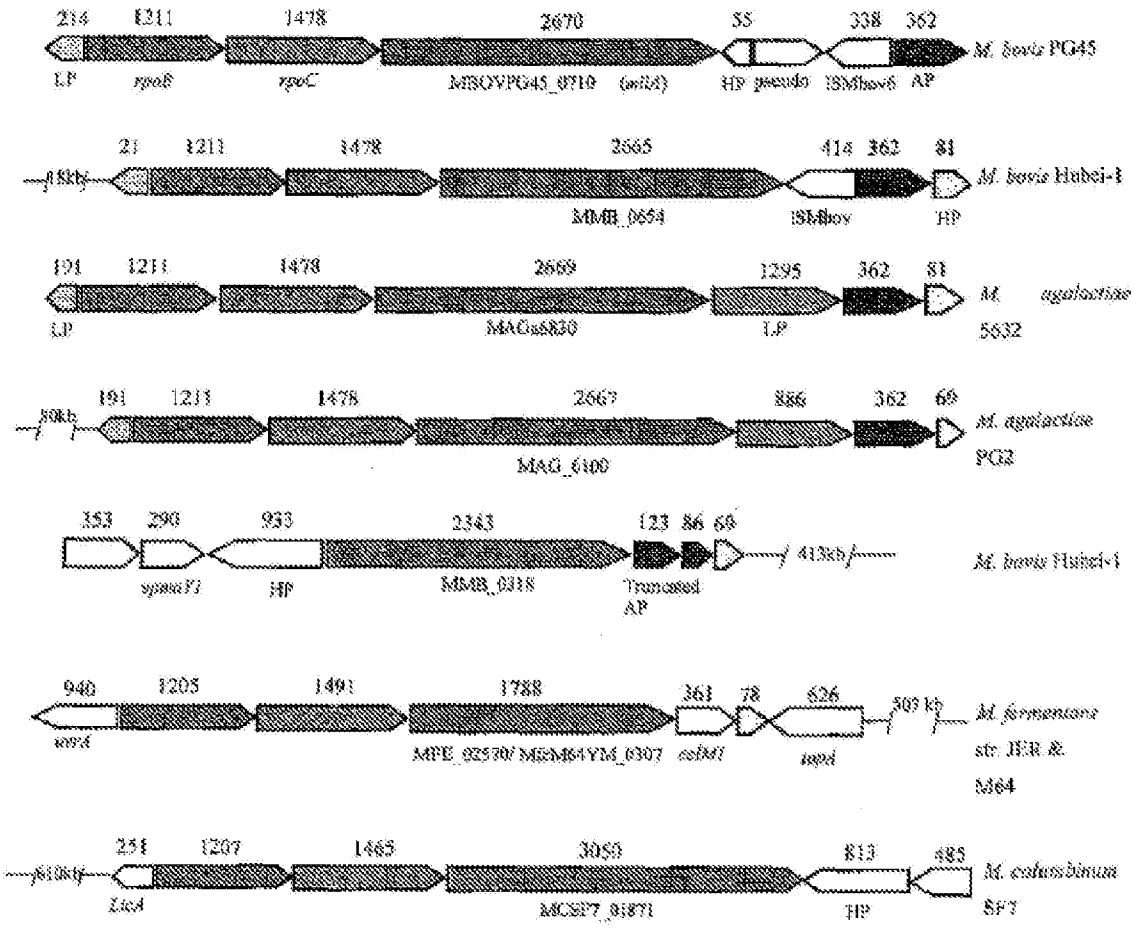
CTG GTT CCG CGT GGA TCC CCG GAA TTC CCG GGT CGA CTC GAG CGG CCG CAT CGT GAC TGA
BamH I
EcoR I
Sma I
Sal I
Xho I
Not I
Stop codons
pGEX
4900 bp

Figure 12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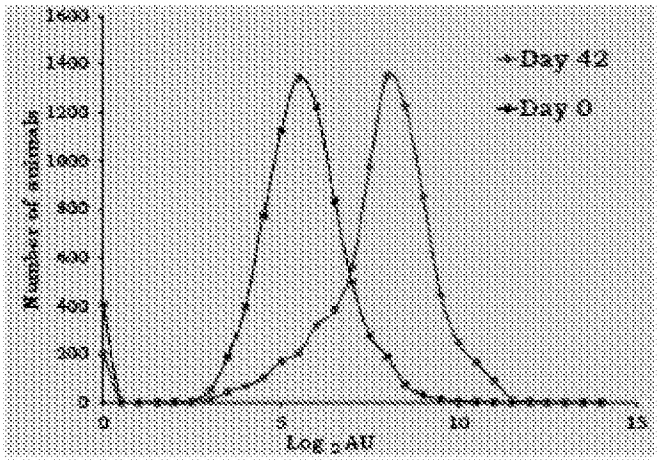
LP rpoB rpoC MSOVPG45_0710 (orfA) HP pseudo ISMbov6 AP
M. bovis PG45
21 1211 1478 2665 414 362 81
MMB_0654 ISMbov HP
M. bovis Hubei-1
191 1211 1478 2669 1295 362 81
LP MAGa6830 LP
M. agalactiae 5632
191 1211 1478 2667 886 362 69
MAG_6100
M. agalactiae PG2
353 290 933 2343 123 86 60
ypmB/1 HP MMB_0318 Truncated AP
M. bovis Hubei-1
940 1205 1491 1788 361 78 626
orf4 MPE_02530/ MSMB64YM_0307 orf41 tnpA
M. fermentans str. JER & M64
251 1207 1465 3050 813 485
LtrA MCSP7_01871 HP
M. columbinum SF7

Figure 13



No. animals	7448
Mean antibody units (AU)	44.6
Standard deviation	33.4
Cut-off value	111.4
Seronegative on day 0	90 %
Seropositive on day 42	73.5 %

Figure 14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2014/050231

A. CLASSIFICATION OF SUBJECT MATTER

G01N 33/569 (2006.01) ***G01N 33/543 (2006.01)*** ***G01N 33/68 (2006.01)*** ***C07K 14/30 (2006.01)*** ***C07K 17/00 (2006.01)*** ***A61K 39/02 (2006.01)*** ***A61P 31/04 (2006.01)***

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)

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 practicable, search terms used)

EPODOC, WPI, MEDLINE, BIOSIS, HCAPLUS, EMBASE, AGRICOLA: mycoplasma, mycoplasma immunogenic lipase, milA, hyopneumoniae P65, lipase, ELISA, immunoassay, vaccine, antigen and like terms

Espacenet, Google Scholar: Inventor/applicant names

GenomeQuest: SEQ ID NOs: 1-8, 15-21

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

[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 application or patent 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
26 November 2014	26 November 2014
Name and mailing address of the ISA/AU	**Authorised officer**
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustralia.gov.au	Jonathan Konrad AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. +61 2 6283 2601

INTERNATIONAL SEARCH REPORT C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		International application No. **PCT/AU2014/050231**
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	US 5,788,962 A (Wise et al.) 04 August 1998 See the whole document, particularly SEQ ID NO: 2, column 5 fourth paragraph to column 6 fourth paragraph and column 10 first paragraph As above	1, 3, 6-14, 16, 18-21, 23, 25-28, 30, 32-34 2, 15, 22, 29
X Y	WO 2003/004051 A2 (PFIZER PRODUCTS INC.) 16 January 2003 See the whole document, particularly the abstract, page 2 second paragraph, pages 3 first two paragraphs, page 7 last two paragraphs and the claims As above	21, 23, 25-28, 30, 32-34 22, 29
Y	GenBank accession no. AAV27666, 11 March 2010 Sequence is 100% identical to present SEQ ID NO: 21 and comprises present SEQ ID NO: 8	2, 15, 22, 29
A	GenBank accession no. ADR24994, 25 January 2011 Sequence is 100% identical to present SEQ ID NO: 15 and comprises present SEQ ID NO: 2	
A	FR 2722509 A1 (INSTITUT PASTEUR) 19 January 1996	
P,A	Adamu, J. Y. et al., 'Membrane proteins of *Mycoplasma bovis* and their role in pathogenesis', Research in Veterinary Science, 2013, published online 27 June 2013, Vol. 95, No. 2, pages 321-325	
P,X	Wawegama, N. K. et al., 'Development of a recombinant protein-based enzyme-linked immunosorbent assay for diagnosis of *Mycoplasma bovis* infection in cattle', Clinical and Vaccine Immunology, 2014, published online 11 December 2013, Vol. 21, No. 2, pages 196-202 See the whole document	1-20, 28-31

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2014/050231

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

   a. (means)

   ☐ on paper

   ☒ in electronic form

   b. (time)

   ☐ in the international application as filed

   ☒ together with the international application in electronic form

   ☐ subsequently to this Authority for the purposes of search

2. ☒ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/AU2014/050231**

This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

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End of Annex

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.