(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
29 December 2011 (29.12.2011)

PCT

(10) International Publication Number
WO 2011/161551 A2

(51) International Patent Classification:
C12N 1/00 (2006.01)

(21) International Application Number:
PCT/IB2011/002341

(22) International Filing Date:
10 June 2011 (10.06.2011)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
1009861.4 11 June 2010 (11.06.2010) GB

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2011/161551 A2

(54) **Title**: OMV VACCINES

(57) **Abstract**: The invention is in the field of outer membrane vesicles (OMV) and their uses. More particularly the present invention provides OMV obtained from a bacterium being an ompA mutant and/or a mutant in one or more components of the Tol-Pal complex and presenting a heterologous antigen on its surface. The heterologous antigen is selected from the group consisting of Chlamydia trachomatis CT823, CT681, CT372, CT443, CT043, CT733, CT279, CT601 and CT153 for the treatment, prevention or diagnosis of Chlamydia infection.

OMV VACCINES

TECHNICAL FIELD

The invention is in the field of outer membrane vesicles that present a heterologous antigen on their surface and their uses.

BACKGROUND

C. trachomatis is a Gram-negative bacterium which is an obligate intracellular pathogen. It is a common cause of urogenital tract infections, which leads to pelvic inflammatory disease (10-20% of cases), infertility and ectopic pregnancy. Such conditions are common in industrialised countries and are caused by serovars D-K.

C. trachomatis is also a leading cause of ocular infections, resulting in trachoma (150 million cases annually) and blindness (6 million cases annually), mainly in developing countries. These conditions are caused by serovars A-C. Infection with serovars L1-L3 of *C. trachomatis* causes lymphogranuloma venereum.

Vaccine development has been identified as essential to controlling infection with *C. trachomatis*. Vaccines against *C. trachomatis* appear to depend on a Th1-polarized cell-mediated immune response, in particular on CD4+ lymphocytes that produce IFN-γ. For example, depletion of CD4+ T cells in mice results in loss of protective immunity [1], and adoptive transfer of *Chlamydia*-specific CD4+ T cells confers protection against challenge with *C. trachomatis* ([2],[3]). Furthermore, recent studies report that *C. trachomatis* infection in mice induces a CD4-Th1 protective immune response, indicating that critical *Chlamydia* antigens are processed and presented via the MHC class II pathway ([4];[5]).

Immune protection against infection with *C. trachomatis* is likely to be mediated by immunization with *C. trachomatis* proteins that are targets of CD4+ T cells and that are capable of inducing B-cell responses. B-cells and antibodies have been shown to be important for enhancing the protective effector T-cell response against primary infection [6]. B-cells and antibodies also play an important role in resolution of secondary infection ([7],[8]).

Neutralizing antibodies have been shown to play an important role in protection against *Chlamydia* infection.

Numerous studies on the most promising vaccine candidate (Major Outer Membrane Protein, MOMP) have shown that an effective vaccine is likely to be based on several *C. trachomatis* antigens. The homologue proteins CT823 of *Chlamydia trachomatis* (Ct) and TC0210 of *Chlamydia muridarum* (Cm) are annotated as serine proteases and share a 93.36 percent sequence identity. Previous studies, with mass spectrometric and cytofluorimetric analysis on CT823 have confirmed its localization on the surface of the bacterium [9]. The CT823 antigen is able to induce a specific CD4-Th1 response in splenocytes isolated from mice infected with *C. trachomatis* and has been predicted to contain MHC class II epitopes ([10];[11]).

It is an object of the invention to provide a vehicle for delivering antigens such as *Chlamydia* CT823 in vaccine formulations.

DISCLOSURE OF THE INVENTION

The present invention provides an outer membrane vesicle (OMV) presenting a heterologous antigen on its surface, wherein the OMV is obtained from a bacterium which is an ompA mutant and/or which is a mutant in one or more components of the Tol-Pal complex. The Tol-Pal complex is shown in Figure 1a.

Gram-negative bacteria naturally shed OMVs which are released into the growth medium. Heterologous antigens are expressed in the Gram-negative bacteria such that they assemble in the membrane that is then released in the culture supernatant. OMVs from Gram-negative bacteria are representative of the outer membrane and periplasmic bacterial compartments and allow the presentation of membrane proteins in their natural composition and conformation. As the OMVs carry the recombinant proteins in the proper conformation, they represent an excellent choice as delivery vehicles for heterologous membrane proteins.

The use of OMVs to express outer membrane proteins is described in WO 2002/062380 (GlaxoSmithKline Biologicals S.A.). WO 2002/062380 discloses a Gram-negative bacterial OMV presenting on its surface one or more outer membrane proteins from *Chlamydia*. Presentation of the PorB outer membrane protein from *C. trachomatis* is said to be preferred. The presentation of PmpG and MOMP outer membrane proteins from *C. trachomatis* is also described.

WO 2002/062380 describes methods to optimize the outer membrane protein (OMP) and LPS composition of OMV ("bleb") vaccines by deleting immunodominant variable

OMPs, as well as non-protective OMPs, by creating conserved OMPs by deletion of variable regions, by upregulating expression of protective OMPs and by eliminating control mechanisms for expression of protective OMPs.

WO 2006/046143 (Novartis Vaccines & Diagnostics, SRL) discloses that disruption of the pathways involved in degradation of peptidoglycan (the murein layer) gives bacteria that release vesicles into their culture medium, and that these vesicles are rich in immunogenic outer membrane proteins and can elicit broad-ranging bactericidal immune responses. In particular, the inventors of WO 2006/046143 found that knocking out the meningococcal mltA homolog gives bacteria that spontaneously release vesicles that are rich in immunogenic outer membrane proteins and that can elicit cross-reactive antibody responses with higher bactericidal titres than OMVs prepared by normal production processes. *E. coli* having a knock out of one or more of the components of the Tol-Pal complex, such as tolA, tolQ, tolB, pal and/or tolR are also described.

The inventors have surprisingly found that the use of the OMVs of the present invention as delivery vehicles enables the antigenicity of promising antigens to be increased compared to when the antigens are delivered in their purified form. For example, it has been found that antigens which are not protective when tested in a *chlamydial* animal model when administered in their purified form, may become protective when presented in an OMV of the invention.

Bacteria

One aspect of the invention relates to a mutant bacterium, which expresses a heterologous antigen.

The invention allows the production of OMVs from a bacterium of choice. The bacterium from which the OMV of the invention is prepared may be Gram-positive, but it is preferably Gram-negative. The bacterium may be any suitable bacterium, for example, *Bordetella pertussis, Borrelia burgdorferi, Brocella melitensis, Brucella ovis, Chlamydia psittaci, Chlamydia trachomatis, Escherischia coli, Haemophilus influenzae, Legionella pneumophila, Neisseria meningitidis or N. gonorrhoeae, Moraxella catarrhalis, Pseudomonas aeruginosa, Yersinia enterocolitica, Shigella flexneri, Treponema, Porphyromonas, Helicobacter or Salmonella enterica serovar typhimurium.*

For example, the bacterium may be from the *Escherichia* genus. In preferred embodiments, the bacterium is *E. coli*. Any suitable *E. coli* strain may be used. For

example, in some embodiments, the *E. coli* is from the BL21 strain, for example, *E. coli* BL21(DE3). The present inventors have surprisingly found that the use of an *E. coli* BL21(DE3) strain is an excellent delivery vehicle for presenting heterologous antigens. In some embodiments, the bacterium is from the K1 or K12 strain.

The bacterium will typically have been generated by mutation of at least one component of the Tol-Pal complex and/or of the OmpA gene in a chosen starting strain.

Where the bacterium is not *E. coli*, the bacterium may have a mutation of the homologue of the ompA gene and/or of one or more components of the homologue of the *E. coli* Tol-Pal complex.

In some embodiments, one or more (e.g. 2, 3, 4, 5) components of the *E. coli* Tol-Pal complex is mutated. A schematic diagram of the Tol-Pal complex is shown in Figure 1a (see also [12], [13], [14]). For example, in some embodiments, any combination of tolA, tolQ, tolB, pal and/or tolR is mutated. For example, one, two, three, four or all of tolA, tolQ, tolB, pal and/or tolR may be mutated. For example, one or more (e.g. 2 or more, 3 or more, 4 or more or all) of tolA, tolQ, tolB, pal and/or tolR may be mutated. Mutation of tolR is preferred.

In one embodiment, the OMV is obtained from a bacterium which is an ompA mutant. In another embodiment, the OMV is obtained from a bacterium which is an ompA mutant and a mutant in at least one component of its Tol-Pal complex, for example, a tolR mutant. In preferred embodiments, the OMV is obtained from a bacterium which is an ompA mutant but which is wild type in the genes encoding the Tol-Pal complex, or which expresses a functional Tol-Pal complex. Preferably, in embodiments in which the bacterium is an ompA mutant, the bacterium is wild type in its tolR gene or expresses a functional TolR protein. In most preferred embodiments, the bacterium is an ompA mutant and all other genes are wild type genes.

Preferably, the bacterium is an *E. coli* ompA mutant or an *E. coli* ompA and tolR mutant. In some embodiments, the bacterium is selected from *E. coli* BL21(DE3)ΔompA, *E. coli* BL21(DE3)ΔompAΔtolR, or *E. coli* BL21(DE3)ΔtolR. The Δ symbol is used herein to refer to a bacterial strain from which the coding sequence of the gene recited after the Δ symbol has been deleted. Thus, a bacterial strain which is "ΔompA" does not comprise the coding sequence for the ompA gene. Likewise, a bacterial strain which is "ΔtolR" does not comprise the coding sequence for the tolR gene. Preferably, the entire coding

sequence is deleted. However, the coding sequence may alternatively be deleted in part. For example, the N-terminal half or the C-terminal half may be deleted.

The present inventors have surprisingly found that *E. coli* ΔtolR mutant strains and *E. coli* ΔompA mutant strains overproduce OMVs relative to wild type *E. coli*. Thus, the mutation of the ompA gene and/or one or more components of the Tol-Pal complex preferably results in the mutant bacterium producing an increased number of OMVs compared to its respective wild type strain which carries a wild type ompA gene and/or Tol-Pal complex. OmpA is an integral membrane protein and is the most abundant of the outer membrane proteins in *E. coli*. It is, therefore, particularly surprising that an *E. coli* lacking the OmpA protein is viable. Indeed, according to Murakami et al. [15], an *E. coli* ompA single mutant cannot promote vesicle release.

Hyperblebbing Gram-negative bacteria from which blebs may more easily be made in higher yield and may be more homogeneous in nature are described in WO 02/062378 (Smithkline Beecham Biologicals S.A.). The blebs are derived from bacteria selected from the group consisting of *Neisseria meningitidis, Neisseria lactamica, Neisseria gonorrhoeae, Helicobacter pylori, Salmonella typhi, Salmonella typhimurium, Vibrio cholerae, Shigella spp., Haemophilus influenzae, Bordetella pertussis, Pseudomonas aeruginosa* and *Moraxella catarrhalis*. Such bacteria have been genetically modified by down-regulation of expression of one or more tol genes and mutations of one or more gene(s) encoding a protein comprising a peptidoglycan-associated site to attenuate the peptidoglycan-binding activity of the protein(s) whilst ensuring that the truncated protein folds correctly in the outer membrane.

The present inventors have surprisingly found that OMVs from *E. coli* ΔompA mutant strains express a higher percentage of outer membrane proteins compared to *E. coli* ΔtolR mutant strains (see Example 9). Thus, in some embodiments, the mutant bacterium expresses a higher percentage of outer membrane proteins compared to its respective wild type strain, more preferably, compared to a tolR mutant strain. In some embodiments, the OMV produced from the bacterium expresses a higher percentage of outer membrane proteins compared to an OMV from the respective wild type strain of the bacterium, more preferably, compared to a tolR mutant from the same strain. For example, the protein composition of the OMV of the invention may comprise 60% or more outer membrane proteins. In some embodiments, the protein composition of the OMV comprises at least 70%, at least 75%, at least 80%, at least 85%, at least 90% or at

least 95% outer membrane proteins. Preferably, the protein composition of the OMV comprises at least 98% or at least 99% outer membrane proteins. Most preferably, the OMV comprises 100% outer membrane proteins. In some embodiments, the protein composition of the OMV comprises 25% or less cytoplasmic proteins (e.g. 20% or less, 15% or less, 10% or less, 5% or less, 3% or less, 2% or less, 1% or less). Preferably, the OMV comprises no cytoplasmic proteins. The percentage of outer membrane proteins and cytoplasmic proteins is preferably assessed according to the method described in Example 9.

The present inventors have also surprisingly found that an OMV of the present invention from an *E. coli* ΔompA mutant strain expresses an increased amount of the heterologous antigen compared to an OMV from an *E. coli* ΔtolR mutant strain (see Example 10). Thus, in some embodiments, the mutant bacterium expresses an increased amount of the heterologous antigen compared to its respective wild type strain, more preferably compared to a tolR mutant strain. In some embodiments, the OMV produced from the bacterium expresses a larger amount of the heterologous antigen compared to an OMV from the respective wild type strain of the bacterium, more preferably, compared to an OMV produced by a tolR mutant from the same strain. For example, in some embodiments, the OMV of the invention expresses 105% or more (for example, 110% or more, 125% or more, 150% or more, 175% or more, 200% or more or 250% or more) heterologous antigen compared to an OMV from the respective wild type strain of the bacterium, or more preferably, compared to an OMV from a tolR mutant from the same strain.

The mutation of the ompA gene and/or one or more components of the Tol-Pal complex is preferably a knock out mutation. For example, part or all of the gene sequence may be deleted such that only a fragment of the protein is expressed or such that no protein is expressed. For example, a fragment of the coding sequence may be deleted, for example, at least 20%, at least 40%, at least 60%, at least 80%, at least 90%, at least 95% of the coding sequence. In some embodiments, the expressed protein lacks one or more than one domain. In preferred embodiments, the mutation is a deletion of the entire coding sequence of the OmpA protein and/or the at least one component of the Tol-Pal complex. In such embodiments, this results in the bacterium not expressing the OmpA protein and/or at least one component of the Tol-Pal complex (preferably TolR).

Consequently, the OMV does not express the OmpA protein and/or the at least one component of the Tol-Pal complex.

The at least one component of the Tol-Pal complex or the OmpA protein may be knocked out by any suitable method. In one embodiment, the at least one component of the Tol-Pal complex and/or the ompA gene is deleted using recombinant DNA techniques, such as homologous recombination techniques. Such techniques are well known in the art (e.g. using Red/ET recombineering technology such as the *E. coli* gene deletion kit from Gene Bridges, GmbH) [16]. For example, the at least one component of the Tol-Pal complex and/or the ompA gene may be replaced with an antibiotic resistance cassette as a selection marker. In some embodiments, the tolR gene is replaced with a kanamycin resistance gene by homologous recombination, as shown in Figure 1b.

In other embodiments, the one or more mutations in at least one component of the Tol-Pal complex and/or the ompA gene, may each independently be a substitution, an insertion or a deletion. For example, each mutation may involve a single amino acid, such as a point mutation. A truncation is an example of a deletion. Truncations may involve deletion of up to 10, up to 20, up to 30 or up to 40 (or more) amino acids at the N-terminus and/or C-terminus. Preferably, such mutations result in the production of a non-functional protein.

Mutant bacteria - expression of other genes

OMVs from a double mutant of *E. coli* that lacks both the Braun lipoprotein (lpp) and OmpA are described in US 6,558,677. In preferred embodiments, the bacterium has a wild type Braun lipoprotein gene. In some embodiments, the bacterium expresses a mutated but functional version of the Braun lipoprotein.

Murakami et al. [15] discloses that an *E. coli* ompA mutant itself could not promote vesicle release; however, ompA, pal and major lipoprrpteoin lpp mutants formed large numbers of vesicles. Thus, the present invention provides that, in some embodiments, the OMV is from a bacterium which is wild type in its pal and major lipoprotein lpp genes. In some embodiments, the bacterium expresses a mutated but functional version of the pal and major lipoprotein genes.

Mutant bacteria - mutations of other genes

In some embodiments, in addition to having a mutation of the ompA gene and/or one or more components of the Tol-Pal complex, the bacterium may have one or more mutations of other gene(s). To reduce pyrogenic activity, for instance, the bacterium should have low endotoxin (lipo-oligosaccharide (LOS) / lipopolysaccharide (LPS)) levels, and this can be achieved by knock out of enzymes involved in LPS biosynthesis. OMVs of the invention preferably contain no more than 20% by weight of LOS/LPS, measured relative to the total protein (*i.e.* there should be at least 4 times more protein than LOS/LPS, by weight). The maximum LOS/LPS level is preferably even lower than 20% *e.g.* 15%, 10%, 5% or lower. Processes for preparing LPS depleted outer membranes from Gram-negative bacteria are disclosed in European Patent No. 0624376.

As well as having mutations or knock outs of particular endogenous genes, the bacterium may express one or more genes that are not endogenous. For example, the invention may use a recombinant strain that expresses new genes relative to the corresponding wild-type strain. Expression of non-endogenous genes in this way can be achieved by various techniques, e.g., chromosomal insertion, knock in mutations, expression from extra-chromosomal vectors e.g. from plasmids, etc.

Further, as well as down-regulating expression of specific proteins, the bacterium may in some embodiments over-express (relative to the corresponding wild-type strain) other immunogens.

Vesicle compositions

The invention provides the OMVs that are spontaneously released into culture medium by bacteria of the invention. These OMVs are distinct from the vesicles that can be prepared artificially from the same bacteria, such as the sarkosyl-extracted OMVs prepared in Adu-Bobie et al [17] from 'ΔGNA33' meningococci. They are also distinct from microvesicles (MVs [18]) and 'native OMVs' ('NOMVs' [19]), although vesicles of the invention seem to be more similar to MVs and NOMVs than to sarkosyl-extracted OMVs. The vesicles are also distinct from blebs, which are outer-membrane protrusions that remain attached to bacteria prior to release as MVs ([20]; [21]).

The vesicles of the invention have a diameter of 50-100nm by electron microscopy, which is smaller than that of artificial meningococcal OMVs (diameter ~270nm, [22]). The diameter is roughly the same as that of artificial OMVs that have been

heat-denatured (~105nm, [22]), but the vesicles of the invention retain antigenicity whereas heat-denatured artificial OMVs lose their antigenicity. Moreover, the OMVs of the invention are substantially free from cytoplasmic contamination.

Unlike the starting culture, the OMV-containing compositions of the invention will generally be substantially free from whole bacteria, whether living or dead. The size of the OMVs of the invention means that they can readily be separated from whole bacteria by filtration through a 0.22μm filter *e.g.* as typically used for filter sterilisation. Thus the invention provides a process for preparing OMVs of the invention, comprising filtering the culture medium from bacteria of the invention through a filter that retards whole bacteria but that lets the OMVs pass through *e.g.* a 0.22μm filter. Although OMVs will pass through standard 0.22μm filters, these can rapidly become clogged by other material, and so it is preferred to perform sequential steps of filter sterilisation through a series of filters of decreasing pore size, finishing with a standard sterilisation filter (*e.g.* a 0.22μm filter). Examples of preceding filters would be those with pore size of 0.8μm, 0.45μm, *etc.* The filtrate can be further treated *e.g.* by ultracentrifugation.

Vesicle combinations

The invention also provides methods for preparing OMVs from more than one bacterial strain, and the OMVs from the different bacteria can be combined. Thus the invention provides a composition comprising a mixture of *n* sets of OMVs of the invention, prepared from *n* different strains of a bacterium. The value of *n* can be 1, 2, 3, 4, 5, *etc.* The different strains can be in the same or different serogroups.

The invention also provides a kit comprising OMVs of the invention prepared from *n* different strains of a bacterium. The OMVs can be kept and stored separately in the kit until they are required to be used together *e.g.* as an admixture, or for simultaneous, separate or sequential use.

The invention also provides a process comprising: preparing *n* sets of OMVs of the invention, one from each of *n* different strains of a bacterium; and combining the *n* sets of OMVs. The different sets can be combined into a kit or into an admixture.

As well as being selected from different strains of a bacterium, such as different *Escherichia* strains, OMVs can be selected from different bacterial genera, or from different pathogens. Thus the invention provides a composition comprising a mixture of *n* sets of OMVs of the invention, prepared from *n* different species of bacteria.

Similarly, the invention provides a kit comprising OMVs of the invention prepared from *n* different species of bacteria, and provides a process comprising the step of preparing *n* sets of OMVs of the invention, one from each of *n* different species of bacteria.

In some embodiments, different heterologous antigens are expressed on the different OMVs.

Heterologous antigens

A "heterologous" antigen is an antigen derived from a pathogenic species that is different from the species of bacterium from which the OMV is obtained, and is preferably an antigen from a pathogen genus different from the genus of bacterium from which the OMV is obtained.

The heterologous antigen is preferably from a bacterium or virus. For example, in some embodiments, the heterologous antigen is a bacterial antigen, such as an antigen from *Chlamydia, Mycobacterium tuberculosis, Mycobacterium leprae, Streptococcus, Pseudomonas, Shigella, Campylobacter, Salmonella, Yersinia pestis, Rickettsia prowazekii, Neisseria or Helicobacter.* In some embodiments, the heterologous antigen is a viral antigen, such as an antigen from a virus of the *Adenoviridae, Picornaviridae, Herpesviridae, Hepadnaviridae, Flaviviridae, Retroviridae, Orthomyxoviridae, Paramyxoviridae, Papovaviridae, Rhabdoviridae* or *Togaviridae* family, for example, an antigen from HIV or influenza.

In preferred embodiments, the heterologous antigen is a *Chlamydial* antigen, for example, an antigen from *C. trachomatis, C. pneumoniae, C. psittaci, C. pecorum, C. muridarum* or *C. suis.* In embodiments in which the heterologous antigen is from *Chlamydia*, the bacterium from which the outer membrane vesicle is obtained is not from the same species of *Chlamydia* and is preferably not from the *Chlamydia* genus.

The human serovariants ("serovars") of *C. trachomatis* are divided into two biovariants ("biovars"). Serovars A-K elicit epithelial infections primarily in the ocular tissue (A-C) or urogental tract (D-K). Serovars L1, L2 and L3 are the agents of invasive lymphogranuloma venereum (LGV).. The heterologous antigen may be selected from any one of serovars A, B, C, D, E, F, G, H, I, J, K, L1, L2 or L3. Preferably, the heterologous antigen is from *C. trachomatis* serovar D, or from another epidemiologically prevalent serotype.

Preferably, the heterologous antigen is a membrane protein, more preferably an outer membrane protein.

Examples of antigens from *C. trachomatis* which are suitable for use in the present invention are CT823, CT601, CT372, CT443, CT043, CT733, CT279, CT153 and MOMP (CT681). Examples of antigens from *C. muridarum* which are suitable for use in the present invention are TC0210, TC0052, TC0106, TC0313, TC0431, TC0551, TC0651, TC0727 and TC0890. Examples of other antigens suitable for use in the invention are TC0660 and TC0741.

In preferred embodiments, the heterologous antigen is CT823 or TC0210. The sequences of CT823 and TC0210 are presented in Figure 9. CT823 from *Chlamydia trachomatis* (Ct) and TC0210 from *Chlamydia muridarum* (Cm) are protein homologues which are annotated as serine proteases and share a 93.36 percent sequence identity. Together with the high temperature requirement A (HtrA) protein of *E. coli* and the homologues in other bacteria and eukaryotes, these proteins constitute the HtrA protease family. The chief role of these proteases is to degrade misfolded proteins in the periplasm ([23]; [24]; [25]). HtrA from *Chlamydia trachomatis* (also referred to herein as "CtHtrA" or "CT823") has been characterised as a serine endoprotease, specific for unfolded proteins, which is temperature activated above 34°C ([26]). Chaperone activity has been observed, although this appears to be target-dependent.

CT823 is an outer membrane protein. In order for a protective immune response to be raised against this heterologous antigen, the heterologous antigen should preferably be presented in its correctly folded state.

The inventors have surprisingly found that expression of TC0210 in a mutant OMV according to the invention results in a neutralising immune response being raised against both *C. muridarum* and *C. trachomatis*. The immune response is improved compared to the response generated by the purified TC0210 antigen (i.e. when it is not expressed in an OMV).

The heterologous antigen is preferably the wild type antigen. However, in some embodiments, the heterologous antigen is a variant of a wild type antigen. For example, the variant may have at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98% or at least 99% sequence identity to the wild type antigen. Methods of determining sequence identity are well known in the art. Identity between heterologous

antigens is preferably determined by the Smith-Waterman homology search algorithm as implemented in the MPSRCH program (Oxford Molecular), using an affine gap search with parameters gap open penalty = 12 and gap extension penalty = 2. Such variants include homologs, orthologs, allelic variants and functional mutants. In embodiments in which the heterologous antigen is a variant of a wild type antigen, the immunogenicity of the variant is preferably the same as or very similar to the immunogenicity of the wild type antigen when tested under the same conditions, such as when used in an ELISA assay or in a neutralization assay.

Where the heterologous antigen is a variant of a wild type antigen, the amino acid sequence of the variant preferably contains fewer than twenty mutations (e.g. 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1) relative to the wild type sequence. Each mutation preferably involves a single amino acid and is preferably a point mutation. The mutations may each independently be a substitution, an insertion or a deletion. Preferred mutations are single amino acid substitutions. The variant may also include one or more (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, *etc.*) single amino acid deletions relative to the wild type sequences. The variant may also include one or more (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, *etc.*) insertions (*e.g.* each of 1, 2, 3, 4 or 5 amino acids) relative to the wild type sequences. Deletions, substitutions or insertions may be at the N-terminus and/or C-terminus, or may be between the two termini. Thus a truncation is an example of a deletion. Truncations may involve deletion of up to 10, up to 20, up to 30, up to 40 (or more) amino acids at the N-terminus and/or C-terminus.

Amino acid substitutions may be to any one of the other nineteen naturally occurring amino acids. In preferred embodiments, one or more mutations is a conservative substitution. In another embodiment, one or more mutations is a non-conservative substitution. A conservative substitution is commonly defined as a substitution introducing an amino acid having sufficiently similar chemical properties, e.g. having a related side chain (*e.g.* a basic, positively charged amino acid should be replaced by another basic, positively charged amino acid), in order to preserve the structure and the biological function of the molecule. Genetically-encoded amino acids are generally divided into four families: (1) acidic *i.e.* aspartate, glutamate; (2) basic *i.e.* lysine, arginine, histidine; (3) non-polar *i.e.* alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar *i.e.* glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine. Phenylalanine, tryptophan, and tyrosine

are sometimes classified jointly as aromatic amino acids. In general, substitution of single amino acids within these families does not have a major effect on the biological activity. Further examples of conservative substitutions that may be used in the invention are presented in Table I.

TABLE 1

Amino Acid	Synonymous Groups	More Preferred Synonymous Groups
Ser	Gly, Ala, Ser, Thr, Pro	Thr, Ser
Arg	Asn, Lys, Gln, Arg, His	Arg, Lys, His
Leu	Phe, Ile, Val, Leu, Met	Ile, Val, Leu, Met
Pro	Gly, Ala, Ser, Thr, Pro	Pro
Thr	Gly, Ala, Ser, Thr, Pro	Thr, Ser
Ala	Gly, Thr, Pro, Ala, Ser	Gly, Ala
Val	Met, Phe, Ile, Leu, Val	Met, Ile, Val, Leu
Gly	Ala, Thr, Pro, Ser, Gly	Gly, Ala
Ile	Phe, Ile, Val, Leu, Met	Ile, Val, Leu, Met
Phe	Trp, Phe, Tyr	Tyr, Phe
Tyr	Trp, Phe, Tyr	Phe, Tyr
Cys	Ser, Thr, Cys	Cys
His	Asn, Lys, Gln, Arg, His	Arg, Lys, His
Gln	Glu, Asn, Asp, Gln	Asn, Gln
Asn	Glu, Asn, Asp, Gln	Asn, Gln
Lys	Asn, Lys, Gln, Arg, His	Arg, Lys, His
Asp	Glu, Asn, Asp, Gln	Asp, Glu
Glu	Glu, Asn, Asp, Gln	Asp, Glu
Met	Phe, Ile, Val, Leu, Met	Ile, Val, Leu, Met
Trp	Trp, Phe, Tyr	Trp

Examples of non-conservative substitutions that may be used in the invention include the substitution of an uncharged polar amino acid with a nonpolar amino acid, the substitution of a nonpolar amino acid with an uncharged polar amino acid, the substitution of an acidic amino acid with a basic amino acid and the substitution of a basic amino acid with an acidic amino acid.

In some embodiments in which the heterologous antigen is a variant of a wild type antigen, the variant may comprise one or more amino acid derivatives. By "amino acid derivative" is intended an amino acid or amino acid-like chemical entity other than one of the 20 genetically encoded naturally occurring amino acids. In particular, the amino acid derivative may contain substituted or non-substituted, linear, branched, or cyclic alkyl moieties, and may include one or more heteroatoms. The amino acid derivatives can be made de novo or obtained from commercial sources (Calbiochem-Novabiochem AG, Switzerland; Bachem, USA).

In some embodiments, the heterologous antigen comprises or consists of a fragment of a wild type antigen or of a variant thereof. The fragment should comprise at least *n* consecutive amino acids from the wild type antigen or from the variant thereof and, depending on the particular sequence, *n* is 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, 10 or more, 11 or more, 12 or more, 15 or more, 20 or more, 30 or more, 40 or more, 50 or more (*e.g.* 60, 90, 120, 150, 180, 210, 240, 270, 300, 330, 360, 390, 420, 450, 480 or more). Such fragments do not comprise the full length sequence of the wild type antigen. In some embodiments, the fragment is 481 amino acids or less in length (for example, 400 or less, 300 or less, 200 or less, 100 or less, 50 or less, 40 or less, 30 or less, 25 or less, 22 or less or 20 or less amino acids in length).

Preferably the fragment comprises one or more epitopes from the wild type antigen. Such epitopes may be defined using epitope mapping techniques such as those described in Example 8. In some embodiments, the heterologous antigen is a fragment of the *C. muridarum* TC0210 antigen. For example, the fragment may comprise or consist of a sequence selected from the group consisting of DYFNDEFFNRFFGLP (SEQ ID NO: 36), SHREQ (SEQ ID NO: 37), ALQKMGVRVQNLTPE (SEQ ID NO: 38), NQVLKNAKGENVLLM (SEQ ID NO: 39), SPMLGYSAPKKDSSTGICLA (SEQ ID NO: 40), EDLLKEVSRGFSKVAAQATP (SEQ ID NO: 41),

TGSQAIASPGNKRGFQENPF (SEQ ID NO: 42), PRPQQRDAVR (SEQ ID 43), IAIGNPFGLQATVTVGVISAKGRNQLHIVD (SEQ ID NO: 44) and NTAIVSGSGGYIGIGFAIPSLMAKRVIDQL (SEQ ID NO: 45). For example, it may comprise or consist of a sequence selected from the group consisting of DYFNDEFFNRFFGLP (SEQ ID NO: 36), SHREQ (SEQ ID NO: 37), ALQKMGVRVQNLTPE (SEQ ID NO: 38) and NQVLKNAKGENVLLM (SEQ ID 39). DYFNDEFFNRFFGLP (SEQ ID NO: 36) is particularly preferred.

In some embodiments, the heterologous antigen is a fragment of the *C. muridarum* TC0210 antigen which comprises or consists of a sequence selected from the group consisting of VAAQATPGVVYIENFPK (SEQ ID NO: 46), GFQENPFDYFNDEFFNRFFGLPSHREQPRPQQR (SEQ ID NO: 47), GTGFIVSEDGYVVTNHHVVEDAGK (SEQ ID 48), TDLAVIKIQAK (SEQ ID NO: 49), VIDQLISDGQVTR (SEQ ID NO: 50), AGLRQEDVIVAYNGKEVESLSALR (SEQ ID NO: 51), FIEIPVTVTQIPAEDGVSALQK (SEQ ID NO: 52), VQNLTPEICK (SEQ ID NO: 53), NAKGENVLLMVSQGEVIR (SEQ ID NO: 54) and GENVLLMVSQGEVIR (SEQ ID NO: 55).

In some embodiments, the heterologous antigen is selected from the group consisting of the corresponding fragments from the *C. trachomatis* CT823 antigen. For example, the fragment may comprise or consist of a sequence selected from the group consisting of DYFNDEFFNRFFGLP (SEQ ID NO: 56), SHREQ (SEQ ID NO: 57), ALQKMGVRVQNITPE (SEQ ID NO: 58), NQVLKNSKGENVLLM (SEQ ID NO: 59), SPMLGYSASKKDSKADICLA (SEQ ID NO: 60), EDLLKEVSRGFSRVAAKATP (SEQ ID NO: 61), TGNQAIASPGNKRGFQENPF (SEQ ID NO: 62), IAIGNPFGLQATVTVGVISAKGRNQLHIVD (SEQ ID NO: 63) and NTAIVSGSGGYIGIGFAIPSLMAKRVIDQL (SEQ ID NO: 64).. For example, it may comprise or consist of a sequence selected from the group consisting of DYFNDEFFNRFFGLP (SEQ ID NO: 56), SHREQ (SEQ ID NO: 57), ALQKMGVRVQNITPE (SEQ ID NO: 58) and NQVLKNSKGENVLLM (SEQ ID NO: 59). DYFNDEFFNRFFGLP (SEQ ID NO: 56) is particularly preferred.

Preferably, the heterologous antigen is immunogenic when it is presented in the OMV. In embodiments in which the heterologous antigen comprises or consists of a fragment of a wild type antigen or of a variant thereof, the fragment is preferably immunogenic. The term "immunogenic", in the context of an immunogenic heterologous antigen,

means that the heterologous antigen is capable of eliciting an immune response, such as a cell-mediated and/or an antibody response, against the pathogen (such as a bacterium or a virus) from which the antigen is derived, for example, against the antigen in the context of the pathogen. Preferably, the immune response is elicited against the wild type pathogen from which the antigen is derived. For example, such an immune response may be elicited when the OMV of the invention is used to immunise a subject (preferably a mammal, more preferably a human or a mouse). In one embodiment, the OMV of the invention is capable of stimulating *in vitro* CD4+ IFN+-γ cells in splenocytes purified from mice infected with the live pathogen (such as *C. trachomatis*) and/or elicits antibodies that recognise the pathogen (such as *C. trachomatis*). The heterologous antigen preferably elicits antibodies that recognise the pathogen from which the heterologous antigen is derived. For example, the heterologous antigen preferably elicits antibodies that can bind to, and preferably neutralise the infection and/or virulence of the pathogen from which the heterologous antigen is derived. Preferred heterologous antigens are those which are recognised by anti-sera upon infection with a pathogen of interest. More preferred are those heterologous antigens which elicit a protective immune response against a pathogen of interest.

In some embodiments, the heterologous antigen is immunogenic when it is presented in the OMV but is not immunogenic when administered in purified form.

In some embodiments, the heterologous antigen presented in the OMV of the invention elicits an immune response which is cross-reactive with an antigen from a different species of the pathogen and thus the heterologous antigen may be used to raise an immune response against that different pathogen species. For example, where the heterologous antigen is from *C. muridarum*, the immune response may cross-react with an antigen from *C. trachomatis* or *C. pneumoniae*. Similarly, where the heterologous antigen is from *C. trachomatis*, the immune response may cross-react with an antigen from C. *pneumoniae* or *C. muridarum*. Further, where the heterologous antigen is from *C. pneumoniae*, the immune response may cross-react with an antigen from *C. trachomatis* or *C. muridarum*.

The heterologous antigen presented on the surface of the OMV is in the form of a polypeptide which comprises or consists of the heterologous antigen. Thus, in some embodiments, the polypeptide contains amino acid sequence N-terminal and/or C-terminal to the heterologous antigen.

Combinations with other antigens

In some embodiments, an OMV of the invention presents only one heterologous antigen on its surface. In other embodiments, an OMV of the invention presents more than one heterologous antigen on its surface, for example two, three, four, five, six or more, ten or more, fifteen or more, etc. For example, the OMV may present TC0210 and an additional heterologous antigen; or CT823 and an additional heterologous antigen. The additional heterologous antigen may be any heterologous antigen as described herein but is preferably a Chlamydia antigen.

In embodiments in which the OMV comprises more than one heterologous antigen, the two or more heterologous antigens may be in the form of separate polypeptides or may be present in the same polypeptide as a fusion protein. For example, the polypeptide may comprise two or more full length antigens. Alternatively, the polypeptide may comprise an epitope string of heterologous antigens which are epitopes from one or more antigens. For example, the epitope string may comprise two or more of the fragments from *C. muridarum* and/or *C. trachomatis* that are described above. The epitopes may be directly linked without any intervening sequence or may be linked by a length of amino acid sequence (for example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or 11 or more amino acids). In some embodiments, an OMV of the invention comprises a combination of heterologous antigens comprising full length wild type antigens or variants thereof and heterologous antigens comprising fragments of wild type antigens or variants thereof.

In some embodiments, a composition is provided which comprises a first OMV of the invention together with one or more additional antigens. The one or more additional antigens may be in the context of an OMV as described herein, or may be administered in an alternative form, for example, as a purified antigen or as its encoding nucleic acid. Preferably the additional antigen is a heterologous antigen in accordance with the present invention which is different from the heterologous antigen presented on the first OMV.

The invention also includes an immunogenic composition comprising a combination of antigens, e.g., *Chlamydia* antigens, said combination comprising an OMV of the invention in combination with one or more additional antigens, such as *Chlamydia* antigens. Also provided is an OMV of the invention for a use as described herein, wherein the OMV is for use in combination with one or more additional *Chlamydia* antigens (or their encoding nucleic acids). The one or more additional antigens (e.g. 2,

3, 4, 5, 6, 7 or more additional antigens) may be administered simultaneously, separately or sequentially with the OMV of the invention, for example as a combined preparation.

Examples of combinations of heterologous antigens for use in the invention include TC0106+TC0431; TC0660+TC0741; TC0551+TC0890; TC0106+TC0210+TC0741+TC0313; and TC0551+TC0890+TC106+TC431.

PCT/IB2010/050988 (Novartis Vaccines and Diagnostics, SRL) discloses that immunization with three combinations of these antigens (TC0106-TC0431; TC0660-TC0741; TC0551-TC0890) provided a significant IFU reduction in the lungs of *C. muridarum* infected mice. The contribution of individual antigens to protection was also assessed in the mouse model, leading to the identification of 4 antigens (TC0106, TC0210, TC0741 and TC0313) which were able to partially reduce the IFU load per lung (approximately 0.5-1 Log). It was then evaluated whether higher protection could be achieved by administering 4-antigen combinations. Compared to the 2-antigen combination, the 4-antigen combination (TC0551+TC0890+TC0106+TC0431) appeared to have an additive protective effect in the reduction of bacteria shed in the lung, (2.2 $\log_{10}$ reduction with P=0.0003). A slightly greater efficacy in accelerating the bacterial clearance was also observed, with 29% of animals resolving the infection completely.

In one embodiment, the one or more additional antigens are *Chlamydia* antigens selected from the antigens presented in Table 2. For example, one or more (for example, all) of the additional antigens are selected from the *Chlamydia trachomatis* antigens listed in Table 2, but may alternatively or additionally be selected from the *Chlamydia pneumoniae* antigens listed in Table 2. In one embodiment, one or more of the one or more additional antigens are selected from CT823, CT372, CT443, CT043, CT153, CT279, CT601, CT711, CT114, CT480, CT456, CT381, CT089, CT734 and CT016. These additional antigens are listed in Table 2 and their sequences are set out in the "Sequences" section that follows Table 2.

In one embodiment, an OMV of the invention is combined with CT089. In another embodiment, an OMV of the invention is combined with CT089 and CT381. Preferred combinations are an OMV of the invention with one or more antigens selected from CT372, CT443, CT601, CT153 and CT279. Another preferred combination includes an OMV of the invention in combination with 1, 2 or 3 of CT456, CT733 and/or CT043 (in particular a combination of all four antigens).

Preferably, CT823 is the heterologous antigen presented by the OMV of the invention. Preferred combinations include CT823+CT089; CT823+CT089+CT381; CT823 with one or more antigens selected from CT372, CT443, CT601, CT153 and CT279 (for example CT823+CT372; CT823+CT443, CT823+CT601, CT823+CT153 and CT823+CT279); CT823 with 1, 2 or 3 of CT456, CT733 and/or CT043 (for example, CT823+CT456; CT823+CT733; CT823+CT043 or more preferably, CT823+CT456+CT733+CT043).

Advantageous combinations of the invention are those in which two or more antigens act synergistically. Thus, the protection against *Chlamydia* achieved by their combined administration exceeds that expected by mere addition of their individual protective efficacy.

The one or more additional *Chlamydia* antigens may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to a sequence presented in Table 2; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of a sequence presented in Table 2, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). These one or more additional *Chlamydia* antigens include variants of a sequence presented in Table 2. Preferred fragments of (b) comprise an epitope from a sequence presented in Table 2. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of a sequence presented in Table 2, while retaining at least one epitope of a sequence presented in Table 2. Other fragments omit one or more protein domains. When an additional *Chlamydia* antigen comprises a sequence that is not identical to a complete sequence from Table 2 (e.g. when it comprises a sequence with less than 100% sequence identity thereto, or when it comprises a fragment thereof), it is preferred in each individual instance that the additional *Chlamydia* antigen can elicit an antibody that recognises a protein having the complete sequence from the Table 2 antigen from which it is derived.

TABLE 2

C.pneumoniae **accession number & annotation**	***C.trachomatis*** **accession number & annotation**	**CT No.**
	Hypothetical protein (AAC67968)	**CT372**
	omcB (AAC68042)	**CT443**

	Hypothetical protein (AAC67634)	CT043
	Hypothetical protein (AAC67744)	CT153
	Nqr3 (AAC67872)	CT279
	papQ (AAC68203)	CT601
	hypothetical protein (AAC68306)	CT711
	hypothetical protein (AAC67705)	CT114
	oppA_4 (AAC68080)	CT480
	hypothetical protein (AAC68056)	CT456
	ArtJ (AAC67977)	CT381
	IcrE (AAC67680)	CT089
	hypothetical protein (AAC68329)	CT734
	hypothetical protein (AAC67606)	CT016
gi|4376729|gb|AAD18590.1| Polymorphic Outer Membrane Protein G Family	gi|3329346|gb|AAC68469.1| Putative Outer Membrane Protein G	
gi|4376729|gb|AAD18590.1| Polymorphic Outer Membrane Protein G Family	gi|3329346|gb|AAC68469.1| Putative Outer Membrane Protein G	
gi|4376731|gb|AAD18591.1| Polymorphic Outer Membrane Protein G/I Family	gi|3329346|gb|AAC68469.1| Putative Outer Membrane Protein G	
gi|4376731|gb|AAD18591.1| Polymorphic Outer Membrane Protein G/I Family	gi|3329350|gb|AAC68472.1| Putative Outer Membrane Protein I	
gi|4376731|gb|AAD18591.1| Polymorphic Outer Membrane Protein G/I Family	gi|3329346|gb|AAC68469.1| Putative Outer Membrane Protein G	
gi|4376733|gb|AAD18593.1| Polymorphic Outer Membrane Protein G Family	gi|3328840|gb|AAC68009.1| Putative outer membrane protein A	
gi|4376731|gb|AAD18591.1| Polymorphic Outer Membrane Protein G/I Family	gi|3329346|gb|AAC68469.1| Putative Outer Membrane Protein G	
gi|4376754|gb|AAD18611.1| Polymorphic Outer Membrane Protein (Frame-shift with C	gi|3329344|gb|AAC68467.1| Putative Outer Membrane Protein E	
gi|4376260|gb|AAD18163.1| Polymorphic Outer Membrane Protein G Family	gi|3329346|gb|AAC68469.1| Putative Outer Membrane Protein G	
gi|4376262|gb|AAD18165.1| hypothetical protein	gi|3328765|gb|AAC67940.1| hypothetical protein	
gi|4376269|gb|AAD18171.1| hypothetical protein	gi|3328825|gb|AAC67995.1| hypothetical protein	
gi|4376270|gb|AAD18172.1| Polymorphic Outer Membrane Protein G Family	gi|3329350|gb|AAC68472.1| Putative Outer Membrane Protein I	
gi|4376272|gb|AAD18173.1| Predicted OMP {leader peptide: outer membrane}	gi|3328772|gb|AAC67946.1| hypothetical protein	CT351
gi|4376273|gb|AAD18174.1| Predicted OMP {leader peptide}	gi|3328771|gb|AAC67945.1| hypothetical protein	CT350
gi|4376296|gb|AAD18195.1| hypothetical protein	gi|3328520|gb|AAC67712.1| Ribulose-P Epimerase	
gi|4376362|gb|AAD18254.1| YbbP family hypothetical protein	gi|3328401|gb|AAC67602.1| hypothetical protein	
gi|4376372|gb|AAD18263.1| Signal Peptidase I	gi|3328410|gb|AAC67610.1| Signal Peptidase I	
gi|4376397|gb|AAD18286.1| CHLPS hypothetical protein	gi|3328506|gb|AAC67700.1| CHLPS hypothetical protein	
gi|4376402|gb|AAD18290.1| ACR family	gi|3328505|gb|AAC67699.1| ACR family	
gi|4376419|gb|AAD18305.1| CT149 hypothetical protein	gi|3328551|gb|AAC67740.1| possible hydrolase	
gi|4376446|gb|AAD18330.1| hypothetical protein	gi|3329261|gb|AAC68390.1| hypothetical protein	
gi|4376466|gb|AAD18348.1| Oligopeptide Binding Protein	gi|3328604|gb|AAC67790.1| Oligopeptide Binding Protein	CT198
gi|4376467|gb|AAD18349.1| Oligopeptide Binding Protein	gi|3328604|gb|AAC67790.1| Oligopeptide Binding Protein	
gi|4376468|gb|AAD18350.1| Oligopeptide Binding Protein	gi|3328539|gb|AAC67730.1| Oligopeptide Binding Protein	
gi|4376469|gb|AAD18351.1| Oligopeptide Binding Protein	gi|3328579|gb|AAC67766.1| Oligopeptide binding protein permease	
gi|4376520|gb|AAD18398.1| Polysaccharide Hydrolase-Invasin Repeat Family	gi|3328526|gb|AAC67718.1| predicted polysaccharide hydrolase-invasin repeat family	
gi|4376567|gb|AAD18441.1| Inclusion Membrane Protein C	gi|3328642|gb|AAC67825.1| Inclusion Membrane Protein C	
gi|4376576|gb|AAD18449.1| Omp85 Analog	gi|3328651|gb|AAC67834.1| Omp85 Analog	CT241
gi|4376577|gb|AAD18450.1| (OmpH-Like Outer Membrane Protein)	gi|3328652|gb|AAC67835.1| (OmpH-Like Outer Membrane Protein)	CT242
gi|4376601|gb|AAD18472.1| Low Calcium Response D	gi|3328486|gb|AAC67681.1| Low Calcium Response D	
gi|4376602|gb|AAD18473.1| Low Calcium Response E	gi|3328485|gb|AAC67680.1| Low Calcium Response E	CT089

gi|4376607|gb|AAD18478.1| Phopholipase D Superfamily	gi|3328479|gb|AAC67675.1| Phopholipase D Superfamily (leader (33) peptide)	
gi|4376615|gb|AAD18485.1| YojL hypothetical protein	gi|3328472|gb|AAC67668.1| hypothetical protein	CT077
gi|4376624|gb|AAD18493.1| Solute Protein Binding Family	gi|3328461|gb|AAC67658.1| Solute Protein Binding Family	
gi|4376639|gb|AAD18507.1| Flagellar Secretion Protein	gi|3328453|gb|AAC67651.1| Flagellar Secretion Protein	
gi|4376664|gb|AAD18529.1| Leucyl Aminopeptidase A	gi|3328437|gb|AAC67636.1| Leucyl Aminopeptidase A	CT045
gi|4376672|gb|AAD18537.1| CBS Domain protein (Hemolysin Homolog)	gi|3328667|gb|AAC67849.1| Hypothetical protein containing CBS domains	
gi|4376679|gb|AAD18543.1| CT253 hypothetical protein	gi|3328664|gb|AAC67846.1| hypothetical protein	
gi|4376696|gb|AAD18559.1| CT266 hypothetical protein	gi|3328678|gb|AAC67859.1| hypothetical protein	CT266
gi|4376717|gb|AAD18579.1| Phospholipase D superfamily	gi|3328698|gb|AAC67877.1| Phospholipase D superfamily	
gi|4376727|gb|AAD18588.1| Polymorphic Outer Membrane Protein G/I Family	gi|3329346|gb|AAC68469.1| Putative Outer Membrane Protein G	
gi|4376728|gb|AAD18589.1| Polymorphic Outer Membrane Protein G Family	gi|3329346|gb|AAC68469.1| Putative Outer Membrane Protein G	
gi|4376729|gb|AAD18590.1| Polymorphic Outer Membrane Protein G Family	gi|3329350|gb|AAC68472.1| Putative Outer Membrane Protein I	
gi|4376731|gb|AAD18591.1| Polymorphic Outer Membrane Protein G/I Family	gi|3329350|gb|AAC68472.1| Putative Outer Membrane Protein I	
gi|4376733|gb|AAD18593.1| Polymorphic Outer Membrane Protein G Family	gi|3328840|gb|AAC68009.1| Putative outer membrane protein A	
gi|4376735|gb|AAD18594.1| Polymorphic Outer Membrane Protein (truncated) A/I Fam	gi|3328840|gb|AAC68009.1| Putative outer membrane protein A	
gi|4376736|gb|AAD18595.1| Polymorphic Outer Membrane Protein G Family	gi|3329346|gb|AAC68469.1| Putative Outer Membrane Protein G	
gi|4376737|gb|AAD18596.1| Polymorphic Outer Membrane Protein H Family	gi|3329347|gb|AAC68470.1| Putative Outer Membrane Protein H	
gi|4376751|gb|AAD18608.1| Polymorphic Outer Membrane Protein E Family	gi|3329344|gb|AAC68467.1| Putative Outer Membrane Protein E	
gi|4376752|gb|AAD18609.1| Polymorphic Outer Membrane Protein E Family	gi|3329344|gb|AAC68467.1| Putative Outer Membrane Protein E	
gi|4376753|gb|AAD18610.1| Polymorphic Outer Membrane Protein E/F Family	gi|3329344|gb|AAC68467.1| Putative Outer Membrane Protein E	
gi|4376757|gb|AAD18613.1| hypothetical protein	gi|3328701|gb|AAC67880.1| PP-loop superfamily ATPase	
gi|4376767|gb|AAD18622.1| Arginine Periplasmic Binding Protein	gi|3328806|gb|AAC67977.1| Arginine Binding Protein	CT381
gi|4376790|gb|AAD18643.1| Heat Shock Protein-70	gi|3328822|gb|AAC67993.1| HSP-70	CT396
gi|4376802|gb|AAD18654.1| CT427 hypothetical protein	gi|3328857|gb|AAC68024.1| hypothetical protein	
gi|4376814|gb|AAD18665.1| CT398 hypothetical protein	gi|3328825|gb|AAC67995.1| hypothetical protein	CT398
gi|4376829|gb|AAD18679.1| polymorphic membrane protein A Family	gi|3328840|gb|AAC68009.1| Putative outer membrane protein A	
gi|4376830|gb|AAD18680.1| polymorphic membrane protein B Family	gi|3328841|gb|AAC68010.1| Putative outer membrane protein B	
gi|4376832|gb|AAD18681.1| Solute binding protein	gi|3328844|gb|AAC68012.1| Solute-binding protein	CT415
gi|4376834|gb|AAD18683.1| (Metal Transport Protein)	gi|3328846|gb|AAC68014.1| (Metal Transport Protein)	
gi|4376847|gb|AAD18695.1| Tail-Specific Protease	gi|3328872|gb|AAC68040.1| Tail-Specific Protease	
gi|4376848|gb|AAD18696.1| 15 kDa Cysteine-Rich Protein	gi|3328873|gb|AAC68041.1| 15kDa Cysteine-Rich Protein	
gi|4376849|gb|AAD18697.1| 60 kDa Cysteine-Rich OMP	gi|3328874|gb|AAC68042.1| 60kDa Cysteine-Rich OMP	CT443
gi|4376850|gb|AAD18698.1| 9 kDa-Cysteine-Rich Lipoprotein	gi|3328876|gb|AAC68043.1| 9kDa-Cysteine-Rich Lipoprotein	CT444
gi|4376878|gb|AAD18723.1| 2-Component Sensor	gi|3328901|gb|AAC68067.1| 2-component regulatory system-sensor histidine kinase	CT467
gi|4376879|gb|AAD18724.1| similarity to CHLPS IncA	gi|3328451|gb|AAC67649.1| hypothetical protein	
gi|4376884|gb|AAD18729.1| CT471 hypothetical protein	gi|3328905|gb|AAC68071.1| hypothetical protein	
gi|4376886|gb|AAD18731.1| YidD family	gi|3328908|gb|AAC68073.1| hypothetical protein	
gi|4376890|gb|AAD18734.1| CT476 hypothetical protein	gi|3328911|gb|AAC68076.1| hypothetical protein	
gi|4376892|gb|AAD18736.1| Oligopeptide Permease	gi|3328913|gb|AAC68078.1| Oligopeptide Permease	
gi|4376894|gb|AAD18738.1| Oligopeptide Binding Lipoprotein	gi|3328915|gb|AAC68080.1| oligopeptide Binding Lipoprotein	

gi|4376900|gb|AAD18743.1| Glutamine Binding Protein	gi|3328922|gb|AAC68086.1| Glutamine Binding Protein	
gi|4376909|gb|AAD18752.1| Protease	gi|6578107|gb|AAC68094.2| Protease	
gi|4376952|gb|AAD18792.1| Apolipoprotein N-Acetyltransferase	gi|3328972|gb|AAC68136.1| Apolipoprotein N-Acetyltransferase	
gi|4376960|gb|AAD18800.1| FKBP-type peptidyl-prolyl cis-trans isomerase	gi|3328979|gb|AAC68143.1| FKBP-type peptidyl-prolyl cis-trans isomerase	**CT541**
gi|4376968|gb|AAD18807.1| CT547 hypothetical protein	gi|3328986|gb|AAC68149.1| hypothetical protein	**CT547**
gi|4376969|gb|AAD18808.1| CT548 hypothetical protein	gi|3328987|gb|AAC68150.1| hypothetical protein	
gi|4376998|gb|AAD18834.1| Major Outer Membrane Protein	gi|3329133|gb|AAC68276.1| Major Outer Membrane Protein	**CT681**
gi|4377005|gb|AAD18841.1| YopC/Gen Secretion Protein D	gi|3329125|gb|AAC68269.1| probable Yop proteins translocation protein	
gi|4377015|gb|AAD18851.1| FHA domain; (homology to adenylate cyclase)	gi|3329115|gb|AAC68259.1| (FHA domain; homology to adenylate cyclase)	
gi|4377033|gb|AAD18867.1| CHLPN 76 kDa Homolog_1 (CT622)	gi|3329069|gb|AAC68226.1| CHLPN 76kDa Homolog	**CT622**
gi|4377034|gb|AAD18868.1| CHLPN 76 kDa Homolog_2 (CT623)	gi|6578109|gb|AAC68227.2| CHLPN 76kDa Homolog	**CT623**
gi|4377035|gb|AAD18869.1| Integral Membrane Protein	gi|3329071|gb|AAC68228.1| Integral Membrane Protein	
gi|4377072|gb|AAD18902.1| CT648 hypothetical protein	gi|3329097|gb|AAC68825.1| hypothetical protein	
gi|4377073|gb|AAD18903.1| CT647 hypothetical protein	gi|3329096|gb|AAC68824.1| hypothetical protein	**CT647**
gi|4377085|gb|AAD18914.1| CT605 hypothetical protein	gi|3329050|gb|AAC68208.1| hypothetical protein	
gi|4377090|gb|AAD18919.1| Peptidoglycan-Associated Lipoprotein	gi|3329044|gb|AAC68202.1| Peptidoglycan-Associated Lipoprotein	**CT600**
gi|4377091|gb|AAD18920.1| macromolecule transporter	gi|3329043|gb|AAC68201.1| component of a macromolecule transport system	
gi|4377092|gb|AAD18921.1| CT598 hypothetical protein	gi|3329042|gb|AAC68200.1| hypothetical protein	
gi|4377093|gb|AAD18922.1| Biopolymer Transport Protein	gi|3329041|gb|AAC68199.1| Biopolymer Transport Protein	**CT597**
gi|4377094|gb|AAD18923.1| Macromolecule transporter	gi|3329040|gb|AAC68198.1| polysaccharide transporter	
gi|4377101|gb|AAD18929.1| CT590 hypothetical protein	gi|3329033|gb|AAC68192.1| hypothetical protein	
gi|4377102|gb|AAD18930.1| CT589 hypothetical protein	gi|3329032|gb|AAC68191.1| hypothetical protein	**CT589**
gi|4377106|gb|AAD18933.1| hypothetical protein	gi|3328796|gb|AAC67968.1| hypothetical protein	
gi|4377111|gb|AAD18938.1| Enolase	gi|3329030|gb|AAC68189.1| Enolase	**CT587**
gi|4377127|gb|AAD18953.1| General Secretion Protein D	gi|3329013|gb|AAC68174.1| Gen. Secretion Protein D	
gi|4377130|gb|AAD18956.1| predicted OMP {leader peptide}	gi|3329010|gb|AAC68171.1| predicted OMP	**CT569**
gi|4377132|gb|AAD18958.1| CT567 hypothetical protein	gi|3329008|gb|AAC68169.1| hypothetical protein	**CT567**
gi|4377133|gb|AAD18959.1| CT566 hypothetical protein	gi|3329007|gb|AAC68168.1| hypothetical protein	
gi|4377140|gb|AAD18965.1| Yop Translocation J	gi|3329000|gb|AAC68161.1| Yop proteins translocation lipoprotein J	**CT559**
gi|4377170|gb|AAD18992.1| Outer Membrane Protein B	gi|3329169|gb|AAC68308.1| Outer Membrane Protein Analog	**CT713**
gi|4377177|gb|AAD18998.1| Flagellar M-Ring Protein	gi|3329175|gb|AAC68314.1| Flagellar M-Ring Protein	
gi|4377182|gb|AAD19003.1| CT724 hypothetical protein	gi|3329181|gb|AAC68319.1| hypothetical protein	
gi|4377184|gb|AAD19005.1| Rod Shape Protein	gi|3329183|gb|AAC68321.1| Rod Shape Protein	
gi|4377193|gb|AAD19013.1| CT734 hypothetical protein	gi|3329192|gb|AAC68329.1| hypothetical protein	
gi|4377206|gb|AAD19025.1| CHLTR possible phosphoprotein	gi|3329204|gb|AAC68339.1| CHLTR possible phosphoprotein	
gi|4377222|gb|AAD19040.1| Muramidase (invasin repeat family)	gi|3329221|gb|AAC68354.1| Muramidase (invasin repeat family)	**CT759**
gi|4377223|gb|AAD19041.1| Cell Division Protein FtsW	gi|3329222|gb|AAC68355.1| Cell Division Protein FtsW	
gi|4377224|gb|AAD19042.1| Peptidoglycan Transferase	gi|3329223|gb|AAC68356.1| Peptidoglycan Transferase	**CT761**
gi|4377225|gb|AAD19043.1| Muramate-Ala Ligase & D-Ala-D-Ala Ligase	gi|3329224|gb|AAC68357.1| UDP-N-acetylmuramate-alanine ligase	
gi|4377248|gb|AAD19064.1| Thioredoxin Disulfide Isomerase	gi|3329244|gb|AAC68375.1| Thioredoxin Disulfide Isomerase	
gi|4377261|gb|AAD19076.1| CT788 hypothetical protein - {leader peptide-periplasmi	gi|3329253|gb|AAC68383.1| {leader (60) peptide-periplasmic}	
gi|4377280|gb|AAD19093.1| Insulinase family/Protease III	gi|3329273|gb|AAC68402.1| Insulinase family/Protease III	
gi|4377287|gb|AAD19099.1| Putative Outer Membrane Protein D Family	gi|3329279|gb|AAC68408.1| Putative Outer Membrane Protein D	
gi|4377306|gb|AAD19116.1| DO Serine Protease	gi|3329293|gb|AAC68420.1| DO Serine Protease	**CT823**
gi|4377342|gb|AAD19149.1| ABC transporter permease	gi|3329327|gb|AAC68451.1| ABC transporter permease —	

	pyrimidine biosynthesis protein	
gi|4377347|gb|AAD19153.1| CT858 hypothetical protein	gi|6578118|gb|AAC68456.2| predicted Protease containing IRBP and DHR domains	
gi|4377353|gb|AAD19159.1| CT863 hypothetical protein	gi|3329337|gb|AAC68461.1| hypothetical protein	
gi|4377367|gb|AAD19171.1| Predicted OMP	gi|3328795|gb|AAC67967.1| hypothetical protein	
gi|4377408|gb|AAD19209.1| hypothetical protein	gi|3328795|gb|AAC67967.1| hypothetical protein	
gi|4377409|gb|AAD19210.1| Predicted Outer Membrane Protein (CT371)	gi|3328795|gb|AAC67967.1| hypothetical protein	
gi|4376411|gb|	gi|3328512|gb|AAC67705.1| hypothetical protein	**CT114**
gi|4376508|gb|	gi|3328585|gb|AAC67772.1| hypothetical protein	**CT181**
gi|4376710|gb|	gi|3328692|gb|AAC67872.1| NADH (Ubiquinone) Oxidoreductase, Gamma	**CT279**
gi|4376777|gb|	gi|3328815|gb|AAC67986.1| hypothetical protein	**CT389**
gi|4376782|gb|	gi|3328817|gb|AAC67988.1| hypothetical protein	**CT391**
gi|4376863|gb|	gi|3328887|gb|AAC68054.1| Arginyl tRNA transferase	**CT454**
gi|4376866|gb|	gi|3328889|gb|AAC68056.1| hypothetical protein	**CT456**
gi|4376972|gb|	gi|3328991|gb|AAC68153.1| D-Ala-D-Ala Carboxypeptidase	**CT551**
gi|4377139|gb|	gi|3329001|gb|AAC68162.1| hypothetical protein	**CT560**
gi|4377154|gb|	gi|3329154|gb|AAC68295.1| hypothetical protein	**CT700**
gi|4377191|gb|AAD19012.1| hypothetical protein	gi|3329191|gb|AAC68328.1| hypothetical protein	**CT733**

The additional *Chlamydia* antigens used in the invention may be present in the composition as individual separate polypeptides. Alternatively, the combination may be present as a hybrid polypeptide in which two or more (*i.e.* 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 or more) of the antigens are expressed as a single polypeptide chain. Hybrid polypeptides offer two principal advantages: first, a polypeptide that may be unstable or poorly expressed on its own can be assisted by adding a suitable hybrid partner that overcomes the problem; second, commercial manufacture is simplified as only one expression and purification need be employed in order to produce two polypeptides which are both antigenically useful. Different hybrid polypeptides may be mixed together in a single formulation. Within such combinations, a *Chlamydia trachomatis* antigen may be present in more than one hybrid polypeptide and/or as a non-hybrid polypeptide. It is preferred, however, that an antigen is present either as a hybrid or as a non-hybrid, but not as both.

Hybrid polypeptides can be represented by the formula NH_2-A-{-X-L-}$_n$-B-COOH, wherein: at least one X is an amino acid sequence of a heterologous antigen as described above; L is an optional linker amino acid sequence; A is an optional N-terminal amino acid sequence; B is an optional C-terminal amino acid sequence; *n* is an integer of 2 or more (*e.g.* 2, 3, 4, 5, 6, *etc.*). Usually *n* is 2 or 3.

If a -X- moiety has a leader peptide sequence in its wild-type form, this may be included or omitted in the hybrid protein. In some embodiments, the leader peptides will be

deleted except for that of the -X- moiety located at the N-terminus of the hybrid protein *i.e.* the leader peptide of X_1 will be retained, but the leader peptides of X_2 ... X_n will be omitted. This is equivalent to deleting all leader peptides and using the leader peptide of X_1 as moiety -A-.

For each *n* instances of {-X-L-}, linker amino acid sequence -L- may be present or absent. For instance, when *n*=2 the hybrid may be NH_2-X_1-L_1-X_2-L_2-COOH, NH_2-X_1-X_2-COOH, NH_2-X_1-L_1-X_2-COOH, NH_2-X_1-X_2-L_2-COOH, *etc.* Linker amino acid sequence(s) -L- will typically be short (*e.g.* 20 or fewer amino acids *i.e.* 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1). Examples comprise short peptide sequences which facilitate cloning, poly-glycine linkers (*i.e.* comprising Gly_n where *n* = 2, 3, 4, 5, 6, 7, 8, 9, 10 or more), and histidine tags (*i.e.* His_n where *n* = 3, 4, 5, 6, 7, 8, 9, 10 or more). Other suitable linker amino acid sequences will be apparent to those skilled in the art. A useful linker is GSGGGG (SEQ ID NO: 65), with the Gly-Ser dipeptide being formed from a *Bam*HI restriction site, thus aiding cloning and manipulation, and the $(Gly)_4$ tetrapeptide being a typical poly-glycine linker.

-A- is an optional N-terminal amino acid sequence. This will typically be short (*e.g.* 40 or fewer amino acids *i.e.* 40, 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1). Examples include leader sequences to direct protein trafficking, or short peptide sequences which facilitate cloning or purification (*e.g.* histidine tags *i.e.* His_n where *n* = 3, 4, 5, 6, 7, 8, 9, 10 or more). Other suitable N-terminal amino acid sequences will be apparent to those skilled in the art. If X_1 lacks its own N-terminus methionine, -A- is preferably an oligopeptide (*e.g.* with 1, 2, 3, 4, 5, 6, 7 or 8 amino acids) which provides a N-terminus methionine.

-B- is an optional C-terminal amino acid sequence. This will typically be short (*e.g.* 40 or fewer amino acids *i.e.* 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1). Examples include sequences to direct protein trafficking, short peptide sequences which facilitate cloning or purification (*e.g.* comprising histidine tags *i.e.* His_n where *n* = 3, 4, 5, 6, 7, 8, 9, 10 or more), or sequences which enhance protein stability. Other suitable C-terminal amino acid sequences will be apparent to those skilled in the art.

Where hybrid polypeptides are used, the individual antigens within the hybrid (*i.e.* individual -X- moieties) may be from one or more strains. Where n=2, for instance, X_2 may be from the same strain as X_1 or from a different strain. Where n=3, the strains might be (i) $X_1=X_2=X_3$ (ii) $X_1=X_2\neq X_3$ (iii) $X_1\neq X_2=X_3$ (iv) $X_1\neq X_2\neq X_3$ or (v) $X_1=X_3\neq X_2$, *etc.*

The invention also provides a kit comprising an OMV of the invention and one or more additional antigens for simultaneous, separate or sequential administration.

The heterologous antigen may be presented by any suitable method. In some embodiments, the coding sequence of the heterologous antigen is fused to a leader peptide sequence, for example, the leader peptide sequence of OmpA, and the fusion is placed under the control of a promoter in a plasmid. The lac promoter is an example of a suitable promoter. Any suitable plasmid may be used, for example, the multicopy plasmid pET. As mentioned above, the heterologous antigen may be presented in the context of a longer polypeptide sequence, for example, with additional sequence N-terminal and/or C-terminal to the heterologous antigen. In such cases, the leader peptides will be fused to the longer polypeptide sequence.

In preferred embodiments, the derived plasmid is used to transform the bacterium and the recombinant clones are grown in liquid cultures. The OMVs released may be purified by any suitable method, for example, centrifugation.

Antibodies

The heterologous antigen preferably induces antibodies able to neutralize infection or virulence of the pathogen from which the antigen is derived. These neutralizing antibodies may be used as a vaccine capable of neutralising the infection or virulence of the pathogen, for example of *Chlamydia*, more particularly, of *C. trachomatis* or *C. pneumoniae*.

According to a further aspect, the invention provides one or more antibodies which bind to a heterologous antigen presented by an OMV of the invention. Preferably, the antibody does not bind to the heterologous antigen when it is not presented in an OMV of the present invention. For example, the antibody preferably does not bind to the heterologous antigen in its purified form. Preferably, the antibody binds to an epitope that is immunoaccessible and in its native conformation when the heterologous antigen is presented in an OMV of the invention but which is not immunoaccessible and/or not in its native conformation when presented by the antigen in its purified form. For example,

the present inventors have found that different epitopes are recognised in TC0210 when it is presented by an OMV of the invention than when it is presented in its purified form (see results of Example 8). Thus, in some embodiments, there is provided an OMV, composition or vaccine of the invention for use in raising antibodies that bind to one or more epitopes in the heterologous antigen that are not immunoaccessible when the heterologous antigen is administered in a purified form.

The term "antibody" includes intact immunoglobulin molecules, as well as fragments thereof which are capable of binding an antigen. These include hybrid (chimeric) antibody molecules ([27], [28]); $F(ab')_2$ and F(ab) fragments and Fv molecules; non-covalent heterodimers ([29]; [30]); single-chain Fv molecules (sFv) [31]; dimeric and trimeric antibody fragment constructs; minibodies [32],[33]; humanized antibody molecules [34],[35],[36]; and any functional fragments obtained from such molecules, as well as antibodies obtained through non-conventional processes such as phage display. Preferably, the antibodies are monoclonal antibodies. Methods of obtaining monoclonal antibodies are well known in the art. Humanised or fully-human antibodies are preferred.

The antibodies may be polyclonal or monoclonal and may be produced by any suitable means. The antibody may include a detectable label.

Also provided is a method for preparing antibodies comprising immunising a mammal (such as a mouse or a rabbit) with an OMV of the invention and obtaining polyclonal antibodies or monoclonal antibodies by conventional techniques. For example, polyclonal antisera may be obtained by bleeding the immunized animal into a glass or plastic container, incubating the blood at 25°C for one hour, followed by incubating at 4°C for 2-18 hours. The serum is recovered by centrifugation (*eg.* 1,000*g* for 10 minutes). Monoclonal antibodies may be prepared using the standard method of Kohler & Milstein [37], or a modification thereof, or by any other suitable method. A polyclonal antibody preparation prepared by this method is also provided.

Antibody titres and specificities can be measured using standard methods available in the art. Other methods of testing the immunogenicity of proteins are also well known in the art.

The antibodies of the invention may be used in combination with one or more antibodies specific for one or more additional antigens (e.g. *Chlamydia* antigens) for use in diagnosis of infections (e.g. *Chlamydia* infections).

Immunogenic compositions and medicaments

The OMV, medicament or bacterium may be in the form of a composition. These compositions may be suitable as immunogenic compositions (*e.g.* vaccines), or as diagnostic reagents. Generally, the composition will comprise multiple copies of the same OMV or bacterium.

It is particularly advantageous to use an OMV of the invention in an immunogenic composition such as a vaccine. Preferably, the final formulation of the vaccine is more stable compared with immunogenic compositions that comprise the heterologous antigen in purified form.

In embodiments in which the immunogenic composition comprises one or more bacteria of the invention, it is preferred that the bacterium is a non-pathogenic bacterium. In such embodiments, the OMVs are generated *in vivo*.

An immunogenic composition of the invention comprises an OMV according to the invention. Immunogenic compositions according to the invention may either be prophylactic (*i.e.* to prevent infection) or therapeutic (*i.e.* to treat infection), but will typically be prophylactic. Where the immunogenic composition is for prophylactic use, the human is preferably a child (*e.g.* a toddler or infant) or a teenager; where the immunogenic composition is for therapeutic use, the human is preferably a teenager or an adult. An immunogenic composition intended for children may also be administered to adults *e.g.* to assess safety, dosage, immunogenicity, *etc.*

In some embodiments, the immunogenic composition is for treatment or prevention of *Chlamydia* infection or an associated condition (*e.g.* trachoma, blindness, cervicitis, pelvic inflammatory disease, infertility, ectopic pregnancy, chronic pelvic pain, salpingitis, urethritis, epididymitis, infant pneumonia, patients infected with cervical squamous cell carcinoma, and/or HIV infection, *etc.*), preferably, *C. trachomatis* infection. In some embodiments, the immunogenic composition is effective against *C. pneumoniae*.

Immunogenic compositions used as vaccines comprise an immunologically effective amount of the OMV of the invention, as well as any other components, as needed. By 'immunologically effective amount', it is meant that the administration of that amount to an individual, either in a single dose or as part of a series, is effective for treatment or prevention. This amount varies depending upon the health and physical condition of the

individual to be treated, age, the taxonomic group of the individual to be treated (*e.g.* non-human primate, primate, *etc.*), the capacity of the individual's immune system to synthesise antibodies, the degree of protection desired, the formulation of the vaccine, the treating doctor's assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials. Where more than one antigen is included in a composition, then two antigens may be present at the same dose as each other or at different doses.

In general, a composition of the invention will comprise a heterologous antigen at a concentration that will be sufficient to elicit an immune response against that antigen. Heterologous antigens in the composition will typically be present at a concentration of at least 1μg/ml each. For example, in some embodiments, one dose of a composition of the invention comprises 1 to 600μg of the heterologous antigen, for example, 1 to 500 μg, 100-500 μg, 100-200 μg, 1 to 300 μg, 1 to 100 μg, 1 to 50 μg, 1 to 35 μg, 1 to 25 μg, 10 to 30 μg, 20 to 30 μg, 23 to 27 μg or 24 to 26 μg of heterologous antigen. Preferably, one dose of a composition of the present invention comprises 25 μg of the heterologous antigen. These dose preferences may be applied to the methods of the invention *mutatis mutandis*.

Dosage treatment can be a single dose schedule or a multiple dose schedule. Multiple doses may be used in a primary immunisation schedule and/or in a booster immunisation schedule. In a multiple dose schedule the various doses may be given by the same or different routes *e.g.* a parenteral prime and mucosal boost, a mucosal prime and parenteral boost, *etc.* Multiple doses will typically be administered at least 1 week apart (*e.g.* about 2 weeks, about 3 weeks, about 4 weeks, about 6 weeks, about 8 weeks, about 10 weeks, about 12 weeks, about 16 weeks, *etc.*).

The pH of a composition will generally be between 5.0 and 8.1, and more typically between 6.0 and 8.0, *e.g.* 6.5 and 7.5, or between 7.0 and 7.8, preferably about 7. pH may be maintained by the use of a buffer.

The composition is preferably sterile. The composition is preferably non-pyrogenic *e.g.* containing <1 EU (endotoxin unit, a standard measure) per dose, and preferably <0.1 EU per dose. The composition is preferably gluten free. The composition may be isotonic with respect to humans.

Immunogenic compositions of the invention will generally be administered directly to a patient. Direct delivery may be accomplished by parenteral injection (*e.g.* subcutaneously, intraperitoneally, intravenously, intramuscularly, or to the interstitial space of a tissue), or mucosally, such as by rectal, oral (*e.g.* tablet, spray), vaginal, topical, transdermal (*See e.g.* [38]) or transcutaneous (See *e.g.* [39] and [40]), intranasal (See *e.g.* [41]), ocular, aural, pulmonary or other mucosal administration.

Pathogen infections (such as *Chlamydia* infections) affect various areas of the body and so the immunogenic compositions of the invention may be prepared in various forms. For example, the compositions may be prepared as injectables, either as liquid solutions or suspensions. Solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection can also be prepared (*e.g.* a lyophilised composition or a spray-freeze dried composition). The composition may be prepared for topical administration *e.g.* as an ointment, cream or powder. The composition may be prepared for oral administration *e.g.* as a tablet or capsule, or as a syrup (optionally flavoured). The composition may be prepared for pulmonary administration *e.g.* as an inhaler, using a fine powder or a spray. The composition may be prepared as a suppository or pessary. The composition may be prepared for nasal, aural or ocular administration *e.g.* as drops.

The invention also provides a delivery device pre-filled with an immunogenic composition of the invention.

The invention also provides a kit comprising a first component and a second component wherein neither the first component nor the second component is a composition of the invention as described herein, but wherein the first component and the second component can be combined to provide a composition of the invention as described herein. The kit may further include a third component comprising one or more of the following: instructions, syringe or other delivery device, adjuvant, or pharmaceutically acceptable formulating solution.

The composition may be in kit form, designed such that a combined composition is reconstituted just prior to administration to a patient. Such kits may comprise one or more OMVs in liquid form and one or more lyophilised agents.

Where a composition is to be prepared extemporaneously prior to use (e.g. where a component is presented in lyophilised form) and is presented as a kit, the kit may

comprise two vials, or it may comprise one ready-filled syringe and one vial, with the contents of the syringe being used to reactivate the contents of the vial prior to injection.

Compositions may thus be pharmaceutically acceptable. They will usually include components in addition to the OMV(s) *e.g.* they typically include one or more pharmaceutical carrier(s) and/or excipient(s). A thorough discussion of such components is available in reference 166.

Compositions will generally be administered to a mammal in aqueous form. Prior to administration, however, the composition may have been in a non-aqueous form. For instance, although some vaccines are manufactured in aqueous form, then filled and distributed and administered also in aqueous form, other vaccines are lyophilised during manufacture and are reconstituted into an aqueous form at the time of use. Thus a composition of the invention may be dried, such as a lyophilised formulation.

The composition may include preservatives such as thiomersal or 2-phenoxyethanol. It is preferred, however, that the vaccine should be substantially free from (*i.e.* less than 5μg/ml) mercurial material *e.g.* thiomersal-free. Vaccines containing no mercury are more preferred. Preservative-free vaccines are particularly preferred.

To control tonicity, it is preferred to include a physiological salt, such as a sodium salt. Sodium chloride (NaCl) is preferred, which may be present at between 1 and 20 mg/ml *e.g.* about 10±2mg/ml NaCl. Other salts that may be present include potassium chloride, potassium dihydrogen phosphate, disodium phosphate dehydrate, magnesium chloride, calcium chloride, *etc.*

Compositions will generally have an osmolality of between 200 mOsm/kg and 400 mOsm/kg, preferably between 240-360 mOsm/kg, and will more preferably fall within the range of 290-310 mOsm/kg.

Compositions may include one or more buffers. Typical buffers include: a phosphate buffer; a Tris buffer; a borate buffer; a succinate buffer; a histidine buffer (particularly with an aluminum hydroxide adjuvant); or a citrate buffer. Buffers will typically be included in the 5-20mM range.

The composition may include material for a single immunisation, or may include material for multiple immunisations (*i.e.* a 'multidose' kit). The inclusion of a preservative is preferred in multidose arrangements. As an alternative (or in addition) to

including a preservative in multidose compositions, the compositions may be contained in a container having an aseptic adaptor for removal of material.

Human vaccines are typically administered in a dosage volume of about 0.5ml, although a half dose (*i.e.* about 0.25ml) may be administered to children.

Immunogenic compositions of the invention may also comprise one or more immunoregulatory agents. Preferably, one or more of the immunoregulatory agents include one or more adjuvants. The adjuvants may include a TH1 adjuvant and/or a TH2 adjuvant, further discussed below.

Thus the invention provides an immunogenic composition comprising a combination of: (1) an OMV of the invention; and (2) an adjuvant, such as an aluminium hydroxide adjuvant (for example, one or more antigens may be adsorbed to aluminium hydroxide).

Adjuvants which may be used in compositions of the invention include, but are not limited to:

A. Mineral-containing compositions

Mineral containing compositions suitable for use as adjuvants in the invention include mineral salts, such as aluminium salts and calcium salts (or mixtures thereof). Calcium salts include calcium phosphate (*e.g.* the "CAP" particles disclosed in ref. 42). Aluminum salts include hydroxides, phosphates, sulfates, *etc.*, with the salts taking any suitable form (*e.g.* gel, crystalline, amorphous, *etc.*). Adsorption to these salts is preferred (*e.g.* all antigens may be adsorbed). The mineral containing compositions may also be formulated as a particle of metal salt [43].

The adjuvants known as aluminum hydroxide and aluminum phosphate may be used. These names are conventional, but are used for convenience only, as neither is a precise description of the actual chemical compound which is present (*e.g.* see chapter 9 of reference 48). The invention can use any of the "hydroxide" or "phosphate" adjuvants that are in general use as adjuvants. The adjuvants known as "aluminium hydroxide" are typically aluminium oxyhydroxide salts, which are usually at least partially crystalline. The adjuvants known as "aluminium phosphate" are typically aluminium hydroxyphosphates, often also containing a small amount of sulfate (*i.e.* aluminium hydroxyphosphate sulfate). They may be obtained by precipitation, and the reaction

conditions and concentrations during precipitation influence the degree of substitution of phosphate for hydroxyl in the salt.

A fibrous morphology (*e.g.* as seen in transmission electron micrographs) is typical for aluminium hydroxide adjuvants. The pI of aluminium hydroxide adjuvants is typically about 11 *i.e.* the adjuvant itself has a positive surface charge at physiological pH. Adsorptive capacities of between 1.8-2.6 mg protein per mg Al^{+++} at pH 7.4 have been reported for aluminium hydroxide adjuvants.

Aluminium phosphate adjuvants generally have a PO_4/Al molar ratio between 0.3 and 1.2, preferably between 0.8 and 1.2, and more preferably 0.95±0.1. The aluminium phosphate will generally be amorphous, particularly for hydroxyphosphate salts. A typical adjuvant is amorphous aluminium hydroxyphosphate with PO_4/Al molar ratio between 0.84 and 0.92, included at 0.6mg Al^{3+}/ml. The aluminium phosphate will generally be particulate (*e.g.* plate-like morphology as seen in transmission electron micrographs). Typical diameters of the particles are in the range 0.5-20μm (*e.g.* about 5-10μm) after any antigen adsorption. Adsorptive capacities of between 0.7-1.5 mg protein per mg Al^{+++} at pH 7.4 have been reported for aluminium phosphate adjuvants.

The point of zero charge (PZC) of aluminium phosphate is inversely related to the degree of substitution of phosphate for hydroxyl, and this degree of substitution can vary depending on reaction conditions and concentration of reactants used for preparing the salt by precipitation. PZC is also altered by changing the concentration of free phosphate ions in solution (more phosphate = more acidic PZC) or by adding a buffer such as a histidine buffer (makes PZC more basic). Aluminium phosphates used according to the invention will generally have a PZC of between 4.0 and 7.0, more preferably between 5.0 and 6.5 *e.g.* about 5.7.

Adsorption of *S.aureus* protein antigens (except IsdA, Sta019 and Sta073) to an aluminium hydroxide adjuvant is advantageous, particularly in a multi-protein combination (in which all antigens may be adsorbed). A histidine buffer can usefully be included in such adjuvanted compositions.

Suspensions of aluminium salts used to prepare compositions of the invention may contain a buffer (*e.g.* a phosphate or a histidine or a Tris buffer), but this is not always necessary. The suspensions are preferably sterile and pyrogen-free. A suspension may include free aqueous phosphate ions *e.g.* present at a concentration between 1.0 and

20 mM, preferably between 5 and 15 mM, and more preferably about 10 mM. The suspensions may also comprise sodium chloride.

The invention can use a mixture of both an aluminium hydroxide and an aluminium phosphate. In this case there may be more aluminium phosphate than hydroxide *e.g.* a weight ratio of at least 2:1 *e.g.* ≥5:1, ≥6:1, ≥7:1, ≥8:1, ≥9:1, *etc.*

The concentration of Al^{+++} in a composition for administration to a patient is preferably less than 10mg/ml *e.g.* ≤5 mg/ml, ≤4 mg/ml, ≤3 mg/ml, ≤2 mg/ml, ≤1 mg/ml, *etc.* A preferred range is between 0.3 and 1mg/ml. A maximum of 0.85mg/dose is preferred.

B. Oil Emulsions

Oil emulsion compositions suitable for use as adjuvants in the invention include squalene-water emulsions, such as MF59 [Chapter 10 of ref. 48; see also ref. 44] (5% Squalene, 0.5% Tween 80, and 0.5% Span 85, formulated into submicron particles using a microfluidizer). Complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA) may also be used.

Various oil-in-water emulsion adjuvants are known, and they typically include at least one oil and at least one surfactant, with the oil(s) and surfactant(s) being biodegradable (metabolisable) and biocompatible. The oil droplets in the emulsion are generally less than 5μm in diameter, and ideally have a sub-micron diameter, with these small sizes being achieved with a microfluidiser to provide stable emulsions. Droplets with a size less than 220nm are preferred as they can be subjected to filter sterilization.

The emulsion can comprise oils such as those from an animal (such as fish) or vegetable source. Sources for vegetable oils include nuts, seeds and grains. Peanut oil, soybean oil, coconut oil, and olive oil, the most commonly available, exemplify the nut oils. Jojoba oil can be used *e.g.* obtained from the jojoba bean. Seed oils include safflower oil, cottonseed oil, sunflower seed oil, sesame seed oil and the like. In the grain group, corn oil is the most readily available, but the oil of other cereal grains such as wheat, oats, rye, rice, teff, triticale and the like may also be used. 6-10 carbon fatty acid esters of glycerol and 1,2-propanediol, while not occurring naturally in seed oils, may be prepared by hydrolysis, separation and esterification of the appropriate materials starting from the nut and seed oils. Fats and oils from mammalian milk are metabolizable and may therefore be used in the practice of this invention. The procedures for separation, purification, saponification and other means necessary for obtaining pure oils from

animal sources are well known in the art. Most fish contain metabolizable oils which may be readily recovered. For example, cod liver oil, shark liver oils, and whale oil such as spermaceti exemplify several of the fish oils which may be used herein. A number of branched chain oils are synthesized biochemically in 5-carbon isoprene units and are generally referred to as terpenoids. Shark liver oil contains a branched, unsaturated terpenoids known as squalene, 2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene, which is particularly preferred herein. Squalane, the saturated analog to squalene, is also a preferred oil. Fish oils, including squalene and squalane, are readily available from commercial sources or may be obtained by methods known in the art. Other preferred oils are the tocopherols (see below). Mixtures of oils can be used.

Surfactants can be classified by their 'HLB' (hydrophile/lipophile balance). Preferred surfactants of the invention have a HLB of at least 10, preferably at least 15, and more preferably at least 16. The invention can be used with surfactants including, but not limited to: the polyoxyethylene sorbitan esters surfactants (commonly referred to as the Tweens), especially polysorbate 20 and polysorbate 80; copolymers of ethylene oxide (EO), propylene oxide (PO), and/or butylene oxide (BO), sold under the DOWFAX™ tradename, such as linear EO/PO block copolymers; octoxynols, which can vary in the number of repeating ethoxy (oxy-1,2-ethanediyl) groups, with octoxynol-9 (Triton X-100, or t-octylphenoxypolyethoxyethanol) being of particular interest; (octylphenoxy)polyethoxyethanol (IGEPAL CA-630/NP-40); phospholipids such as phosphatidylcholine (lecithin); nonylphenol ethoxylates, such as the Tergitol™ NP series; polyoxyethylene fatty ethers derived from lauryl, cetyl, stearyl and oleyl alcohols (known as Brij surfactants), such as triethyleneglycol monolauryl ether (Brij 30); and sorbitan esters (commonly known as the SPANs), such as sorbitan trioleate (Span 85) and sorbitan monolaurate. Non-ionic surfactants are preferred. Preferred surfactants for including in the emulsion are Tween 80 (polyoxyethylene sorbitan monooleate), Span 85 (sorbitan trioleate), lecithin and Triton X-100.

Mixtures of surfactants can be used *e.g.* Tween 80/Span 85 mixtures. A combination of a polyoxyethylene sorbitan ester such as polyoxyethylene sorbitan monooleate (Tween 80) and an octoxynol such as t-octylphenoxypolyethoxyethanol (Triton X-100) is also suitable. Another useful combination comprises laureth 9 plus a polyoxyethylene sorbitan ester and/or an octoxynol.

Preferred amounts of surfactants (% by weight) are: polyoxyethylene sorbitan esters (such as Tween 80) 0.01 to 1%, in particular about 0.1 %; octyl- or nonylphenoxy polyoxyethanols (such as Triton X-100, or other detergents in the Triton series) 0.001 to 0.1 %, in particular 0.005 to 0.02%; polyoxyethylene ethers (such as laureth 9) 0.1 to 20 %, preferably 0.1 to 10 % and in particular 0.1 to 1 % or about 0.5%.

Preferred emulsion adjuvants have an average droplets size of <1μm *e.g.* ≤750nm, ≤500nm, ≤400nm, ≤300nm, ≤250nm, ≤220nm, ≤200nm, or smaller. These droplet sizes can conveniently be achieved by techniques such as microfluidisation.

Specific oil-in-water emulsion adjuvants useful with the invention include, but are not limited to:

- A submicron emulsion of squalene, Tween 80, and Span 85. The composition of the emulsion by volume can be about 5% squalene, about 0.5% polysorbate 80 and about 0.5% Span 85. In weight terms, these ratios become 4.3% squalene, 0.5% polysorbate 80 and 0.48% Span 85. This adjuvant is known as 'MF59' [45-47], as described in more detail in Chapter 10 of ref. 48 and chapter 12 of ref. 49. The MF59 emulsion advantageously includes citrate ions *e.g.* 10mM sodium citrate buffer.

- An emulsion of squalene, a tocopherol, and polysorbate 80 (Tween 80). The emulsion may include phosphate buffered saline. It may also include Span 85 (*e.g.* at 1%) and/or lecithin. These emulsions may have from 2 to 10% squalene, from 2 to 10% tocopherol and from 0.3 to 3% Tween 80, and the weight ratio of squalene:tocopherol is preferably ≤1 as this provides a more stable emulsion. Squalene and Tween 80 may be present volume ratio of about 5:2 or at a weight ratio of about 11:5. One such emulsion can be made by dissolving Tween 80 in PBS to give a 2% solution, then mixing 90ml of this solution with a mixture of (5g of DL-α-tocopherol and 5ml squalene), then microfluidising the mixture. The resulting emulsion may have submicron oil droplets *e.g.* with an average diameter of between 100 and 250nm, preferably about 180nm. The emulsion may also include a 3-de-O-acylated monophosphoryl lipid A (3d-MPL). Another useful emulsion of this type may comprise, per human dose, 0.5-10 mg squalene, 0.5-11 mg tocopherol, and 0.1-4 mg polysorbate 80 [50].

- An emulsion of squalene, a tocopherol, and a Triton detergent (*e.g.* Triton X-100). The emulsion may also include a 3d-MPL (see below). The emulsion may contain a phosphate buffer.

- An emulsion comprising a polysorbate (*e.g.* polysorbate 80), a Triton detergent (*e.g.* Triton X-100) and a tocopherol (*e.g.* an α-tocopherol succinate). The emulsion may include these three components at a mass ratio of about 75:11:10 (*e.g.* 750μg/ml polysorbate 80, 110μg/ml Triton X-100 and 100μg/ml α-tocopherol succinate), and these concentrations should include any contribution of these components from antigens. The emulsion may also include squalene. The emulsion may also include a 3d-MPL (see below). The aqueous phase may contain a phosphate buffer.

- An emulsion of squalane, polysorbate 80 and poloxamer 401 ("Pluronic™ L121"). The emulsion can be formulated in phosphate buffered saline, pH 7.4. This emulsion is a useful delivery vehicle for muramyl dipeptides, and has been used with threonyl-MDP in the "SAF-1" adjuvant [51] (0.05-1% Thr-MDP, 5% squalane, 2.5% Pluronic L121 and 0.2% polysorbate 80). It can also be used without the Thr-MDP, as in the "AF" adjuvant [52] (5% squalane, 1.25% Pluronic L121 and 0.2% polysorbate 80). Microfluidisation is preferred.

- An emulsion comprising squalene, an aqueous solvent, a polyoxyethylene alkyl ether hydrophilic nonionic surfactant (*e.g.* polyoxyethylene (12) cetostearyl ether) and a hydrophobic nonionic surfactant (*e.g.* a sorbitan ester or mannide ester, such as sorbitan monoleate or 'Span 80'). The emulsion is preferably thermoreversible and/or has at least 90% of the oil droplets (by volume) with a size less than 200 nm [53]. The emulsion may also include one or more of: alditol; a cryoprotective agent (*e.g.* a sugar, such as dodecylmaltoside and/or sucrose); and/or an alkylpolyglycoside. The emulsion may include a TLR4 agonist [54]. Such emulsions may be lyophilized.

- An emulsion of squalene, poloxamer 105 and Abil-Care [55]. The final concentration (weight) of these components in adjuvanted vaccines are 5% squalene, 4% poloxamer 105 (pluronic polyol) and 2% Abil-Care 85 (Bis-PEG/PPG-16/16 PEG/PPG-16/16 dimethicone; caprylic/capric triglyceride).

- An emulsion having from 0.5-50% of an oil, 0.1-10% of a phospholipid, and 0.05-5% of a non-ionic surfactant. As described in reference 56, preferred phospholipid components are phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidylglycerol, phosphatidic acid, sphingomyelin and cardiolipin. Submicron droplet sizes are advantageous.

- A submicron oil-in-water emulsion of a non-metabolisable oil (such as light mineral oil) and at least one surfactant (such as lecithin, Tween 80 or Span 80). Additives may be included, such as QuilA saponin, cholesterol, a saponin-lipophile conjugate (such as GPI-0100, described in reference 57, produced by addition of aliphatic amine to desacylsaponin via the carboxyl group of glucuronic acid), dimethyidioctadecylammonium bromide and/or N,N-dioctadecyl-N,N-bis (2-hydroxyethyl)propanediamine.

- An emulsion in which a saponin (*e.g.* QuilA or QS21) and a sterol (*e.g.* a cholesterol) are associated as helical micelles [58].

- An emulsion comprising a mineral oil, a non-ionic lipophilic ethoxylated fatty alcohol, and a non-ionic hydrophilic surfactant (*e.g.* an ethoxylated fatty alcohol and/or polyoxyethylene-polyoxypropylene block copolymer) [59].

- An emulsion comprising a mineral oil, a non-ionic hydrophilic ethoxylated fatty alcohol, and a non-ionic lipophilic surfactant (*e.g.* an ethoxylated fatty alcohol and/or polyoxyethylene-polyoxypropylene block copolymer) [59].

In some embodiments an emulsion may be mixed with antigen extemporaneously, at the time of delivery, and thus the adjuvant and antigen may be kept separately in a packaged or distributed vaccine, ready for final formulation at the time of use. In other embodiments an emulsion is mixed with antigen during manufacture, and thus the composition is packaged in a liquid adjuvanted form,. The antigen will generally be in an aqueous form, such that the vaccine is finally prepared by mixing two liquids. The volume ratio of the two liquids for mixing can vary (*e.g.* between 5:1 and 1:5) but is generally about 1:1. Where concentrations of components are given in the above descriptions of specific emulsions, these concentrations are typically for an undiluted composition, and the concentration after mixing with an antigen solution will thus decrease.

Where a composition includes a tocopherol, any of the α, β, γ, δ, ε or ξ tocopherols can be used, but α-tocopherols are preferred. The tocopherol can take several forms *e.g.* different salts and/or isomers. Salts include organic salts, such as succinate, acetate, nicotinate, *etc.* D-α-tocopherol and DL-α-tocopherol can both be used. Tocopherols are advantageously included in vaccines for use in elderly patients (*e.g.* aged 60 years or older) because vitamin E has been reported to have a positive effect on the immune response in this patient group [60]. They also have antioxidant properties that may help to stabilize the emulsions [61]. A preferred α-tocopherol is DL-α-tocopherol, and the preferred salt of this tocopherol is the succinate. The succinate salt has been found to cooperate with TNF-related ligands *in vivo*.

C. Saponin formulations [chapter 22 of ref. 48]

Saponin formulations may also be used as adjuvants in the invention. Saponins are a heterogeneous group of sterol glycosides and triterpenoid glycosides that are found in the bark, leaves, stems, roots and even flowers of a wide range of plant species. Saponin from the bark of the *Quillaia saponaria* Molina tree have been widely studied as adjuvants. Saponin can also be commercially obtained from *Smilax ornata* (sarsaprilla), *Gypsophilla paniculata* (brides veil), and *Saponaria officianalis* (soap root). Saponin adjuvant formulations include purified formulations, such as QS21, as well as lipid formulations, such as ISCOMs. QS21 is marketed as Stimulon™.

Saponin compositions have been purified using HPLC and RP-HPLC. Specific purified fractions using these techniques have been identified, including QS7, QS17, QS18, QS21, QH-A, QH-B and QH-C. Preferably, the saponin is QS21. A method of production of QS21 is disclosed in ref. 62. Saponin formulations may also comprise a sterol, such as cholesterol [63].

Combinations of saponins and cholesterols can be used to form unique particles called immunostimulating complexes (ISCOMs) [chapter 23 of ref. 48]. ISCOMs typically also include a phospholipid such as phosphatidylethanolamine or phosphatidylcholine. Any known saponin can be used in ISCOMs. Preferably, the ISCOM includes one or more of QuilA, QHA & QHC. ISCOMs are further described in refs. 63-65. Optionally, the ISCOMS may be devoid of additional detergent [66].

A review of the development of saponin based adjuvants can be found in refs. 67 & 68.

D. Virosomes and virus-like particles

Virosomes and virus-like particles (VLPs) can also be used as adjuvants in the invention. These structures generally contain one or more proteins from a virus optionally combined or formulated with a phospholipid. They are generally non-pathogenic, non-replicating and generally do not contain any of the native viral genome. The viral proteins may be recombinantly produced or isolated from whole viruses. These viral proteins suitable for use in virosomes or VLPs include proteins derived from influenza virus (such as HA or NA), Hepatitis B virus (such as core or capsid proteins), Hepatitis E virus, measles virus, Sindbis virus, Rotavirus, Foot-and-Mouth Disease virus, Retrovirus, Norwalk virus, human Papilloma virus, HIV, RNA-phages, Qß-phage (such as coat proteins), GA-phage, fr-phage, AP205 phage, and Ty (such as retrotransposon Ty protein p1). VLPs are discussed further in refs. 69-74. Virosomes are discussed further in, for example, ref. 75

E. Bacterial or microbial derivatives

Adjuvants suitable for use in the invention include bacterial or microbial derivatives such as non-toxic derivatives of enterobacterial lipopolysaccharide (LPS), Lipid A derivatives, immunostimulatory oligonucleotides and ADP-ribosylating toxins and detoxified derivatives thereof.

Non-toxic derivatives of LPS include monophosphoryl lipid A (MPL) and 3-O-deacylated MPL (3dMPL). 3dMPL is a mixture of 3 de-O-acylated monophosphoryl lipid A with 4, 5 or 6 acylated chains. A preferred "small particle" form of 3 De-O-acylated monophosphoryl lipid A is disclosed in ref. 76. Such "small particles" of 3dMPL are small enough to be sterile filtered through a 0.22µm membrane [76]. Other non-toxic LPS derivatives include monophosphoryl lipid A mimics, such as aminoalkyl glucosaminide phosphate derivatives *e.g.* RC-529 [77,78].

Lipid A derivatives include derivatives of lipid A from *Escherichia coli* such as OM-174. OM-174 is described for example in refs. 79 & 80.

Immunostimulatory oligonucleotides suitable for use as adjuvants in the invention include nucleotide sequences containing a CpG motif (a dinucleotide sequence containing an unmethylated cytosine- linked by a phosphate bond to a guanosine). Double-stranded RNAs and oligonucleotides containing palindromic or poly(dG) sequences have also been shown to be immunostimulatory.

The CpG's can include nucleotide modifications/analogs such as phosphorothioate modifications and can be double-stranded or single-stranded. References 81, 82 and 83 disclose possible analog substitutions *e.g.* replacement of guanosine with 2'-deoxy-7-deazaguanosine. The adjuvant effect of CpG oligonucleotides is further discussed in refs. 84-89.

The CpG sequence may be directed to TLR9, such as the motif GTCGTT or TTCGTT [90]. The CpG sequence may be specific for inducing a Th1 immune response, such as a CpG-A ODN, or it may be more specific for inducing a B cell response, such a CpG-B ODN. CpG-A and CpG-B ODNs are discussed in refs. 91-93. Preferably, the CpG is a CpG-A ODN.

Preferably, the CpG oligonucleotide is constructed so that the 5' end is accessible for receptor recognition. Optionally, two CpG oligonucleotide sequences may be attached at their 3' ends to form "immunomers". See, for example, refs. 90 & 94-, 96.

A useful CpG adjuvant is CpG7909, also known as ProMune™ (Coley Pharmaceutical Group, Inc.). Another is CpG1826. As an alternative, or in addition, to using CpG sequences, TpG sequences can be used [97], and these oligonucleotides may be free from unmethylated CpG motifs. The immunostimulatory oligonucleotide may be pyrimidine-rich. For example, it may comprise more than one consecutive thymidine nucleotide (*e.g.* TTTT, as disclosed in ref. 97), and/or it may have a nucleotide composition with >25% thymidine (*e.g.* >35%, >40%, >50%, >60%, >80%, *etc.*). For example, it may comprise more than one consecutive cytosine nucleotide (*e.g.* CCCC, as disclosed in ref. 97), and/or it may have a nucleotide composition with >25% cytosine (*e.g.* >35%, >40%, >50%, >60%, >80%, *etc.*). These oligonucleotides may be free from unmethylated CpG motifs. Immunostimulatory oligonucleotides will typically comprise at least 20 nucleotides. They may comprise fewer than 100 nucleotides.

A particularly useful adjuvant based around immunostimulatory oligonucleotides is known as IC-31™ [98]. Thus an adjuvant used with the invention may comprise a mixture of (i) an oligonucleotide (*e.g.* between 15-40 nucleotides) including at least one (and preferably multiple) CpI motifs (*i.e.* a cytosine linked to an inosine to form a dinucleotide), and (ii) a polycationic polymer, such as an oligopeptide (*e.g.* between 5-20 amino acids) including at least one (and preferably multiple) Lys-Arg-Lys tripeptide sequence(s). The oligonucleotide may be a deoxynucleotide comprising 26-mer

sequence 5'-$(IC)_{13}$-3' (SEQ ID NO: 66). The polycationic polymer may be a peptide comprising 11-mer amino acid sequence KLKLLLLLKLK (SEQ ID NO: 67). The oligonucleotide and polymer can form complexes *e.g.* as disclosed in references 99 & 100.

Bacterial ADP-ribosylating toxins and detoxified derivatives thereof may be used as adjuvants in the invention. Preferably, the protein is derived from *E.coli* (*E.coli* heat labile enterotoxin "LT"), cholera ("CT"), or pertussis ("PT"). The use of detoxified ADP-ribosylating toxins as mucosal adjuvants is described in ref. 101 and as parenteral adjuvants in ref. 102. The toxin or toxoid is preferably in the form of a holotoxin, comprising both A and B subunits. Preferably, the A subunit contains a detoxifying mutation; preferably the B subunit is not mutated. Preferably, the adjuvant is a detoxified LT mutant such as LT-K63, LT-R72, and LT-G192. The use of ADP-ribosylating toxins and detoxified derivatives thereof, particularly LT-K63 and LT-R72, as adjuvants can be found in refs. 103-110. A useful CT mutant is or CT-E29H [111]. Numerical reference for amino acid substitutions is preferably based on the alignments of the A and B subunits of ADP-ribosylating toxins set forth in ref. 112, specifically incorporated herein by reference in its entirety.

F. Human immunomodulators

Human immunomodulators suitable for use as adjuvants in the invention include cytokines, such as interleukins (*e.g.* IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-12 [113], *etc.*) [114], interferons (*e.g.* interferon-γ), macrophage colony stimulating factor, and tumor necrosis factor. A preferred immunomodulator is IL-12.

G. Bioadhesives and Mucoadhesives

Bioadhesives and mucoadhesives may also be used as adjuvants in the invention. Suitable bioadhesives include esterified hyaluronic acid microspheres [115] or mucoadhesives such as cross-linked derivatives of poly(acrylic acid), polyvinyl alcohol, polyvinyl pyrollidone, polysaccharides and carboxymethylcellulose. Chitosan and derivatives thereof may also be used as adjuvants in the invention [116].

H. Microparticles

Microparticles may also be used as adjuvants in the invention. Microparticles (*i.e.* a particle of ~100nm to ~150μm in diameter, more preferably ~200nm to ~30μm in

diameter, and most preferably ~500nm to ~10μm in diameter) formed from materials that are biodegradable and non-toxic (*e.g.* a poly(α-hydroxy acid), a polyhydroxybutyric acid, a polyorthoester, a polyanhydride, a polycaprolactone, *etc.*), with poly(lactide-co-glycolide) are preferred, optionally treated to have a negatively-charged surface (*e.g.* with SDS) or a positively-charged surface (*e.g.* with a cationic detergent, such as CTAB).

I. Liposomes (Chapters 13 & 14 of ref. 48)

Examples of liposome formulations suitable for use as adjuvants are described in refs. 117-119.

J. Polyoxyethylene ether and polyoxyethylene ester formulations

Adjuvants suitable for use in the invention include polyoxyethylene ethers and polyoxyethylene esters [120]. Such formulations further include polyoxyethylene sorbitan ester surfactants in combination with an octoxynol [121] as well as polyoxyethylene alkyl ethers or ester surfactants in combination with at least one additional non-ionic surfactant such as an octoxynol [122]. Preferred polyoxyethylene ethers are selected from the following group: polyoxyethylene-9-lauryl ether (laureth 9), polyoxyethylene-9-steoryl ether, polyoxytheylene-8-steoryl ether, polyoxyethylene-4-lauryl ether, polyoxyethylene-35-lauryl ether, and polyoxyethylene-23-lauryl ether.

K. Phosphazenes

A phosphazene, such as poly[di(carboxylatophenoxy)phosphazene] ("PCPP") as described, for example, in references 123 and 124, may be used.

L. Muramyl peptides

Examples of muramyl peptides suitable for use as adjuvants in the invention include N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-normuramyl-L-alanyl-D-isoglutamine (nor-MDP), and N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-*sn*-glycero-3-hydroxyphosphoryloxy)-ethylamine MTP-PE).

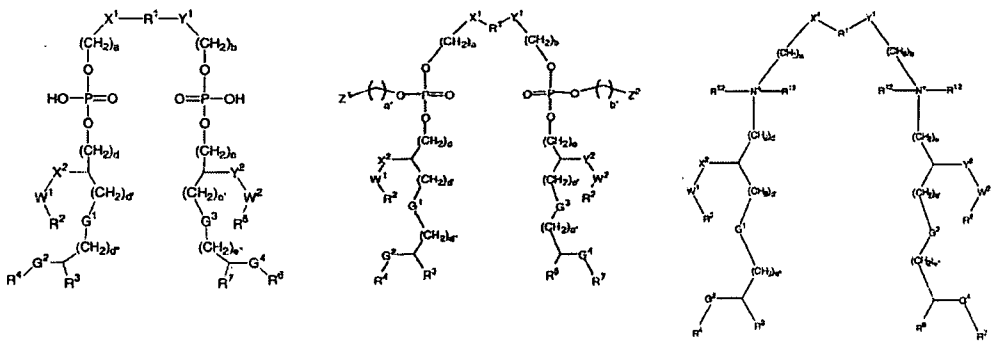
M. Imidazoquinolone Compounds.

Examples of imidazoquinolone compounds suitable for use adjuvants in the invention include Imiquimod ("R-837") [125,126], Resiquimod ("R-848") [127], and their analogs; and salts thereof (*e.g.* the hydrochloride salts). Further details about immunostimulatory imidazoquinolines can be found in references 128 to 132.

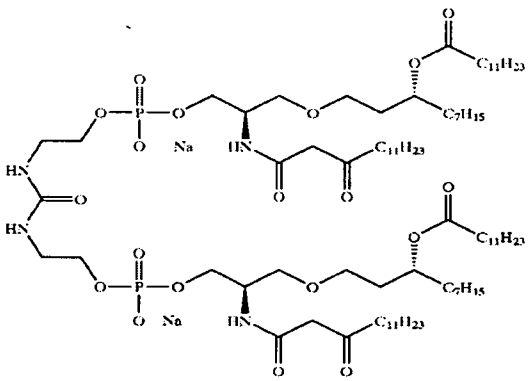
N. Substituted ureas

Substituted ureas useful as adjuvants include compounds of formula I, II or III, or salts thereof:

I II III



as defined in reference 133, such as 'ER 803058', 'ER 803732', 'ER 804053', ER 804058', 'ER 804059', 'ER 804442', 'ER 804680', 'ER 804764', ER 803022 or 'ER 804057' *e.g.*:



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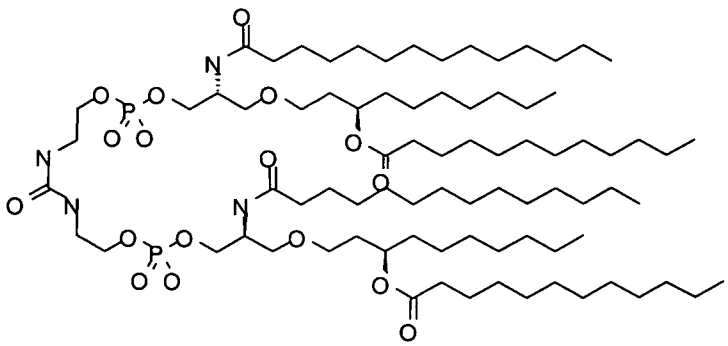

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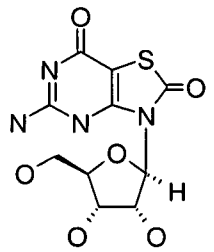
O. Further adjuvants

Further adjuvants that may be used with the invention include:

- Cyclic diguanylate ('c-di-GMP'), which has been reported as a useful adjuvant for *S.aureus* vaccines [134].
- A thiosemicarbazone compound, such as those disclosed in reference 135. Methods of formulating, manufacturing, and screening for active compounds are also described in reference 135. The thiosemicarbazones are particularly effective in the stimulation of human peripheral blood mononuclear cells for the production of cytokines, such as TNF-α.
- A tryptanthrin compound, such as those disclosed in reference 136. Methods of formulating, manufacturing, and screening for active compounds are also described in reference 136. The thiosemicarbazones are particularly effective in the stimulation of human peripheral blood mononuclear cells for the production of cytokines, such as TNF-α.
- A nucleoside analog, such as: (a) Isatorabine (ANA-245; 7-thia-8-oxoguanosine):

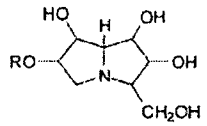


and prodrugs thereof; (b) ANA975; (c) ANA-025-1; (d) ANA380; (e) the compounds disclosed in references 137 to 139Loxoribine (7-allyl-8-oxoguanosine) [140].

- Compounds disclosed in reference 141, including: Acylpiperazine compounds, Indoledione compounds, Tetrahydraisoquinoline (THIQ) compounds, Benzocyclodione compounds, Aminoazavinyl compounds, Aminobenzimidazole quinolinone (ABIQ) compounds [142,143], Hydrapthalamide compounds, Benzophenone compounds, Isoxazole compounds, Sterol compounds, Quinazilinone compounds, Pyrrole compounds [144], Anthraquinone

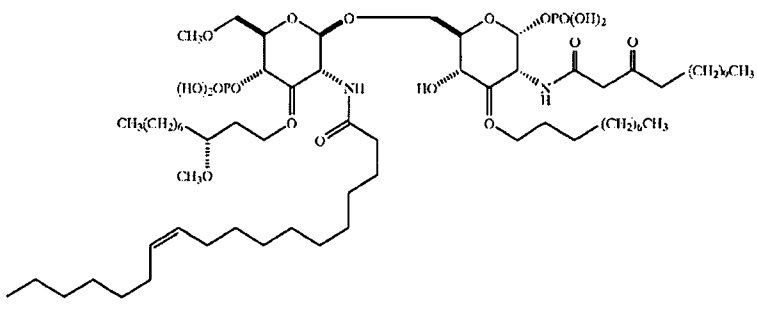
compounds, Quinoxaline compounds, Triazine compounds, Pyrazalopyrimidine compounds, and Benzazole compounds [145].

- Compounds containing lipids linked to a phosphate-containing acyclic backbone, such as the TLR4 antagonist E5564 [146,147]:
- A polyoxidonium polymer [148,149] or other N-oxidized polyethylene-piperazine derivative.
- Methyl inosine 5'-monophosphate ("MIMP") [150].
- A polyhydroxlated pyrrolizidine compound [151], such as one having formula:



where R is selected from the group comprising hydrogen, straight or branched, unsubstituted or substituted, saturated or unsaturated acyl, alkyl (*e.g.* cycloalkyl), alkenyl, alkynyl and aryl groups, or a pharmaceutically acceptable salt or derivative thereof. Examples include, but are not limited to: casuarine, casuarine-6-α-D-glucopyranose, 3-*epi*-casuarine, 7-*epi*-casuarine, 3,7-di*epi*-casuarine, *etc.*

- A CD1d ligand, such as an α-glycosylceramide [152-159] (*e.g.* α-galactosylceramide), phytosphingosine-containing α-glycosylceramides, OCH, KRN7000 [(2S,3S,4R)-1-O-(α-D-galactopyranosyl)-2-(N-hexacosanoylamino)-1,3,4-octadecanetriol], CRONY-101, 3"-O-sulfo-galactosylceramide, *etc.*
- A gamma inulin [160] or derivative thereof, such as algammulin.



Adjuvant combinations

The invention may also comprise combinations of one or more of the adjuvants identified above. For example, the following adjuvant compositions may be used in the invention: (1) a saponin and an oil-in-water emulsion [161]; (2) a saponin (*e.g.* QS21) + a non-toxic LPS derivative (*e.g.* 3dMPL) [162]; (3) a saponin (*e.g.* QS21) + a non-toxic LPS derivative (*e.g.* 3dMPL) + a cholesterol; (4) a saponin (*e.g.* QS21) + 3dMPL + IL-12 (optionally + a sterol) [163]; (5) combinations of 3dMPL with, for example, QS21 and/or oil-in-water emulsions [164]; (6) SAF, containing 10% squalane, 0.4% Tween 80™, 5% pluronic-block polymer L121, and thr-MDP, either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion. (7) Ribi™ adjuvant system (RAS), (Ribi Immunochem) containing 2% squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of monophosphorylipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL + CWS (Detox™); and (8) one or more mineral salts (such as an aluminum salt) + a non-toxic derivative of LPS (such as 3dMPL).

Other substances that act as immunostimulating agents are disclosed in chapter 7 of ref. 48.

The use of an aluminium hydroxide and/or aluminium phosphate adjuvant is particularly preferred, and antigens are generally adsorbed to these salts. Calcium phosphate is another preferred adjuvant. Other preferred adjuvant combinations include combinations of Th1 and Th2 adjuvants such as CpG & alum or resiquimod & alum. A combination of aluminium phosphate and 3dMPL may be used.

To improve thermal stability, a composition may include a temperature protective agent. This component may be particularly useful in adjuvanted compositions (particularly those containing a mineral adjuvant, such as an aluminium salt). As described in reference 165, a liquid temperature protective agent may be added to an aqueous vaccine composition to lower its freezing point *e.g.* to reduce the freezing point to below 0°C. Thus the composition can be stored below 0°C, but above its freezing point, to inhibit thermal breakdown. The temperature protective agent also permits freezing of the composition while protecting mineral salt adjuvants against agglomeration or sedimentation after freezing and thawing, and may also protect the composition at elevated temperatures *e.g.* above 40°C. A starting aqueous vaccine and the liquid

temperature protective agent may be mixed such that the liquid temperature protective agent forms from 1-80% by volume of the final mixture. Suitable temperature protective agents should be safe for human administration, readily miscible/soluble in water, and should not damage other components (*e.g.* antigen and adjuvant) in the composition. Examples include glycerin, propylene glycol, and/or polyethylene glycol (PEG). Suitable PEGs may have an average molecular weight ranging from 200-20,000 Da. In a preferred embodiment, the polyethylene glycol can have an average molecular weight of about 300 Da ('PEG-300').

The invention provides an immunogenic composition comprising: (i) one or more OMV(s) of the invention and (ii) a temperature protective agent. This composition may be formed by mixing (i) an aqueous composition comprising one or more OMV(s) of the invention, with (ii) a temperature protective agent. The mixture may then be stored *e.g.* below 0°C, from 0-20°C, from 20-35°C, from 35-55°C, or higher. It may be stored in liquid or frozen form. The mixture may be lyophilised. The composition may alternatively be formed by mixing (i) a dried composition comprising one or more OMV(s) of the invention, with (ii) a liquid composition comprising the temperature protective agent. Thus component (ii) can be used to reconstitute component (i).

The compositions of the invention may elicit both a cell mediated immune response as well as a humoral immune response. This immune response will preferably induce long lasting (*e.g.* neutralising) antibodies and a cell mediated immunity that can quickly respond upon exposure to the pathogen (e.g. to *Chlamydia*).

Two types of T cells, CD4 and CD8 cells, are generally thought necessary to initiate and/or enhance cell mediated immunity and humoral immunity. CD8 T cells can express a CD8 co-receptor and are commonly referred to as Cytotoxic T lymphocytes (CTLs). CD8 T cells are able to recognized or interact with antigens displayed on MHC Class I molecules.

CD4 T cells can express a CD4 co-receptor and are commonly referred to as T helper cells. CD4 T cells are able to recognize antigenic peptides bound to MHC class II molecules. Upon interaction with a MHC class II molecule, the CD4 cells can secrete factors such as cytokines. These secreted cytokines can activate B cells, cytotoxic T cells, macrophages, and other cells that participate in an immune response. Helper T

cells or CD4+ cells can be further divided into two functionally distinct subsets: TH1 phenotype and TH2 phenotypes which differ in their cytokine and effector function.

Activated TH1 cells enhance cellular immunity (including an increase in antigen-specific CTL production) and are therefore of particular value in responding to intracellular infections. Activated TH1 cells may secrete one or more of IL-2, IFN-γ, and TNF-β. A TH1 immune response may result in local inflammatory reactions by activating macrophages, NK (natural killer) cells, and CD8 cytotoxic T cells (CTLs). A TH1 immune response may also act to expand the immune response by stimulating growth of B and T cells with IL-12. TH1 stimulated B cells may secrete IgG2a.

Activated TH2 cells enhance antibody production and are therefore of value in responding to extracellular infections. Activated TH2 cells may secrete one or more of IL-4, IL-5, IL-6, and IL-10. A TH2 immune response may result in the production of IgG1, IgE, IgA and memory B cells for future protection.

An enhanced immune response may include one or more of an enhanced TH1 immune response and a TH2 immune response.

A TH1 immune response may include one or more of an increase in CTLs, an increase in one or more of the cytokines associated with a TH1 immune response (such as IL-2, IFN-γ, and TNF-β), an increase in activated macrophages, an increase in NK activity, or an increase in the production of IgG2a. Preferably, the enhanced TH1 immune response will include an increase in IgG2a production.

A TH1 immune response may be elicited using a TH1 adjuvant. A TH1 adjuvant will generally elicit increased levels of IgG2a production relative to immunization of the antigen without adjuvant. TH1 adjuvants suitable for use in the invention may include for example saponin formulations, virosomes and virus like particles, non-toxic derivatives of enterobacterial lipopolysaccharide (LPS), immunostimulatory oligonucleotides. Immunostimulatory oligonucleotides, such as oligonucleotides containing a CpG motif, are preferred TH1 adjuvants for use in the invention.

A TH2 immune response may include one or more of an increase in one or more of the cytokines associated with a TH2 immune response (such as IL-4, IL-5, IL-6 and IL-10), or an increase in the production of IgG1, IgE, IgA and memory B cells. Preferably, the enhanced TH2 immune resonse will include an increase in IgG1 production.

A TH2 immune response may be elicited using a TH2 adjuvant. A TH2 adjuvant will generally elicit increased levels of IgG1 production relative to immunization of the antigen without adjuvant. TH2 adjuvants suitable for use in the invention include, for example, mineral containing compositions, oil-emulsions, and ADP-ribosylating toxins and detoxified derivatives thereof. Mineral containing compositions, such as aluminium salts are preferred TH2 adjuvants for use in the invention.

Preferably, the invention includes a composition comprising a combination of a TH1 adjuvant and a TH2 adjuvant. Preferably, such a composition elicits an enhanced TH1 and an enhanced TH2 response, i.e., an increase in the production of both IgG1 and IgG2a production relative to immunization without an adjuvant. Still more preferably, the composition comprising a combination of a TH1 and a TH2 adjuvant elicits an increased TH1 and/or an increased TH2 immune response relative to immunization with a single adjuvant (*i.e.*, relative to immunization with a TH1 adjuvant alone or immunization with a TH2 adjuvant alone).

The immune response may be one or both of a TH1 immune response and a TH2 response. Preferably, immune response provides for one or both of an enhanced TH1 response and an enhanced TH2 response.

The enhanced immune response may be one or both of a systemic and a mucosal immune response. Preferably, the immune response provides for one or both of an enhanced systemic and an enhanced mucosal immune response. Preferably the mucosal immune response is a TH2 immune response. Preferably, the mucosal immune response includes an increase in the production of IgA.

Methods of treatment, and administration of the vaccine

The invention also provides a method for raising an immune response in a mammal comprising the step of administering an effective amount of an OMV or immunogenic composition of the invention. The immune response is preferably protective and preferably involves antibodies and/or cell-mediated immunity. The method may raise a booster response.

The invention also provides a method for immunising a mammal against *Chlamydia* infection, preferably against *C. trachomatis* infection, comprising the step of administering an effective amount of an OMV or immunogenic composition of the invention.

The invention also provides an OMV of the invention in combination with an additional antigen for combined use as a medicament for simultaneous, separate or sequential administration, *e.g.* for use in raising an immune response in a mammal.

The invention also provides an OMV, antibody or immunogenic composition of the invention for use in therapy.

The invention also provides the use of an OMV of the invention in the manufacture of a medicament for raising an immune response in a mammal. By raising an immune response in the mammal by these uses and methods, the mammal can be protected against infection by the pathogen, for example, against *Chlamydia* infection. More particularly, the mammal may be protected against infection by *Chlamydia trachomatis*. The invention is effective against *Chlamydia* of various different serotypes, but can be particularly useful in protecting against disease resulting from *Chlamydia* infection by strains in serovar D.

Thus, according to a further aspect, the invention also provides an OMV, antibody or immunogenic composition according to the invention for use as a medicament (*e.g.* a vaccine) or a diagnostic reagent. In one embodiment, the OMV, antibody or immunogenic composition is used for treatment, prevention or diagnosis of *Chlamydia* infection (preferably *C. trachomatis*) in a mammal. The invention also provides a method of treating, preventing of diagnosing *Chlamydia* infection (preferably, *C. trachomatis* infection) in a patient (preferably a mammal), comprising administering a therapeutically effective amount of an OMV or antibody of the invention.

Preferably, the OMV or antibody according to the invention is for use in the treatment or prevention of *Chlamydia* infection or an associated condition (*e.g.* trachoma, blindness, cervicitis, pelvic inflammatory disease, infertility, ectopic pregnancy, chronic pelvic pain, salpingitis, urethritis, epididymitis, infant pneumonia, cervical squamous cell carcinoma, HIV infection, *etc.*), preferably, *C. trachomatis* infection (such as an ocular condition, urogenital tract condition or invasive lymphogranumoa venereum that is caused by *C. trachomatis).* The immunogenic composition may additionally or alternatively be effective against *C. pneumoniae.*

In some embodiments, the OMV of the present invention is for use in raising neutralising antibodies against infection by a particular pathogen. The pathogen is preferably a bacteria or a virus. Examples of suitable bacteria are *chlamydia*,

Streptococcus, Salmonella, E. coli and *Helicobacter.* Examples of suitable viruses are *HIV, influenza* and *Epstein Barr virus.* Preferably, the OMV is for use in raising neutralising antibodies against *Chlamydia*, for example, against *C. trachomatis*, C. *pneumoniae* or *C. muridarum.*

The mammal is preferably a human. Vaccines prepared according to the invention may be used to treat both children and adults. Where the vaccine is for prophylactic use, the human is preferably a child (*e.g.* a toddler or infant) or a teenager; where the vaccine is for therapeutic use, the human is preferably a teenager or an adult. A vaccine intended for children may also be administered to adults *e.g.* to assess safety, dosage, immunogenicity, *etc.* Thus a human patient may be less than 1 year old, 1-5 years old, 5-15 years old, 15-55 years old, or at least 55 years old. Preferred patients for receiving the vaccines are people going through puberty, teenagers, sexually active people, the elderly (*e.g.* ≥50 years old, ≥60 years old, and preferably ≥65 years), the young (*e.g.* ≤5 years old), hospitalised patients, healthcare workers, armed service and military personnel, pregnant women, the chronically ill, or immunodeficient patients. The vaccines are not suitable solely for these groups, however, and may be used more generally in a population.

Vaccines produced by the invention may be administered to patients at substantially the same time as (*e.g.* during the same medical consultation or visit to a healthcare professional or vaccination centre) other vaccines *e.g.* at substantially the same time as a human papillomavirus vaccine such as Cervarix® or Gardasil®; a tetanus, diphtheria and acellular pertussis vaccine such as TDaP, DTaP or Boostrix®; a rubella vaccine such as MMR; or a tubercolosis vaccine such as the BCG. Examples of other vaccines that the vaccine produced by the invention may be administered at substantially the same time as are a measles vaccine, a mumps vaccine, a varicella vaccine, a MMRV vaccine, a diphtheria vaccine, a tetanus vaccine, a pertussis vaccine, a DTP vaccine, a conjugated *H.influenzae* type b vaccine, an inactivated poliovirus vaccine, a hepatitis B virus vaccine, a meningococcal conjugate vaccine (such as a tetravalent A-C-W135-Y vaccine), a respiratory syncytial virus vaccine, *etc.*

In a preferred embodiment, the OMV of the invention is used to elicit antibodies that are capable of neutralising the infectivity or virulence of *Chlamydia*, for example, of *Chlamydia* trachomatis. Neutralizing antibodies may be used as a vaccine capable of neutralising infectious EB. In one embodiment, the OMV of the invention is used to

elicit antibodies that are capable of neutralising *Chlamydia* infectivity and/or virulence. Thus, the invention also provides the antibodies of the invention for neutralising *Chlamydia* infectivity and/or virulence.

The invention also provides the use of an OMV or antibody of the invention in the manufacture of: (i) a medicament for treating or preventing bacterial infection; (ii) a diagnostic reagent for detecting the presence of bacteria or of antibodies raised against bacteria; and/or (iii) a reagent which can raise antibodies against bacteria. Said bacteria is preferably a *Chlamydia*, e.g. *Chlamydia trachomatis* or *Chlamydia pneumoniae*, but is preferably *Chlamydia trachomatis*.

Detection and diagnostic methods

The invention provides a method for detecting a pathogen, such as a *Chlamydia* bacterium (e.g. *C. trachomatis*) in a sample. The method can involve detecting the presence or absence of an antigen from the pathogen or of nucleic acid encoding an antigen from the pathogen. The method can be used for microbiological testing, clinical or non-clinical diagnosis, *etc.* Detection of the antigen may involve *e.g.* contacting the sample with an antibody of the invention, such as a labelled antibody of the invention. Detection of the nucleic acid antigen may involve any convenient method *e.g.* based on nucleic acid hybridisation, such as by using northern or southern blots, nucleic acid microarrays or 'gene chips', amplification reactions (*e.g.* PCR, SDA, SSSR, LCR, TMA, NASBA, *etc.*).

The invention also provides a method for detecting if a patient has been infected with a pathogen (e.g. a *Chlamydia* bacterium such as *C. trachomatis*), comprising a step of detecting in a sample taken from the patient the presence or absence of an antibody according to the invention. Detection of the antigen may involve, for example, contacting the sample with an antibody of the invention.

Presence of the antigen (e.g. the CT823), or of nucleic acid encoding the antigen (e.g. the CT823 antigen), or of an antibody (e.g. an anti-CT823 antibody), indicates the presence of the pathogen (e.g. *C. trachomatis*) in the sample. In a clinical diagnostic setting, therefore, the results of the method may be used to educate or dictate a therapeutic strategy for a patient *e.g.* a choice of antibiotics, *etc.*

The invention also provides a process for detecting an antigen (e.g. CT823), comprising the steps of: (a) contacting an antibody (e.g. an anti-CT823 antibody) with a biological

sample under conditions suitable for the formation of an antibody-antigen complex; and (b) detecting the complex.

The invention also provides a process for detecting antibodies (e.g. anti-CT823 antibodies), comprising the steps of: (a) contacting an antigen with a biological sample (*e.g.* a blood or serum sample) under conditions suitable for the formation of an antibody-antigen complexes; and (b) detecting the complexes.

Also provided is a method for diagnosing an infection by a pathogen (e.g. *Chlamydia* infection), comprising:

(a) raising an antibody against a heterologous antigen presented in the context of an OMV according to the invention;

(b) contacting the antibody of step (a) with a biological sample suspected of being infected with the pathogen (e.g. *Chlamydia*) under conditions suitable for the formation of antibody-antigen complexes; and

(c) detecting said complexes, wherein detection of said complex is indicative of pathogen infection (e.g. *Chlamydia* infection).

OMVs of the invention can be used in immunoassays to detect antibody levels (or, conversely, antibodies of the invention can be used to detect protein levels). Immunoassays based on well defined, recombinant antigens can be developed to replace invasive diagnostics methods. Antibodies to proteins within biological samples, including for example, blood or serum samples, can be detected. Design of the immunoassays is subject to a great deal of variation, and a variety of these are known in the art. Protocols for the immunoassay may be based, for example, upon competition, or direct reaction, or sandwich type assays. Protocols may also, for example, use solid supports, or may be by immunoprecipitation. Most assays involve the use of labeled antibody or polypeptide; the labels may be, for example, fluorescent, chemiluminescent, radioactive, or dye molecules. Assays which amplify the signals from the probe are also known; examples of which are assays which utilize biotin and avidin, and enzyme-labeled and mediated immunoassays, such as ELISA assays.

Kits suitable for immunodiagnosis and containing the appropriate labeled reagents are constructed by packaging the appropriate materials, including the compositions of the invention, in suitable containers, along with the remaining reagents and materials (for

example, suitable buffers, salt solutions, *etc.*) required for the conduct of the assay, as well as suitable set of assay instructions.

Testing efficacy of compositions

The efficacy of the immunogenic compositions of the present invention can be evaluated in *in vitro* and *in vivo* animal models prior to host, e.g., human, administration. For example, *in vitro* neutralization by Peterson et al (1988) is suitable for testing vaccine compositions directed toward *Chlamydia trachomatis*.

One way of checking efficacy of therapeutic treatment involves monitoring infection (e.g. *C. trachomatis* infection) after administration of the compositions of the invention. One way of checking efficacy of prophylactic treatment involves monitoring immune responses both systemically (such as monitoring the level of IgG1 and IgG2a production) and mucosally (such as monitoring the level of IgA production) against the antigens (e.g. against the *Chlamydia trachomatis* antigens) in the compositions of the invention after administration of the composition. Typically, serum pathogen (e.g. *Chlamydia*) specific antibody responses are determined post-immunisation but pre-challenge whereas mucosal pathogen (e.g. *Chlamydia*) specific antibody responses are determined post-immunisation and post-challenge.

One example of such an *in vitro* test is described as follows. Hyper-immune antisera is diluted in PBS containing 5% guinea pig serum, as a complement source. *Chlamydia trachomatis* (10^4 IFU; inclusion forming units) are added to the antisera dilutions. The antigen-antibody mixtures are incubated at 37°C for 45 minutes and inoculated into duplicate confluent Hep-2 or HeLa cell monolayers contained in glass vials (e.g., 15 by 45 mm), which have been washed twice with PBS prior to inoculation. The monolayer cells are infected by centrifugation at 1000X g for 1 hour followed by stationary incubation at 37°C for 1 hour. Infected monolayers are incubated for 48 or 72 hours, fixed and stained with *Chlamydia* specific antibody, such as anti-MOMP. Inclusion-bearing cells are counted in ten fields at a magnification of 200X. Neutralization titer is assigned on the dilution that gives 50% inhibition as compared to control monolayers/IFU.

Another way of assessing the immunogenicity of the compositions of the present invention is to express the proteins recombinantly for screening patient sera or mucosal secretions by immunoblot and/or microarrays. A positive reaction between the protein

and the patient sample indicates that the patient has mounted an immune response to the heterologous antigen in question. This method may also be used to identify immunodominant antigens and/or epitopes within antigens.

The efficacy of vaccine compositions can also be determined *in vivo* by challenging animal models of infection (e.g. *Chlamydia trachomatis* infection), *e.g.*, guinea pigs or mice, with the vaccine compositions. For example, *in vivo* vaccine composition challenge studies in the guinea pig model of *Chlamydia trachomatis* infection can be performed. A description of one example of this type of approach follows. Female guinea pigs weighing 450 – 500 g are housed in an environmentally controlled room with a 12 hour light-dark cycle and immunized with vaccine compositions via a variety of immunization routes. Post-vaccination, guinea pigs are infected in the genital tract with the agent of guinea pig inclusion conjunctivitis (GPIC), which has been grown in HeLa or McCoy cells (Rank et al. (1988)). Each animal receives approximately $1.4x10^7$ inclusion forming units (IFU) contained in 0.05 ml of sucrose-phosphate-glutamate buffer, pH 7.4 (Schacter, 1980). The course of infection monitored by determining the percentage of inclusion-bearing cells by indirect immunofluorescence with GPIC specific antisera, or by Giemsa-stained smear from a scraping from the genital tract (Rank et al 1988). Antibody titers in the serum is determined by an enzyme-linked immunosorbent assay.

Alternatively, *in vivo* vaccine compositions challenge studies can be performed in the murine model of *Chlamydia trachomatis* (Morrison et al 1995). A description of one example of this type of approach is as follows. Female mice 7 to 12 weeks of age receive 2.5 mg of depo-provera subcutaneously at 10 and 3 days before vaginal infection. Post-vaccination, mice are infected in the genital tract with 1,500 inclusion-forming units of *Chlamydia trachomatis* contained in 5ml of sucrose-phosphate-glutamate buffer, pH 7.4. The course of infection is monitored by determining the percentage of inclusion-bearing cells by indirect immunofluorescence with *Chlamydia trachomatis* specific antisera, or by a Giemsa-stained smear from a scraping from the genital tract of an infected mouse. The presence of antibody titers in the serum of a mouse is determined by an enzyme-linked immunosorbent assay.

General

The practice of the present invention will employ, unless otherwise indicated, conventional methods of chemistry, biochemistry, molecular biology, immunology and pharmacology, within the skill of the art. Such techniques are explained fully in the literature. See, *e.g.*, references 166-173, *etc.*

Where the invention concerns an "epitope", this epitope may be a B-cell epitope and/or a T-cell epitope. Such epitopes can be identified empirically (*e.g.* using PEPSCAN [174,175] or similar methods), or they can be predicted (*e.g.* using the Jameson-Wolf antigenic index [176], matrix-based approaches [177], MAPITOPE [178], TEPITOPE [179,180], neural networks [181], OptiMer & EpiMer [182, 183], ADEPT [184], Tsites [185], hydrophilicity [186], antigenic index [187] or the methods disclosed in references 188-192, *etc.*). Epitopes are the parts of an antigen that are recognised by and bind to the antigen binding sites of antibodies or T-cell receptors, and they may also be referred to as "antigenic determinants".

Where an antigen "domain" is omitted, this may involve omission of a signal peptide, of a cytoplasmic domain, of a transmembrane domain, of an extracellular domain, *etc.*

The term "comprising" encompasses "including" as well as "consisting" *e.g.* a composition "comprising" X may consist exclusively of X or may include something additional *e.g.* X + Y.

The term "about" in relation to a numerical value x is optional and means, for example, $x \pm 10\%$.

References to a percentage sequence identity between two amino acid sequences means that, when aligned, that percentage of amino acids are the same in comparing the two sequences. This alignment and the percent homology or sequence identity can be determined using software programs known in the art, for example those described in section 7.7.18 of ref. 193. A preferred alignment is determined by the Smith-Waterman homology search algorithm using an affine gap search with a gap open penalty of 12 and a gap extension penalty of 2, BLOSUM matrix of 62. The Smith-Waterman homology search algorithm is disclosed in ref. 194.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1A shows the components of the Tol-Pal complex;

Figure 1B is a schematic representation of knocking out the tolR gene in the chromosomal DNA of *E. coli* IHE 3034 using homologous recombination and replacing it with the kanamycin resistance cassette;

Figure 2 shows the protein content released into the culture supernatant from wild type *E. coli* (left hand lane) and from the *E. coli* ΔTolR strain (right hand lane);

Figure 3 is an SDS-PAGE gel that shows total proteins from *E. coli* ΔTolR expressing TC0106 (lane 1), TC0052 (lane 2), TC0727 (lane 3) and TC0210 (lane 4). Low molecular weight markers are provided on the left hand side of the gel. The circles in lanes 1, 2 and 4 indicate expression of the TC0106, TC0052 and TC0210 proteins, respectively;

Figure 4 is a schematic representation of an OMV preparation protocol;

Figure 5A shows Western blot results for (i) TC0052 (41 kDa), (ii) TC0106 (47 kDa), (iii) TC0210 (53 kDa) and (iv) TC0727 (58 kDa). For Western blot (i), the following protein samples were analyzed: *E. coli* BL21(DE3)ΔTolR expressing the heterologous antigen (lane 1); total proteins contained in OMV expressing the heterologous antigen (lane 2); and proteins contained in OMV prepared from *E. coli* BL21(DE3)ΔTolR not expressing chlamydial antigen (lane 3). For each of Western blots (ii), (iii) and (iv), the following protein samples were analyzed: recombinant protein purified from expressing *E. coli* clones (lane 1); *E. coli* BL21(DE3)ΔTolR expressing the heterologous antigen (lane 2); total proteins contained in OMV expressing the heterologous antigen (lane 3); and proteins contained in OMV prepared from *E. coli* BL21(DE3)ΔTolR not expressing chlamydial antigen (lane 4). Each Western blot was incubated with sera obtained by immunizing mice with the respective purified chlamydial recombinant antigen. The arrows indicate the bands corresponding to the respective heterologous antigens.

Figure 5B shows Western blot results for TC0313 and TC0890. The samples that were analyzed are as follows: molecular weight standard markers (left hand lane); recombinant TC0313His (23kDa) purified from expressing *E. coli* clones (lane 1); total TC0313 protein contained in OMV expressing the heterologous antigen (lane 2); proteins contained in OMV prepared from *E. coli* BL21(DE3)ΔTolR not expressing chlamydial antigen (lane 3); recombinant TC0890His (19kDa) purified from expressing *E. coli* clones (lane 4); total TC0890 protein contained in OMV expressing the heterologous antigen (lane 5); proteins contained in OMV prepared from *E. coli*

BL21(DE3)ΔTolR not expressing chlamydial antigen (lane 6). Lanes 1, 2 and 3 were incubated with sera obtained by immunizing mice with purified TC0313 recombinant antigen (23kDa), whilst lanes 4, 5 and 6 were incubated with sera obtained by immunizing mice with purified TC0890 recombinant antigen (19kDa). The lanes are numbered from left to right. The arrows indicate the bands corresponding to the heterologous antigens of 23kDa and 19kDa respectively;

Figure 5C shows Western blot results from left to right for (i) TC0651 (53kDa), (ii) TC0551 (32kDa) and (iii) TC0431 (90kDa), respectively. For TC0651, lane 1 shows proteins contained in OMV prepared from *E. coli* BL21(DE3)ΔTolR not expressing chlamydial antigen whilst lane 2 shows total TC0651 protein contained in OMV expressing the heterologous antigen. For TC0551, lane 1 shows recombinant TC0551His (32kDa) purified from expressing *E. coli* clones, lane 2 shows TC0551 contained in OMV prepared from *E. coli* BL21(DE3)ΔTolR expressing TC0551 and lane 3 shows proteins contained in OMV prepared from *E. coli* BL21(DE3)ΔTolR not expressing chlamydial antigen. For TC0431, lane 1 shows recombinant TC0431His (90kDa) purified from expressing *E. coli* clones, lane 2 shows proteins contained in OMV prepared from *E. coli* BL21(DE3)ΔTolR not expressing chlamydial antigen and lane 3 shows TC0431 contained in OMV prepared from *E. coli* BL21(DE3)ΔTolR expressing TC0431;

Figure 5D shows Western blot results from left to right for (i) TC0431 (90kDa), and (ii) TC0052 (41 kDa). For each Western blot, lane 1 was loaded with an OMV preparation prepared from E.coli BL21(DE3)ΔTolR expressing chlamydial antigen, while lane 2 was loaded with an OMV preparation prepared from E.coli BL21(DE3)ΔTolR not expressing chlamydial antigens (used as a negative control). Figure 5D (i) shows total TC0431 protein contained in OMV expressing the heterologous antigen (see lane 1).

Figure 6 shows the *E. coli* growth curve for the various mutant *E. coli* ΔTolR prepared in Example 3. Time in hours is indicated along the X axis; OD600 is indicated along the Y axis. The growth curve of *E. coli* expressing the TC0210 is indicated with an arrow. (—◆—) is TC0210pET BL21(DE3)ΔTolR; (—■—) is TC0052pET BL21(DE3)ΔTolR; (—△—) is TC0106pET BL21(DE3)ΔTolR; (—✕—) is TC0313pET BL21(DE3)ΔTolR; (—✱—) is TC0431pET BL21(DE3)ΔTolR; (—●—)is TC0551pET BL21(DE3)ΔTolR;

(—+—) is TC0651pET BL21(DE3)ΔTolR; (▬) is TC0727pET BL21(DE3)ΔTolR and (▬) is TC0890pET BL21(DE3)ΔTolR.

Figure 7 is a schematic diagram of shaving and mass spectrometry experiments carried out to confirm the presence of the heterologous antigen in the OMV preparation. The diagram shows (starting from top left and progressing in a clockwise direction): i) shaving the OMV, ii) Recovery of supernatants, iii) DTT + detergent 100°C for 10 mins, iv) Recovery of peptides; v) Separation of the peptides by RP chromatography and peptide fragmentation by MS/MS ESI-Q-TOF, and vi) Protein identification;

Figure 8 shows the sequence of the TC0210 antigen from *C. muridarum*. The peptide recognised in the shaving and mass spectrometry analysis is highlighted in bold and underlined;

Figure 9 is a sequence alignment of *C. muridarum* TC0210 (query sequence) and *C. trachomatis* CT823 (subject sequence);

Figure 10A shows a FACS analysis of antibody binding the surface of chlamydial whole cells;

Figure 10B shows the results of a protein microarray experiment in which chlamydial antigens were expressed in *E. coli*, affinity purified and spotted on glass slides in a microarray format. Two groups of human sera were used for each antigen: MIF positive sera (left hand bar) and MIF negative sera (right hand bar). Antigens are listed on the X axis. From left to right, the antigens listed on the X axis are CT681His, CT823His, CT443His, CT456GST, CT110GST, CT622His, CT089His, CT415GST, CT119GST, CT049GST, CT589GST, CT372GST, CT077His, CT559His, CT322His, CT316His, CT350GST, CT381His, CT355GST, CT389GST, CT127GST, CT157GST, CT567His, CT266His, CT470His, CT398His and CT114His. The % sera is on the Y axis. Each vertical bar represents the mean intensity obtained with the sera (MIF positive vs MIF negative sera) on single antigens. The data second from the left relates to CT823.

Figure 10C shows antigens that give a high signal in a sign-bkg assay. The antigens are listed on the X axis and the signal is given on the Y axis. Chlamydial protein microchips were analysed by immunostaining with sera from mice that were (from left to right) i) never immunized (preimmune sera), ii) immunized with heat inactivated EBs (anti-heat inactivated EBs pre-challenge), iii) immunized with heat inactivated EBs and then challenged with live EBs (anti-heat inactivated EBs post-challenge), iv) immunised with

live EBs (anti-live EBs pre-challenge); and v) immunised with live EBs and then re-challenged again with live EBs (anti-live EBs post-challenge). The antibodies are at a dilution of 1:100. Antigens giving a low signal of less than 800 are not shown. From left to right, the antigens are: CT016His, CT045His, CT077His, CT242His, CT266His, CT396His, CT443His, CT444His, CT480His, CT547His, CT587His, CT600His, CT823His, CT859His, ctEBpel ctEBsup, ctGst381, ctGst398, ctGst480, GstNN and NNhis;

Figure 10 D shows Western blot results with sera obtained from mice immunised with from left to right (i) TC0210His (ii) OMV preparation from BL21(DE3)ΔTolR TC0210 and (iii) OMV preparation from BL21(DE3)ΔTolR, respectively. 200ng of recombinant HtrA (lane 1) and C. muridarum EBs (approximately 10^4 EBs) (lane 2) were dissolved in loading buffer and size-separated by SDS-PAGE (4–12% gel) under reducing conditions and electroblotted onto nitrocellulose membranes. Membranes were saturated overnight with Milk Marwell 10% in PBS (phosphate Buffer) 0.1% Triton, and then were incubated with sera obtained from mice immunised with, from left to right, (i) TC0210His, (ii) OMV preparation from BL21(DE3)ΔTolR TC0210, and (iii) OMV preparation from BL21(DE3)ΔTolR, respectively. An anti-mouse horseradish peroxidase conjugated IgG (Amersham Bioscience TM) was used as the secondary antibody.

Figure 10E shows a FACS analysis of antibody binding the surface of chlamydial whole cells. The peaks, from left to right correspond to purified *C. muridarum* EBs incubated with (i) anti-His sera (filled peak, far left), (ii) serum obtained from mice immunized with OMV preparation from BL21(DE3)ΔTolR (peak second from left), (iii) serum obtained from mice immunized with TC210 (peak second from right), or (iv) BL21(DE3)ΔTolR TC0210 (far right peak).

Figure 11 is a schematic diagram of an immunisation protocol for BALB/c mice;

Figure 12 is a graph showing the results of a *C. muridarum* neutralisation assay using sera of BALB/c mice that had been immunised with OMVs from *E. coli* BL21(DE3)ΔtolR presenting OMV (5 μg) (— -□— -), OMV-TC0210 (5 μg) (-·-·-●-·-·-), OMV (50 μg) (- - -◇- - -), OMV-TC0210 (50 μg) (——■——),and MOMP (——○——). Serum dilution is presented along the X axis and % of neutralisation is presented on the Y axis.

Figure 13 is the results of two Western blots. The membranes were incubated with sera obtained by immunizing mice with PBS + alum + 50μg OMV preparation from *E. coli* BL21(DE3)ΔTolR (left Western blot) or PBS + alum + 50μg OMV preparation from *E. coli* BL21(DE3)ΔTolR-TC0210. In each Western blot, molecular weight markers are presented on the left hand side and lane 1 is loaded with 200ng of TC0210His (53 kDa) purified protein. The arrow shows the band corresponding to the 53 kDa protein TC0210His;

Figure 14 is a bar chart showing the results of an ELISA assay. The four serum samples that were used are from mice immunised with (from left to right) 5μg OMV + alum; 50μg OMV + alum; 5μg OMV-TC0210 + alum; 50μg OMV-TC0210 + alum. Three results are provided for each serum sample as follows (from left to right): IgG2a, IgG1 and IgGtot. ELISA titer is shown on the Y axis;

Figure 15 is a graph showing the results of a *C. muridarum* neutralisation assay using sera of BALB/c mice that had been immunised with OMVs from *E. coli* BL21(DE3)ΔtolR presenting TC0210 (50 μg) (—■—); with purified recombinant TC0210His (20 μg) (—▲—); with MOMP (20 μg) (—◆—) and with the OMVs alone (50 μg) (—▲—). Results are the mean of 6 independent experiments. Serum dilution is presented along the X axis and % of neutralisation is presented on the Y axis.

Figure 16 is a graph showing the results of a *C. trachomatis* neutralisation assay using sera of BALB/c mice that had been immunised with: MOMP from *C. trachomatis* (20 μg) (—◆—); OMV-TC0210 (50 μg) (—■—); MOMP *C. muridarum* (20 μg) (—◆—); CT823-His (20 μg) (—●—); TC0210-His (20 μg) (—▲—) and OMV (50 μg) (—▲—). Serum dilution is presented on the X axis and % of neutralisation is presented on the Y axis. The results are the mean of two experiments;

Figure 17a is a schematic diagram of a CD1 mice immunization protocol;

Figure 17b is the results of 6 Western Blots in which 200ng of purified TC0210His recombinant protein was loaded for each. From left to right, the membranes were incubated, respectively, with sera obtained from mice immunised with: Group 1 (50 μg OMV + alum); Group 2 (50 μg OMV-TC0210 + alum); Group 3 (20 μg TC0210His + 50 μg OMV + alum); Group 4 (1 μg TC0210His + 50 μg OMV + alum); Group 5 (1 μg TC0210 + alum) and Group 6 (20 μg TC0210His + alum);

Figure 17c shows the Western Blots for (i) Group 2 and (ii) Group 4 in more detail. The arrow indicates the 53kDa TC0210 protein. A serum dilution of 1:200 was used;

Figure 18 is a bar chart showing the results of an ELISA assay. The ELISA titer is presented along the Y axis. The immunization groups are presented along the X axis. From left to right, the bars represent sera from mice immunised with: i) 50 μg OMV-TC0210 + alum; ii) 20 μg TC0210His + 50 μg OMV + alum; iii) 20 μg TC0210His + alum; 1 μg TC0210His + alum; 1 μg TC0210His + 50 μg OMV + alum, vi) 50 μg OMV;

Figure 19 is a graph showing the results of a *C. muridarum* neutralization assay using sera of CD1 mice that had been immunised with a) MOMP (20 μg); b) OMVs from *E. coli* BL21(DE3)ΔtolR presenting TC0210 (50 μg); c) OMVs from *E. coli* BL21(DE3)ΔtolR which do not express any heterologous antigens (50 μg); d) purified recombinant TC0210-His (20 μg); e) OMVs 50 μg plus TC0210HIS (1 μg). Serum dilution is presented along the X axis and % of neutralisation is presented along the Y axis. The results are the mean of 6 independent experiments;

Figure 20 shows the results of the epitope mapping experiment and shows the different epitopes of the TC0210 antigen that were recognised by sera of mice immunized with OMV-TC0210 (top), TC0210His (middle) or OMV (bottom);

Figure 21 shows two SDS PAGE gels in which were loaded: (i) 20 μg of an *E. coli* BL21(DE3)ΔtolR OMV preparation (left hand gel); and (ii) 20 μg of an *E. coli* BL21(DE3)ΔompA OMV preparation (right hand gel);

Figure 22 is an SDS PAGE gel showing the protein content of *E. coli* BL21(DE3)ΔompA and the protein content of *E. coli* BL21(DE3)ΔompA expressing TC0210. The lanes were loaded with 5 μg, 10 μg and 20 μg, respectively. The TC0210 band is marked by the arrow;

Figure 23 are the results of a mass spectrometry experiment carried out to confirm the presence of the TC0210 antigen in the OMV preparation.

MODES FOR CARRYING OUT THE INVENTION

Example 1: Materials and methods

The following materials and methods are used in the examples unless stated otherwise:

1) Bacterial strains, cultures, and reagents:

Chlamydia muridarum strain Nigg and *C. trachomatis* serovar D strain D/UW-3/CX were grown on confluent monolayers of LLC-MK2 (ATCC CCL7) in Earle's minimal essential medium (EMEM) as described previously [195]; [196]. Purification of *C. trachomatis* and *C. muridarum* EBs was carried out by Renografin density gradient centrifugation as described previously [195]. *Escherichia coli* BL21(DE3) was grown aerobically in Luria broth (LB) medium (Difco) at 37°C. When appropriate, ampicillin (100 µg/ml) and isopropyl-β-D-galactopyranoside (IPTG; 1 mM) were added to the medium. Unless specified, all chemicals used in this study were purchased from Sigma. Restriction enzymes and DNA modification enzymes were from New England Biolabs.

2) Gene cloning and protein purification:

To produce recombinant proteins such as CT823, TC0210, TC0727, TC0651, TC0313, TC0106, TC0551, TC0431, TC0890, CT681 (*C. trachomatis* MOMP [$MOMP_{Ct}$]) and TC0052 (*C. muridarum* MOMP [$MOMP_{Cm}$]), genes were PCR amplified from *C. trachomatis* and *C. muridarum* chromosomal DNA using specific primers annealing at the 5' and 3' ends of either gene and cloned into plasmid pET21b$^+$ (Invitrogen) so as to fuse a six-histidine tag sequence at the 3' end. Cloning and purification of His fusions were performed as already described [196]. TC0727, TC0651, TC0106, TC0551, TC0431, TC0890, $MOMP_{Ct}$ and $MOMP_{Cm}$ expressed as His fusion proteins were purified from the insoluble protein fraction, while TC0313, CT823 and TC0210 expressed as His fusion proteins were purified from the soluble protein fraction according to the manufacturer's procedure.

3) Construction of BL21(DE3) ΔtolR deletion mutant:

The ΔtolR mutant was produced by replacing tolR coding sequence with a kanamycin resistance ("kmr") cassette. To this aim, a three-step PCR protocol was used to fuse the *tolR* upstream and downstream regions to the kmr gene. Briefly, the 528-bp upstream and 466-bp downstream regions of the *tolR* gene were amplified from *E. coli* BL21(DE3) genomic DNA with the specific primer pairs UpF (TCTGGAATCGAACTCTCTCG) (SEQ ID NO: 68)/UpR-kan (ATTTTGAGACACAACGTGGCTTTCATGGCTTACCCCTTGTTG) (SEQ ID NO: 69); DownF-kan (TTCACGAGGCAGACCTCATAAACATCTGCGTTTCCCTTG) (SEQ ID NO: 70)/ DownR (TTGCTTCTGCTTTAACTCGG) (SEQ ID 71), respectively. In parallel, the kmr cassette was amplified from plasmid pUC4K using the

primers kan-F (ATGAGCCATATTCAACGGGAAAC) (SEQ ID NO: 72) and kan-R (TTAGAAAAACTCATCGAGCATCAAA) (SEQ ID NO: 73). Finally, the three amplified fragments were fused together by mixing 100 ng of each in a PCR containing the UpF/DownR primers. The linear fragment obtained, in which the kmr gene was flanked by the *tolR* upstream and downstream regions, was used to transform the BL21(DE3) *E. coli* strain (made electrocompetent by three washing steps in cold water), and *tolR* mutants were selected by plating transformed bacteria on Luria-Bertani (LB) plates containing 30ug/ml of kanamycin.

Recombination BL21(DE3) cells were produced by using the highly proficient homologous recombination system (*red* operon) [197]. Briefly, electrocompetent bacterial cells were transformed with 5ug of plasmid pAJD434 by electroporation (5.9 ms at 2.5 kV). Bacteria were then grown for 1 h at 37 °C in 1 ml of SOC broth and then plated on LB plates containing trimethoprim (100ug/ml). Expression of the *red* genes carried by pAJD434 was induced by adding 0.2% L-arabinose to the medium. The gene deletion of the *tolR* gene was confirmed by PCR genomic DNA amplification using primers pairs UpF /Kan-R; Kan-F/Kan-R; Kan-F/DownR. The deletion was confirmed also using the primers tolR-F (CGGACCCGTATTCTTAAC) (SEQ ID NO: 74) and tolR-R (GCCTTCGCTTTAGCATCT) (SEQ ID NO: 75) annealing further upstream and downstream from the 5'- and 3'-flanking regions, respectively.

4) Construction of BL21(DE3) ΔompA deletion mutant:

The ΔompA mutant was produced by replacing ompA coding sequence with a Chloramphenicol resistance ("Cmr") cassette using specific primers. The procedure is the same as that utilized to produce BL21(DE3)ΔtolR (see section 3) above). In particular, primers used to amplify the about 530 bp upstream and about 470 bp downstream regions of the *ompA* gene were amplified from BL21(DE3) genomic DNA with the specific primer pairs ompA_Up for: (GATCGGTTGGTTGGCAGAT) (SEQ ID NO: 76)/ ompA cm_Up-rev: (CACCAGGATTTATTTATTCTGCGTTTTTGCGCCTCGTTATCAT) (SEQ ID NO: 77); ompA cm_Down for: (TACTGCGATGAGTGGCAGGCGCAGGCTTAAGTTCTCGTC) (SEQ ID NO: 78)/ ompA Down rev: (AAAATCTTGAAAGCGGTTGG) (SEQ ID NO: 79); CMr FOR: (CGCAGAATAAATAAATCCTGGTG) (SEQ ID NO: 80)/ CMr REV: (CCTGCCACTCATCGCAGTA) (SEQ ID NO: 81). Finally the three amplified

fragments were fused together by mixing 100 ng of each in a PCR containing the ompA_Up for/ ompA Down rev primers.

The linear fragment obtained, in which the Cmr gene was flanked by the *ompA* upstream and downstream regions, was used to transform the BL21(DE3) *E. coli* strain (made electrocompetent by three washing steps in cold water). *ompA* mutants were selected by plating transformed bacteria on Luria-Bertani (LB) plates containing 20ug/ml of Chloramphenicol.

The gene deletion of the *ompA* gene was confirmed by PCR genomic DNA amplification using primers specifically annealing to Cmr cassette (CMr FOR/ CMr REV), or ompA_Up for/ CMr REV, or using primers specific for ompA in order to further verify the deletion of this gene (ompA FOR: (ATGAAAAAGACAGCTATCGC) (SEQ ID NO: 82) / ompA REV: (TTAAGCCTGCGGCTGAGTT) (SEQ ID NO: 83).

5) Expression of *chlamydial* antigens on BL21(DE3) ΔtolR or on BL21(DE3) ΔompA:

To express the 9 *Chlamydia muridarum* antigens (TC0052, TC0106, TC0210, TC0313, TC0431, TC0551, TC0651, TC0727, TC0890) on the outer membrane of *E. coli* mutant strains, genes coding for *chlamydial* antigens were fused in frame to the *E. coli* OmpA leader peptide. These fusions were then inserted in pET 21b (Invitrogen), a plasmid previously modified for cloning with the pipe method [198], by using specific primers annealing to the six-histidine tag sequence at the 3' end and to the gene coding for the *E. coli* OmpA leader peptide at the 5' end. The fusions were placed under the control of a lac promoter in the multicopy plasmid (pET). The obtained plasmid is called pET-TC0xyz. Plasmids were transformed in *E. coli* HK100 cells, made $CaCl_2$ competent after several successive washes in cold, $MgCl_2$ and $CaCl_2$ solutions [198].

Cells were plated on LB containing 100ug/ml of Amp. at 37°C O.N. Positives clones were grown in LB in order to produce mini preparations of plasmids (by using Qiagen mini prep kit).

BL21(DE3) *ΔtolR* and BL21(DE3) *ΔompA E. coli* strain (made electrocompetent by three washing steps in cold water) were transformed with 10ng of each pET-TC0xyz plasmid mini preparation. Bacteria were grown for 1 h at 37 °C in 1 ml of SOC broth and then plated on LB plates containing Amp. (100ug/ml) and incubated O.N. at 37°C.

6) OMV preparation:

BL21(DE3)ΔtolR and BL21(DE3)*ΔompA E. coli* cells expressing or not expressing chlamydial antigens were inoculated from fresh plate into 500ml of LB (Luria Bertani broth) + Amp (100ug/ml) and were incubated at 37°C with shaking (200 r.p.m.) and growth. The induction of recombinant protein expression is made by addition of IPTG 0.1mM at O.D.=0.4. Bacteria culture were grown until at 37°C the O.D.=1. At that point, culture media were filtered through a 0.22 µm pore-size filter (Millipore, Bedford, MA). The filtrates were subjected to high speed centrifugation (200,000 x *g* for 90 min), and the pellets containing the OMVs were washed with PBS and finally resuspended with PBS [199].

7) Western blot analysis:

20ug of OMV preparations and 200ng of TC0210 His were respectively size-separated by SDS-PAGE (4–12% gel) under reducing conditions and electroblotted onto nitrocellulose membranes. Membranes were saturated overnight with Milk Marwell 10% in PBS (phosphate Buffer) 0,1% Triton, shaking at 4°C. Then, membranes were incubated with specific mouse sera at RT for 2 hours (sera dilution 1:200). Anti mouse horseradish peroxidase conjugated IgG (Amersham Biosciences) was used as secondary antibody. Colorimetric staining was performed with the Opti 4CN substrate kit (Bio-Rad).

8) ELISA assay:

IgG directed to recombinant purified TC0210 were assayed by enzyme-linked immunosorbent assay (ELISA). Individual wells of micro-ELISA plates (Nunc Maxisorp) were coated with 1 µg of recombinant protein in PBS (pH 7.4) at 4 °C overnight. The plates were washed, treated for 1h at 37 °C with PBS–1%BSA, and 100 µl aliquots of antisera at different serial dilutions in PBS–0.1% Tween were added to the wells. After incubation for 2h at 37 °C, plates were again washed and incubated for 1h at 37 °C with alkaline-phosphatase conjugated goat anti-mouse IgG (Sigma) diluted 1:2500 in PBS–Tween. Thereafter 100 µl of PNPP (Sigma) were added to the samples and incubated for 30 min. at room temperature. Optical densities were read at 405 nm and the sera–antibody titers were defined as the serum dilution yielding an OD value of 0.5.

9) Neutralization assay:

Sera obtained by immunizing mice (BALB/c or CD1) with OMV-TC0210 were tested *in vitro* for the neutralization activity. *In vitro* neutralization assays were performed on LLC-MK2 (Rhesus monkey kidney) epithelial cell cultures. Three serial dilutions of each sera pool were tested by diluting them 1:30, 1:90, 1:270 in Sucrose phosphate Buffer (SP). Also, purified infectious *C. muridarum* EB were diluted in the same Buffer to contain $3x10^{(5)}$ IFU/ml, and 15ul of EB suspension were added to each serum dilution in a final volume of 150ul. Antibody-EB interaction was allowed by incubating for 30 min. at 37 °C. Also, EB were incubated without sera as an infection control. 100ul of each reaction mix, including EB diluted in SP without sera, were used to inoculate LLC-MK2 confluent monolayers (in duplicate for each serum dilution) in a 96-well tissue culture plate, and centrifuged at 2000g for 1hour at 37°C. After centrifugation, Earle's minimal essential medium containing Earle's salts, 10% fetal bovine serum and 1ug/ml cycloheximide were added. Infected cultures were incubated at 37°C for 24 hours, while in neutralization assays, in which EB of *C. trachomatis* were used for infection, infected cultures were incubated for 48 hours. Then, cell cultures were fixed by adding 100ul of methanol for 5 minutes and the *chlamydial* inclusions were detected by staining with a mouse anti-*Chlamydia* fluorescein-conjugated monoclonal antibody (Merifluor *Chlamydia*, Meridian Diagnostics, Inc.). Finally, all inclusions for each well were counted at of 10x magnification.

Calculations of the infectivity reduction by each sera pool were carried out using pre immune sera neutralization titers as basal levels.

10) Epitope mapping:

On a nitrocellulose membrane 95 synthetic peptides of TC0210 were spotted. Each peptide is constituted by 15 amino acids and overlaps 10mers with the following peptide. Three membranes, made with the same design, were washed with TBS (Tris-HCl 50mM, NaCl 137mM, KCl 2,7mM) containing 0,05% of tween 20 (T-TBS), and then were incubated overnight at 4 °C with 2% milk Marwell in TBS (MBS). Then, on the three membranes, different sera pools (sera dilution 1:100) were tested respectively: sera of mice immunized with a) OMV expressing TC0210, b) TC0210His, and c) OMV alone.

Finally, an anti-mouse horseradish peroxidase conjugated IgG (Amersham Biosciences) was used as a secondary antibody (sera dilution 1:5000). Colorimetric staining was performed with the Opti 4CN substrate kit (Bio-Rad).

Example 2: Construction of an *E. coli* BL21(DE3)ΔtolR deletion mutant

BL21 (DE3)ΔtolR is a mutant *E. coli* strain in which the ΔtolR mutation was introduced by replacing the tolR coding sequence with a Kanamycin resistance cassette. This strain is able to release a large quantity of outer membrane vesicles in the culture supernatant. It was found that the protein content released in the culture supernatant from the ΔtolR mutant strain is higher compared to that released from the wild type strain (see Figure 2).

Example 3: Expression of *chlamydial* antigens in BL21(DE3)ΔtolR or in BL21(DE3)ΔompA

To allow presentation of each of the 9 *Chlamydia muridarum* antigens (TC0052, TC0106, TC0210, TC0313, TC0431, TC0551, TC0651, TC0727 and TC0890 - see Table 3 below) on the outer membrane of *E. coli* mutant strains, genes coding for *chlamydial* antigens were fused in frame to the *E. coli* OmpA leader peptide.

TABLE 3:

***Chlamydia* promising antigens**

C. muridarum	ANNOTATION	*C. trachomatis* homolog
TC0052	MOMP	CT681
TC0651	hypothetical protein	CT372
TC0727	60Kda Cystein Rich OMP	CT443
TC0210	DO serine protease	CT823
TC0313	hypothetiçal protein	CT043
TC0106	hypothetical protein	CT733
TC0551	Na(+)-translocating NADH-quinone reductase	CT279
TC0431	MAC-Perforine protein	CT153
TC0890	Invasine repeat family phosphatase	CT601

The fusions were placed under the control of a *lac* promotor in the multicopy plasmid pET21b+ (Novagen). The obtained plasmid is called pET- TC0xyz.

The 9 different pET-TC0xyz plasmids were transformed in *E. coli* BL21(DE3)*ΔtolR* and *E. coli* BL21(DE3)*ΔompA* strains. The SDS PAGE gel analysis in Figure 3 shows that the expression of chlamydial antigen was clearly visible in the culture total extracts of *E. coli BL21(DE3)ΔtolR* expressing TC0106, TC0052 and TC0210 (see lanes 1, 2 and 4 of Figure 3). On the contrary, expression of TC0727 in *E. coli* was not clearly visible (see lane 3 of Figure 3).

Example 4: OMV preparation

A schematic diagram of OMV preparation is shown in Figure 4. Bacteria were induced when they reached an OD600 of 0.4. The bacteria were grown to OD600 = 1 and were then centrifuged at 6,000g. Culture media of BL21(DE3)ΔtolR and pET-TC0xyz BL21(DE3)ΔtolR strains were filtered through a 0.22 μm filter, centrifuged at high-speed (200,000 x g, 120 min.) and the OMVs were recovered in the pellet were washed with phosphate and were then resuspended in phosphate to obtain the OMVs [199].

Example 5: Analysis of OMV preparations

Western blot analysis was used to determine whether the OMV preparations expressed the heterologous antigens. Six out of the nine recombinant OMVs were shown to carry the heterologous proteins. Figures 5A and 5B indicate that each of TC0052, TC0106, TC0210, TC0727, TC0313 and TC0890 were expressed in the recombinant OMVs. On the contrary, Figure 5C shows that TC0651, TC0551 and TC0431 were not expressed in the recombinant OMVs.

However, in follow up experiments TC0431 was in fact shown to be expressed in the recombinant OMVs but at a low level as can be seen from Figure 5D (i) wherein the amount of OMV preparations loaded onto the gel was doubled in order to obtain detectable levels of the antigen. On the other hand, the expression of TC0052 was not detected in the follow up experiments (see Figure 5D (ii)). The reason for this apparent variability is believed to be the result of instability of engineered strains encoding TC0052, such instability leading to lysis of the bacteria during growth in culture.

The growth curve of *E. coli* BL21(DE3)ΔtolR prepared in Example 3 expressing each of the 9 *chlamydial* antigens is shown in Figure 6. *E. coli* expressing TC0210 had the greatest rate of growth. The rate of growth of *E. coli* expressing TC0313, TC0890, TC0431 or TC0106 was similar but was slower than the rate of growth of *E. coli* expressing TC0210. The rate of growth of *E. coli* expressing TC0727 was slower than *E. coli* expressing any of TC0313, TC0890, TC0431 or TC0106. *E. coli* expressing TC0052, TC0551 or TC0651 had the slowest rate of growth.

TC0210-OMV was found to be the best preparation in terms of yield and quantity of *Chlamydia* antigen that was expressed. Mass spectrometry confirmed the presence of the TC0210 peptide on the surface of TC0210-OMV preparations after the shaving of the same OMV preparation (the technique of *Chlamydia* shaving is described in WO 2007/110700 and a schematic diagram is shown in Figure 7). The peptide identified in the shaving experiment corresponds to amino acids 65-75 of the full length TC0210 protein. The matched peptide is shown in **bold and underlined** (see also Figure 8):

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  1 MMKRLLCVLL STSVFSSPML GYSAPKKDSS TGICLAASQS DRELSQEDLL
 51 KEVSRGFSKV AAQATPGVVY IENFPKTGSQ AIASPGNKRG FQENPFDYFN
101 DEFFNRFFGL PSHREQPRPQ QRDAVRGTGF IVSEDGYVVT NHHVVEDAGK
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151 IHVTLHDGQK YTAKIIGLDP KTDLAVIKIQ AKNLPFLTFG NSDQLQIGDW
201 SIAIGNPFGL QATVTVGVIS AKGRNQLHIV DFEDFIQTDA AINPGNSGGP
251 LLNIDGQVIG VNTAIVSGSG GYIGIGFAIP SLMAKRVIDQ LISDGQVTRG
301 FLGVTLQPID SELAACYKLE KVYGALITDV VKGSPAEKAG LRQEDVIVAY
351 NGKEVESLSA LRNAISLMMP GTRVVLKVVR EGKFIEIPVT VTQIPAEDGV
401 SALQKMGVRV QNLTPEICKK LGLASDTRGI FVVSVEAGSP AASAGVVPGQ
451 LILAVNRQRV SSVEELNQVL KNAKGENVLL MVSQGEVIRF VVLKSDE
```

TC0210 has the properties that are shown in Table 4.

TABLE 4

TC0210				
C. muridarum / *C. trachomatis* antigen	*C. muridarum* / *C. trachomatis* % Similarity	Protein name/ annotation	Novartis scientific data	Other data from literature
TC0210/CT823	91.8	HtrA / DO serine protease	Surface exposed Antibody and CD4-th1 inducer in mouse and humans Highly homologous among the 8 major serovars	Serine endoprotease, temperature-activated shows both chaperon and protease activities (Huston et al., FEBS Lett., 2007); Immunogenic in humans (Sanchez-Campillo et al., Electrophoresis, 1999)

A sequence alignment of *C. muridarum* TC0210 with *C. trachomatis* CT823 is provided in Figure 9. TC0210 is 93% identical to CT823.

Figure 10B shows the results of a protein microarray experiment in which the chlamydial antigens were expressed in *E. coli*, affinity purified and spotted on glass slides in a microarray format. The instruments and the protocols were as described in Bombaci, M. et al [200]. The slides were immuno-stained with hundreds of different human sera and the staining intensity of every spot was measured. The proteins recognised with a fluorescence intensity of more than 1000 are shown. Two groups of human sera were used for each antigen: MIF positive sera (left hand bar) and MIF negative sera (right hand bar) (where "MIF" stands for the Micro Immuno Fluorescence

assay [201], which is used to check if the patient sera contains antibodies against the pathogen (MIF positive) or not (MIF negative)).

The analysis of the protein chip shown in Figure 10B using MIF positive and MIF negative sera shows that there are three groups of chlamydial antigens: one group of antigens is recognized mainly by MIF positive sera (CT681His, CT823His, CT443His, CT456GST, CT110GST, CT622His, CT089His), another group is recognized both by MIF positive and MIF negative sera (CT415GST, CT119GST, CT049GST, CT589GST, CT372GST, CT077His, CT559His, CT322His, CT316His, CT350GST, CT381His, CT355GST, CT389GST, CT127GST, CT157GST, CT567His and CT266His), and a third group of antigens is mainly recognized by MIF negative sera (CT470His, CT398His and CT114His). The CT823 antigen is clearly recognized by MIF positive sera but not by MIF negative sera, suggesting that CT823 is a chlamydial antigen capable of eliciting a humoral immune response during natural infections in man. Thus, CT823 is immunogenic in humans.

This is supported by Figure 10A, which shows that CT823 is exposed on the EB surface (KS=19.1, see Figure 10A). The assay was performed as previously described [196]. A mouse polyclonal antibody serum was used which had been prepared by immunizing mice with TC0210His. Background control sera were from mice immunized with *E. coli* contaminant proteins. The shift between the background control histogram and the immune serum testing histogram was taken as a measure of antibody binding to the EB cell surface. The Kolmogorov–Smirnov (K–S) two-sample test I.T. was performed on the two overlapped histograms. The $D/s(n)$ value (an index of dissimilarity between the two curves) is reported as the "K-S score". A K-S of 19.1 was obtained (T(X) = 1146; Max T value = 1719; Max. difference = 78.5% at value 19.1; Confidence in difference is greater than 99.9%; SED percentage positive: 82.6; Population comparison data 0.06)

Figure 10C shows that the CT823 antigen is one of three antigens strongly recognized by sera from mice immunized with whole *C. trachomatis* EBs. This suggests that native CT823 is contained in EBs and in an amount that is sufficient to elicit an antibody response in mice.

Recombinant HtrA and the native HtrA contained in the *C. muridarum* EBs was also recognized by a mouse polyclonal antibody serum which has been prepared by

immunizing mice with OMV preparation from BL21(DE3)ΔTolR TC0210 as shown by Western Blot in Figure 10D.

The FACS analysis described above for Figure 10A, was repeated using mouse polyclonal antibody serum obtained from mice immunized with OMV preparation from BL21(DE3)ΔTolR TC0210. Having previously demonstrated in Figure 10A that flow cytometry can be used to follow antibody binding the surface of EBs, the data presented in Figure 10E showed that the polyclonal antibodies raised against TC0210 in the context of the OMV were able to recognize the HtrA exposed on the *C. muridarum* EBs' surface (Figure 10E).

Example 6: Immunization of BALB mice with OMV-TC0210:

Mice were immunised according to the schedule shown in Figure 11. Specifically, BALB mice (5/6 weeks old) were immunised intramuscularly at days 1, 20 and 35 before PBMC were taken at day 45 and sera collected at day 60. The mice were divided into 5 groups, with 12 mice for each group, and immunisation was carried out according to the following scheme: GROUP 1: PBS + Alum; GROUP 2: PBS + Alum + 5μg OMV preparation from *E. coli* BL21(DE3)ΔTolR; GROUP 3: PBS + Alum + 5μg OMV preparation from BL21(DE3)ΔTolR TC0210; GROUP 4: PBS + Alum + 50μg OMV preparation from BL21(DE3)ΔTolR; and GROUP 5: PBS + Alum + 50μg OMV preparation from BL21(DE3)ΔTolR TC0210.

Western Blot and ELISA assay

Following immunisation of the mice, the mice sera were tested by Western blot analysis and ELISA in order to evaluate the production of anti-TC0210 antibodies. For each Western blot, the purified *C. muridarum* recombinant protein TC0210 was loaded. Figure 13 shows that the sera of mice immunized with OMV preparations expressing TC0210 were able to recognize the recombinant protein, while the sera of mice immunized with OMV preparations without TC0210 were not.

The ELISA results of Figure 14 show that for mice immunised with 5 μg OMV-TC0210 (Group 3 mice), an equal amount of IgG1 and IgG2a antibodies were raised. However, for mice immunised with 50 μg OMV-TC0210 (Group 5 mice), more IgG2a than IgG1 antibodies were raised. The total amount of IgG antibodies raised was about the same in both cases. Thus, the 50 μg dose works better in terms of quality of elicited antibodies (as shown by the neutralization titer). More IgG antibodies were raised in mice immunised with OMVs expressing TC0210 than were raised in mice immunised with OMVs alone + Alum.

Neutralization of infection with E.B. of C. muridarum.

There is much evidence to support an important role for neutralizing antibodies in the protection against *Chlamydia* infection. In order to evaluate this, a neutralization assay was performed using sera of immunized mice with the OMV preparation expressing the *chlamydial* antigen TC0210. The results are shown in Figure 15, which shows the percentage of neutralization against infection with *C. muridarum* with respect to three different sera dilutions (mean of six independent experiments).

Sera of mice immunized with 50μg OMVs expressing TC0210 (Group 5 mice) are able to neutralize *in vitro C. muridarum* infection with a titer of 1:90 (the neutralization titer is defined as the serum dilution able to reduce EB infection by 50%) (see (—■—) line). This sera is almost as potent at neutralising *C. muridarum in vitro* as the sera obtained by immunizing mice with purified recombinant MOMP (positive control - see the (—◆—) line). In contrast, sera of mice immunized with purified recombinant TC0210 are not able to neutralize the *C. muridarum* infection; in fact neutralization percentages are very low also at minimal serum dilution (1:30) (see the (··△··) line). The (—▲—) line shows the percentage of neutralization relative to OMV without *chlamydial* antigens. Thus, the neutralisation percentage for purified recombinant TC0210 is very similar for the

neutralisation percentage obtained for OMV alone. These calculations have been done versus pre-immune sera. This is one of the first examples in which antibodies directed against a *chlamydial* antigen, other than MOMP, have been able to neutralize *chlamydial* infectivity *in vitro*. Surprisingly, these data show that the TC0210 antigen, which is not protective when tested in a *chlamydial* animal model when administered in its purified form, becomes protective when presented in an OMV of the invention.

The neutralisation assay was repeated again and the results are shown in Figure 12. The results in Figure 12 support the findings discussed above.

Neutralization of infection with E.B. of C. trachomatis.

CT823 is the *C. trachomatis* homologous protein to TC0210. The ability of the anti OMV-TC0210 sera to neutralize *in vitro* infection by *Chlamydia trachomatis* was tested.

Purified *C. trachomatis* EBs were incubated with mouse sera at three different dilutions at 37°C for 30 min. Residual infectivity was determined on LLC-MK2 cells by counting IFU/cs. Neutralization percentages were measured in two independently performed neutralization assays and calculated versus preimmune sera Figure 16 shows the results. Anti OMV-TC0210 mouse polyclonal serum (see (—■—) line) is able to neutralize *C. trachomatis* efficiently *in vitro (*with a similar titer observed by infecting with E.B. of *C. muridarum).* Indeed, anti-OMV-TC0210 mouse polyclonal serum was found to be almost as potent at neutralising *C. trachomatis* infectivity as the sera of mice immunised with MOMP from *C. trachomatis* (see the (—◆—) line, which is the positive control). The neutralizing percentage of this serum is also higher than that obtained after immunization with the homologue recombinant purified protein of *C. trachomatis,* CT823 (see (—◎—) line). The (—◆—) line represents the neutralizing percentage of sera immunized with MOMP of *C. muridarum*; this serum does not neutralize the *chlamydial* infection. Likewise, sera of mice immunized with OMV (see (—▲—) line) or with TC0210His (see (—△—) line) did not neutralize the *chlamydial* infection. It was found that OMV-TC0210 specific-antibody neutralises *C. trachomatis* infection *in vitro* at the same level observed for *C. muridarum.* Thus, an immune response raised against the *C. muridarum* antigen may also neutralise infection against *C. trachomatis.*

Example 7: Immunization of CD1 mice with OMV-TC0210:

In order to confirm the neutralization results of Example 6, immunization was repeated in CD1 mice (5-6 weeks old). Groups of CD1 mice (5 mice in each group) were

immunised according to the following immunisation scheme: Group 1: 50μg OMV + Alum; Group 2: 50 μg OMV-TC0210 + Alum; Group 3: 20μg TC0210His + 50μg OMV + Alum; Group 4: 1μg TC0210His + 50μg OMV + Alum; Group 5: 1 μg TC0210His + Alum; Group 6: 20μg TC0210His + Alum. The scheme was devised also to test whether there is an adjuvant effect of OMV (see Groups 3 and 4). Mice were immunized on days 1, 20 and 40. Sera were collected for the neutralization assay on day 60 (see Figure 17A).

Immunogenicity comparison between TC0210 expressed on the OMV and the recombinant form.

Western blots were performed using sera of mice immunized with 50μg of OMV-TC0210 (Group 2) and sera of mice immunized with 1μg of TC0210His plus 50μg of OMV without *chlamydial* antigens (Group 4), in order to compare the immunogenicity between TC0210 expressed on the OMV and the recombinant form. The quantity of chlamydial antigen present on the *E. coli* OMV surface is estimated to be 1% of the total content of *E. coli* proteins (about 0.5ug of chlamydial protein in 50ug of OMVs).

The Western blot results of Fig. 17B show that sera of mice immunized with OMV-TC0210, or with TC0210-His, produce antibodies against the chlamydial antigen. However, as shown in Fig. 17B, following immunization of mice with a combination of 1ug of TC0210-His plus 50ug of OMV not expressing a chlamydial antigen, antibody production against TC0210 is not visible. In this case, 1ug of TC0210His alone is immunogenic but 1ug of recombinant protein TC0210-His in combination with OMV is not immunogenic. Thus, it seems that the presence of the separate OMVs inhibits the immunogenicity of the purified protein when 1ug purified protein is used. However, the use of a lower concentration of protein (0.5ug TC0210) expressed in the OMV system is immunogenic. The combination of 20ug TC0210-His plus 50ug OMV is suitable to elicit antibody production.

The Western Blot results of Fig. 17C further underline differences between sera produced by immunising with 50ug OMV-TC210 and sera produced by immunising with 1ug TC0210-His plus 50ug OMV. The Western blot results of Figure 17B and 17C show that only sera of mice immunized with OMV-TC0210 produce antibodies against this antigen. This suggests that the neutralizing effect elicited by sera obtained by

immunising with OMV-TC0210 is not caused by an adjuvant effect of OMVs, but by the OMV's capability to present TC0210 in its natural conformation and composition.

ELISA titers

Figure 18 shows the quantitative analysis of antibody production by ELISA. ELISA microplates were coated with 0.5μg of TC0210His in PBS and stored at 4 °C overnight. Plates were saturated with PBS-1%BSA, and then antisera, at different serial dilutions in PBS–0.1% Tween, were added to the wells. Plates were then incubated with alkaline-phosphatase conjugated goat anti-mouse IgG (Sigma). After that 100 μl of PNPP (Sigma) were added to the samples. Optical densities were read at 405 nm and the serum-antibody titer was defined as the serum dilution yielding an OD value of 0.5. Total IgG titers of each mice group immunized with OMV expressing TC0210, or TC0210-His recombinant purified protein (1 μg or 50μg) plus OMV, or with TC0210-His (1 μg or 50μg) alone.

From left to right, the bars in Figure 18 correspond to mice Groups 2, 3, 6, 5, 4 and 1, respectively.

Although antibody titres of sera from mice immunised with OMVs expressing TC0210 are lower than those obtained with TC0210His or with the same recombinant protein + OMVs, the antibodies that are present in sera from mice immunised with OMVs expressing TC0210 are better in terms of neutralizing activity (see below).

Although not yet investigated, it is reasonable that the ELISA results would have been different if Chlamydia EBs had been used for the coating, as these include TC0210 in its native conformation, instead of the recombinant protein as used in the present experiment. In fact, a coating with EBs would allow the detection of antibodies raised against conformational epitopes.

Neutralisation assay

Figure 19 shows *in vitro* neutralization of *C. muridarum* infectivity on LL-CMK2 cells. Purified EBs were incubated with mouse sera at three different dilutions at 37 °C for 30 min. Residual infectivity was determined on LLC-MK2 cells by counting IFU/cs. Neutralization percentages were measured in six independently performed neutralization assays using sera from CD1 mice immunized with 50ug of recombinant OMV expressing TC0210 (Group 2: —■—), with 20μg of TC0210-His purified protein (Group

6: —○—). Sera of mice immunized with OMV alone (Group 1: —▲—) and with recombinant TC0210-His (1 µg) plus OMV alone (Group 4: —◆—), were included as negative controls, while sera of mice immunized with recombinant MOMP (—◆—) were used as the positive control. Data shown are the means and standard deviations of 12 samples.

Sera of mice immunized with OMV expressing TC0210 (—■—) were found to neutralize the *Chlamydial muridarum* infection as efficiently as MOMP (—◆—), while sera of mice immunized with OMV without *chlamydial* antigen or TC0210His do not.

Also the neutralization results indicate that OMVs do not have an adjuvant effect. Instead, the result is due to the capability of recombinant OMV-TC0210 to present the heterologous antigen in its natural conformation and composition.

Example 8: Epitope mapping analysis on TC0210

Epitope mapping experiments were performed in order to verify if there were some differences in terms of linear epitopes recognized between sera of mice immunized with OMV-TC0210 and sera of mice immunized with TC0210His. 95 overlapping synthetic peptides covering the full length of the TC0210 antigen were spotted on three membranes, respectively. Each peptide is constituted by 15 amino acids and overlaps 10mers with the following peptide. On the three membranes, different sera pools were tested: sera of mice immunized a) with OMV expressing TC0210, b) with TC0210His, c) with OMV alone (sera of mice immunized with OMV without the *chlamydial* antigen were used as a negative control), respectively. An anti-mouse horseradish peroxidase conjugated IgG was used as the secondary antibody.

Figure 20 shows that sera of mice immunized with OMV expressing TC0210 (top membrane) recognize different epitopes compared to sera of mice immunized with TC0210 His recombinant purified protein (middle membrane). The sera of mice immunized with OMV-TC0210 recognised the following epitope with high specificity: DYFNDEFFNRFFGLP (SEQ ID NO: 36). The sera of mice immunised with OMV-TC0210 recognised the following epitopes with medium specificity: SHREQ (SEQ ID NO: 37), ALQKMGVRVQNLTPE (SEQ ID NO: 38) and NQVLKNAKGENVLLM (SEQ ID NO: 39). The sera of mice immunised with OMV-TC0210 recognised the following epitopes with low specificity: SPMLGYSAPKKDSSTGICLA (SEQ ID NO: 40); EDLLKEVSRGFSKVAAQATP (SEQ ID NO: 41); TGSQAIASPGNKRGFQENPF

(SEQ ID NO: 42); PRPQQRDAVR (SEQ ID NO: 43), IAIGNPFGLQATVTVGVISAKGRNQLHIVD (SEQ ID NO: 44) and NTAIVSGSGGYIGIGFAIPSLMAKRVIDQL (SEQ ID NO:45).

Sera of mice immunised with TC0210His recognised the following epitope with high specificity: NKRGFQENPFDYFNDEFFNRFFGLP (SEQ ID NO: 84). Sera of mice immunised with TC0210His recognised the following epitope with medium specificity: SHREQ.

The epitope GENVLLMVSQGEVIR (SEQ ID NO: 55) indicated inside the box on the OMV-TC0210 membrane of figure 20 is the same epitope found in the shaving on the *C. trachomatis* E.B. (see GENVLLMVSQGDVVR (SEQ ID NO: 86 in present application), which corresponds to SEQ ID 43 from WO 2007/110700, Novartis Vaccines and Diagnostics, SRL), while the epitope TPGVVYIENFPK (SEQ ID NO: 85), indicated in the other box for the OMV-TC0210 membrane, is the same as the epitope found in the shaving on the OMV-TC0210 preparation that is described in Example 5.

It is hypothesized that sera produced by immunising with OMV-TC0210 and sera produced by immunising with TC0210 would be able to recognise the matching epitopes in CT823. Thus, it is hypothesised that the sera of mice immunized with OMV-TC0210 would presumably recognise the following CT823 epitope with high specificity: DYFNDEFFNRFFGLP (SEQ ID NO: 56). The sera of mice immunised with OMV-TC0210 would presumably recognise the following CT823 epitopes with medium specificity: SHREQ (SEQ ID NO: 57), ALQKMGVRVQNITPE (SEQ ID NO: 58) and NQVLKNSKGENVLLM (SEQ ID NO: 59). The sera of mice immunised with OMV-TC0210 would presumably recognise the following CT823 epitopes with low specificity: SPMLGYSASKKDSKADICLA (SEQ ID NO: 60), EDLLKEVSRGFSRVAAKATP (SEQ ID NO: 61), TGNQAIASPGNKRGFQENPF (SEQ ID NO: 62), IAIGNPFGLQATVTVGVISAKGRNQLHIVD (SEQ ID NO: 63) and NTAIVSGSGGYIGIGFAIPSLMAKRVIDQL (SEQ ID NO: 64).. Sera of mice immunised with TC0210-His would presumably recognise the following CT823 epitope with high specificity: DYFNDEFFNRFFGLP. Sera of mice immunised with TC0210-His would presumably recognise the following CT823 epitope with medium specificity: SHREQ (SEQ ID NO: 57).

Example 9: Increasing the quantity of outer membrane proteins on the OMV

OmpA is involved in the structural maintenance of the membrane system. Probably for this reason, the absence of this protein destabilizes the bacterial outer membrane resulting in the release of an abundant quantity of OMV. Release of OMV in the culture supernatant of BL21(DE3)ΔompA mutant strain was observed here. The OMV preparation has been made as previously described and shown in the schematic diagram of Figure 7. Shaving was performed on the OMVs in order to analyze their quality. The results are presented in Figure 21. The 29 proteins (all outer membrane proteins) identified in the *E. coli* BL21(DE3)ΔompA OMV in the shaving and mass spectrometry analysis are: Hypothetical lipoprotein yiaD precursor [Escherichia coli CFT073], transport channel [Escherichia coli W3110], periplasmic chaperone [Escherichia coli K12], Putative toxin of osmotically regulated toxin-antitoxin system associated with programmed cell death [Escherichia coli cell death [Escherichia coli CFT073], murein lipoprotein [Escherichia coli K12], FKBP-type peptidyl-prolyl cis-trans isomerase (rotamase) [Escherichia coli K12], hypothetical protein b0177 [Escherichia coli K12], hypothetical protein ECP_0753 [Escherichia coli 536], maltoporin precursor [Escherichia coli K12], nucleoside channel, receptor of phage T6 and colicin K [Escherichia coli K12], peptidoglycan-associated outer membrane lipoprotein [Escherichia coli K12], Lipoprotein-34 precursor [Escherichia coli CFT073], scaffolding protein for murein synthesizing machinery [Escherichia coli K12], minor lipoprotein [Escherichia coli K12], predicted lipoprotein [Escherichia coli K12], Outer membrane lipoprotein slyB precursor [Escherichia coli CFT073], outer membrane protein X [Escherichia coli K12], maltose regulon periplasmic protein [Escherichia coli K12], maltose ABC transporter periplasmic protein [Escherichia coli K12], Hypothetical lipoprotein yajG precursor [Escherichia coli CFT073], long-chain fatty acid outer membrane transporter [Escherichia coli K12], Hypothetical protein ybaY precursor [Escherichia coli CFT073], predicted outer membrane lipoprotein [Escherichia coli K12], translocation protein TolB precursor [Escherichia coli K12], hypothetical protein b3147 [Escherichia coli K12], predicted iron outer membrane transporter [Escherichia coli K12], oligopeptide transporter subunit [Escherichia coli K12], hypothetical protein c4729 [Escherichia coli CFT073], hypothetical protein b1604 [Escherichia coli K12].

It was surprisingly found that all proteins present in this new OMV preparation are outer membrane proteins. This result underlines that the quality of the new OMV preparation is better than that obtained from the *E. coli* BL21(DE3)ΔtolR mutant strain, in which

some cytoplasmic proteins were also found. Specifically, in the *E. coli* BL21(DE3)ΔtolR mutant strain, only about 75% of the 100 OMV proteins were outer membrane proteins.

Example 10: Expression of *chlamydial* antigens in the BL21(DE3)ΔompA mutant strain.

Chlamydial antigens were expressed in the *E. coli* BL21(DE3)ΔompA strain in order to verify if there is an increase in the quantity of *chlamydial* antigens in the derived recombinant OMV.

OMVs were prepared from culture supernatants of BL21(DE3)ΔompA strain expressing TC0210 as previously described and shown in the schematic diagram of Figure 4. Different amounts of each OMV preparation were loaded onto 4-12% polyacrylamide gel.

As shown in Figure 22, in these new OMV preparations from the BL21(DE3)ΔompA strain, it was possible to identify the presence of TC0210 directly on SDS PAGE (data confirmed by MASS spectrometry analysis – see the band indicated with the arrow on Figure 22). The TC0210 band is clearly visible at all concentrations. In contrast, in OMV preparations obtained from the *E. coli* BL21(DE3)ΔtolR mutant strain, the presence of TC0210 was observed only by Western blot analysis. This confirms that the quantity of expressed TC0210 antigen is remarkably increased in the BL21(DE3)ΔompA strain relative to the BL21(DE3)ΔtolR mutant strain. Mass spectrometry confirmed the presence of the TC0210 peptide on the TC0210-OMV preparations (Figure 23). The identified peptides are shown in bold. Specifically, the following peptides were identified:

VAAQATPGVVYIENFPK (SEQ ID NO: 46), GFQENPFDYFNDEFFNRFFGLPSHREQPRPQQR (SEQ ID NO: 47), GTGFIVSEDGYVVTNHHVVEDAGK (SEQ ID NO: 48), TDLAVIKIQAK (SEQ ID NO: 49), VIDQLISDGQVTR (SEQ ID NO: 50), AGLRQEDVIVAYNGKEVESLSALR (SEQ ID NO: 51), FIEIPVTVTQIPAEDGVSALQK (SEQ ID NO: 52), VQNLTPEICK (SEQ ID NO: 53), and NAKGENVLLMVSQGEVIR (SEQ ID NO: 54).

The inventors have surprisingly found that BL21(DE3)ΔompA mutant strains generate an increased quantity of heterologous antigen on their OMVs relative to OmpA wild type strains. OmpA is the most abundant protein on *E. coli* outer membrane. The inventors have found that, in the OMV, the deletion of this protein improves the relative abundance of *chlamydial* antigen with respect to the *E. coli* outer membrane proteins.

This increased amount of expressed *chlamydial* antigen suggests that OMVs from ΔompA strains, for example, BL21(DE3)ΔompA-TC0210, are good candidates for raising neutralizing antibodies in mice.

It will be understood that the invention has been described by way of example only and modifications may be made whilst remaining within the scope and spirit of the invention.

SEQUENCE

SEQ ID NO:1 – CT823 protein sequence

MMKRLLCVLLSTSVFSSPMLGYSASKKDSKADICLAVSSGDQEVSQEDLLKEVSRGFSRVAAKATPGVVYI
ENFPKTGNQAIASPGNKRGFQENPFDYFNDEFFNRFFGLPSHREQQRPQQRDAVRGTGFIVSEDGYVVTNH
HVVEDAGKIHVTLHDGQKYTAKIVGLDPKTDLAVIKIQAEKLPFLTFGNSDQLQIGDWAIAIGNPFGLQAT
VTVGVISAKGRNQLHIVDFEDFIQTDAAINPGNSGGPLLNINGQVIGVNTAIVSGSGGYIGIGFAIPSLMA
KRVIDQLISDGQVTRGFLGVTLQPIDSELATCYKLEKVYGALVTDVVKGSPAEKAGLRQEDVIVAYNGKEV
ESLSALRNAISLMMPGTRVVLKIVREGKTIEIPVTVTQIPTEDGVSALQKMGVRVQNITPEICKKLGLAAD
TRGILVVAVEAGSPAASAGVAPGQLILAVNRQRVASVEELNQVLKNSKGENVLLMVSQGDVVRFIVLKSDE

SEQ ID NO:2 – CT823 nucleotide sequence

ATGATGAAAAGATTATTATGTGTGTTGCTATCGACATCAGTTTTCTCTTCGCCAATGCTAGGCTATAGTGC
GTCAAAGAAAGATTCTAAGGCTGATATTTGTCTTGCAGTATCCTCAGGAGATCAAGAGGTTTCACAAGAAG
ATCTGCTCAAAGAAGTATCCCGAGGATTTTCTCGGGTCGCTGCTAAGGCAACGCCTGGAGTTGTATATATA
GAAAATTTTCCTAAAACAGGGAACCAGGCTATTGCTTCTCCAGGAAACAAAAGAGGCTTTCAAGAGAACCC
TTTTGATTATTTTAATGACGAATTTTTTAATCGATTTTTTGGATTGCCTTCGCATAGAGAGCAGCAGCGTC
CGCAGCAGCGTGATGCTGTAAGAGGAACTGGGTTCATTGTTTCTGAAGATGGTTATGTTGTTACTAACCAT
CATGTAGTCGAGGATGCAGGAAAAATTCATGTTACTCTCCACGACGGACAAAAATACACAGCTAAGATCGT
GGGGTTAGATCCAAAAACAGATCTTGCTGTGATCAAAATTCAAGCGGAGAAATTACCATTTTTGACTTTTG
GGAATTCTGATCAGCTGCAGATAGGTGACTGGGCTATTGCTATTGGAAATCCTTTTGGATTGCAAGCAACG
GTCACTGTCGGGGTCATTAGTGCTAAAGGAAGAAATCAGCTACATATTGTAGATTTCGAAGACTTTATTCA
AACAGATGCTGCCATTAATCCTGGGAATTCAGGCGGTCCATTGTTAAACATCAATGGTCAAGTTATCGGGG
TTAATACTGCCATTGTCAGTGGTAGCGGGGGATATATTGGAATAGGGTTTGCTATTCCTAGCTTGATGGCT
AAACGAGTCATTGATCAATTGATTAGTGATGGGCAGGTAACAAGAGGCTTTTTGGGAGTTACCTTGCAACC
GATAGATTCTGAATTGGCTACTTGTTACAAATTGGAAAAAGTGTACGGAGCTTTGGTGACGGATGTTGTTA
AAGGTTCTCCAGCAGAAAAAGCAGGGCTGCGCCAAGAAGATGTCATTGTGGCTTACAATGGAAAAGAAGTA
GAGTCTTTGAGTGCGTTGCGTAATGCCATTTCCCTAATGATGCCAGGGACTCGTGTTGTTTTAAAAATCGT
TCGTGAAGGGAAAACAATCGAGATACCTGTGACGGTTACACAGATCCCAACAGAGGATGGCGTTTCAGCGT
TGCAGAAGATGGGAGTCCGTGTTCAGAACATTACTCCAGAAATTTGTAAGAAACTCGGATTGGCAGCAGAT
ACCCGAGGGATTCTGGTAGTTGCTGTGGAGGCAGGCTCGCCTGCAGCTTCTGCAGGCGTCGCTCCTGGACA
GCTTATCTTAGCGGTGAATAGGCAGCGAGTCGCTTCCGTTGAAGAGTTAAATCAGGTTTTGAAAAACTCGA
AAGGAGAGAATGTTCTCCTTATGGTTTCTCAAGGAGATGTGGTGCGATTCATCGTCTTGAAATCAGACGAG
TAG

SEQ ID NO: 3- CT733 nucleotide sequence

ATGTTAATAAACTTTACCTTTCGCAACTGTCTTTTGTTCCTTGTCACACTGTCTAGTGTCCCTGTTTTCTC
AGCACCTCAACCTCGCGGAACGCTTCCTAGCTCGACCACAAAAATTGGATCAGAAGTTTGGATTGAACAAA
AAGTCCGCCAATATCCAGAGCTTTTATGGTTAGTAGAGCCGTCCTCTACGGGAGCCTCTTTAAAATCTCCT
TCAGGAGCCATCTTTTCTCCAACATTATTCCAAAAAAAGGTCCCTGCTTTCGATATCGCAGTGCGCAGTTT
GATTCACTTACATTTATTAATCCAGGGTTCCCGCCAAGCCTATGCTCAACTGATCCAACTACAGACCAGCG
AATCCCCTCTAACATTTAAGCAATTCCTTGCATTGCATAAGCAATTAACTCTATTTTTAAATTCCCCTAAG
GAATTTTATGACTCTGTTAAAGTGTTAGAGACAGCTATCGTCTTACGTCACTTAGGCTGTTCAACTAAGGC
TGTTGCTGCGTTTAAACCTTATTTCTCAGAAATGCAAAGAGAGGCTTTTTACACTAAGGCTCTGCATGTAC
TACACACCTTCCCAGAGCTAAGCCCATCATTTGCTCGCCTCTCTCCGGAGCAGAAAACTCTCTTCTTCTCC
TTGAGAAAATTGGCGAATTACGATGAGTTACTCTCGCTGACGAACACCCCAAGTTTTCAGCTTCTGTCTGC
TGGGCGCTCGCAACGAGCTCTTTTAGCTCTGGACTTGTACCTCTATGCTTTGGATTCCTGTGGAGAACAGG
GGATGTCCTCTCAATTCCACACAAACTTCGCACCTCTACAGTCCATGTTGCAACAATACGCTACTGTAGAA
GAGGCCTTTTCTCGTTATTTTACTTACCGAGCTAATCGATTAGGATTTGATGGCTCTTCTCGATCCGAGAT
GGCTTTAGTAAGAATGGCCACCTTGATGAACTTGTCTCCTTCCGAAGCTGCGATTTTAACCACAAGCTTCA
AAACCCTTCCTACAGAAGAAGCGGATACTTTGATCAATAGTTTCTATACCAATAAGGGCGATTCGTTGGCT
CTTTCTCTGCGAGGGTTGCCTACACTTGTATCCGAACTGACGCGAACTGCCCATGGCAATACCAATGCAGA
AGCTCGATCTCAGCAAATTTATGCAACTACCCTATCGCTAGTAGTAAAGAGTCTGAAAGCGCACAAAGAAA
TGCTAAACAAGCAAATTCTTTCTAAGGAAATTGTTTTAGATTTCTCAGAAACTGCAGCTTCTTGCCAAGGA
TTGGATATCTTTTCCGAGAATGTCGCTGTTCAAATTCACTTAAATGGAACCGTTAGTATCCATTTATAA

SEQ ID NO: 4 - CT733 protein sequence

MLINFTFRNCLLFLVTLSSVPVFSAPQPRGTLPSSTTKIGSEVWIEQKVRQYPELLWLVEPSSTGASLKSP
SGAIFSPTLFQKKVPAFDIAVRSLIHLHLLIQGSRQAYAQLIQLQTSESPLTFKQFLALHKQLTLFLNSPK
EFYDSVKVLETAIVLRHLGCSTKAVAAFKPYFSEMQREAFYTKALHVLHTFPELSPSFARLSPEQKTLFFS
LRKLANYDELLSLTNTPSFQLLSAGRSQRALLALDLYLYALDSCGEQGMSSQFHTNFAPLQSMLQQYATVE
EAFSRYFTYRANRLGFDGSSRSEMALVRMATLMNLSPSEAAILTTSFKTLPTEEADTLINSFYTNKGDSLA
LSLRGLPTLVSELTRTAHGNTNAEARSQQIYATTLSLVVKSLKAHKEMLNKQILSKEIVLDFSETAASCQG
LDIFSENVAVQIHLNGTVSIHL

SEQ ID NO:5 – CT153 nucleotide sequence

ATGACTAAGCCTTCTTTCTTATACGTTATTCAACCTTTTTCCGTATTTAATCCACGATTAGGACGTTTCTC
TACAGACTCAGATACTTATATCGAAGAAGAAAACCGCCTAGCATCGTTCATTGAGAGTTTGCCACTGGAGA
TCTTCGATATACCTTCTTTCATGGAAACCGCGATTTCCAATAGCCCCTATATTTTATCTTGGGAGACAACT
AAAGACGGCGCTCTGTTCACTATTCTTGAACCCAAACTCTCAGCTTGCGCAGCCACTTGCCTGGTAGCCCC
TTCTATACAAATGAAATCCGATGCGGAGCTCCTAGAAGAAATTAAGCAAGCGTTATTACGCAGCTCTCATG
ACGGTGTGAAATATCGCATCACCAGAGAATCCTTCTCTCCAGAAAAGAAAACTCCTAAGGTTGCTCTAGTC
GATGACGATATTGAATTGATTCGCAATGTCGACTTTTTGGGTAGAGCTGTTGACATTGTCAAATTAGACCC
TATTAATATTCTGAATACCGTAAGCGAAGAGAATATTCTAGATTACTCTTTTACAAGAGAAACGGCTCAGC
TGAGCGCGGATGGTCGTTTTGGTATTCCTCCAGGGACTAAGCTATTCCCTAAACCTTCTTTTGATGTAGAA
ATCAGTACCTCCATTTTCGAAGAAACAACTTCATTTACTCGAAGTTTTTCTGCATCGGTTACTTTTAGTGT
ACCAGACCTCGCGGCGACTATGCCTCTTCAAAGCCCTCCCATGGTAGAAAATGGTCAAAAAGAAATTTGTG
TCATTCAAAAACACTTATTCCCAAGCTACTCTCCTAAACTAGTCGATATTGTTAAACGATACAAAAGAGAG
GCTAAGATCTTGATTAACAAGCTTGCCTTTGGAATGTTATGGCGACATCGGGCTAAAAGCCAAATCCTCAC
CGAGGGAAGCGTACGTCTAGACTTACAAGGATTCACAGAATCGAAGTACAATTACCAGATTCAAGTAGGAT
CCCATACGATTGCAGCTGTATTAATCGATATGGATATTTCCAAGATTCAATCCAAATCAGAACAAGCTTAT
GCAATTAGGAAAATCAAATCAGGCTTTCAACGTAGCTTGGATGACTATCATATTTATCAAATTGAAAGAAA
ACAAACCTTTTCTTTTTCTCCGAAGCATCGCAGCCTCTCATCCACATCCCATTCCGAAGATTCTGATTTGG
ATCTTTCTGAAGCAGCCGCCTTTTCAGGAAGTCTTACCTGCGAGTTTGTAAAAAAAAGCACTCAACATGCC
AAGAATACCGTCACATGTTCCACAGCCGCTCATTCCCTATACACACTCAAAGAAGATGACAGCTCGAACCC
CTCTGAAAAACGATTAGATAGTTGTTTCCGCAATTGGATTGAAAACAAACTAAGCGCCAATTCTCCAGATT
CCTGGTCAGCGTTTATTCAAAAATTCGGAACACACTATATTGCATCAGCAACTTTTGGAGGGATAGGTTTC
CAAGTGCTCAAACTATCTTTTGAACAGGTGGAGGATCTACATAGCAAAAAGATCTCCTTAGAAACCGCAGC
AGCCAACTCTCTATTAAAAGGTTCTGTATCCAGCAGCACAGAATCTGGATACTCCAGCTATAGCTCCACGT
CTTCTTCTCATACGGTATTTTTAGGAGGAACGGTCTTACCTTCGGTTCATGATGAACGTTTAGACTTTAAA
GATTGGTCGGAAAGTGTGCACCTGGAACCTGTTCCTATCCAGGTTTCTTTACAACCTATAACGAATTTACT
AGTTCCTCTCCATTTTCCTAATATCGGTGCTGCAGAGCTCTCTAATAAACGAGAATCTCTTCAACAAGCGA
TTCGAGTCTATCTCAAAGAACATAAAGTAGATGAGCAAGGAGAACGTACTACATTTACATCAGGAATCGAT
AATCCTTCTTCCTGGTTTACCTTAGAAGCTGCCCACTCTCCTCTTATAGTCAGTACTCCTTACATTGCTTC
GTGGTCTACGCTTCCTTATTTGTTCCCAACATTAAGAGAACGTTCTTCGGCAACCCCTATCGTTTTCTATT
TTTGTGTAGATAATAATGAACATGCTTCGCAAAAAATATTAAACCAATCGTATTGCTTCCTCGGGTCCTTG
CCTATTCGACAAAAAATTTTTGGTAGCGAATTTGCTAGTTTCCCCTATCTATCTTTCTATGGAAATGCAAA
AGAGGCGTACTTTGATAACACGTACTACCCAACGCGTTGTGGGTGGATTGTTGAAAAGTTAAATACTACAC
AAGATCAATTCCTCCGGGATGGAGACGAGGTGCGACTAAAACATGTTTCCAGCGGAAAGTATCTAGCAACA
ACTCCTCTTAAGGATACCCATGGTACACTCACGCGTACAACGAACTGTGAAGATGCTATCTTTATTATTAA
AAAATCTTCAGGTTATTGA

SEQ ID NO:6 - CT153 protein sequence

MTKPSFLYVIQPFSVFNPRLGRFSTDSDTYIEEENRLASFIESLPLEIFDIPSFMETAISNSPYILSWETT
KDGALFTILEPKLSACAATCLVAPSIQMKSDAELLEEIKQALLRSSHDGVKYRITRESFSPEKKTPKVALV
DDDIELIRNVDFLGRAVDIVKLDPINILNTVSEENILDYSFTRETAQLSADGRFGIPPGTKLFPKPSFDVE
ISTSIFEETTSFTRSFSASVTFSVPDLAATMPLQSPPMVENGQKEICVIQKHLFPSYSPKLVDIVKRYKRE
AKILINKLAFGMLWRHRAKSQILTEGSVRLDLQGFTESKYNYQIQVGSHTIAAVLIDMDISKIQSKSEQAY
AIRKIKSGFQRSLDDYHIYQIERKQTFSFSPKHRSLSSTSHSEDSDLDLSEAAAFSGSLTCEFVKKSTQHA
KNTVTCSTAAHSLYTLKEDDSSNPSEKRLDSCFRNWIENKLSANSPDSWSAFIQKFGTHYIASATFGGIGF
QVLKLSFEQVEDLHSKKISLETAAANSLLKGSVSSSTESGYSSYSSTSSSHTVFLGGTVLPSVHDERLDFK
DWSESVHLEPVPIQVSLQPITNLLVPLHFPNIGAAELSNKRESLQQAIRVYLKEHKVDEQGERTTFTSGID
NPSSWFTLEAAHSPLIVSTPYIASWSTLPYLFPTLRERSSATPIVFYFCVDNNEHASQKILNQSYCFLGSL

PIRQKIFGSEFASFPYLSFYGNAKEAYFDNTYYPTRCGWIVEKLNTTQDQFLRDGDEVRLKHVSSGKYLAT
TPLKDTHGTLTRTTNCEDAIFIIKKSSGY

SEQ ID NO:7 - CT601 nucleotide sequence

ATGCTCGCTAATCGCTTATTCTTAATAACCCTTTTAGGGTTAAGTTCGTCTGTTTACGGCGCAGGTAAAGC
ACCGTCTTTGCAGGCTATTCTAGCCGAAGTCGAAGACACCTCCTCTCGTCTACACGCTCATCACAATGAGC
TTGCTATGATCTCTGAACGCCTCGATGAGCAAGACACGAAACTACAGCAACTTTCGTCAACACAAGATCAT
AACCTACCTCGACAAGTTCAGCGACTAGAAACGGACCAAAAAGCTTTGGCAAAAACACTGGCGATTCTTTC
GCAATCCGTCCAAGATATTCGGTCTTCTGTACAAAATAAATTACAAGAAATCCAACAAGAACAAAAAAAAT
TAGCACAAAATTTGCGAGCGCTTCGTAACTCTTTACAAGCTCTCGTTGATGGCTCTTCTCCAGAAAATTAT
ATTGATTTCCTAACTGGTGAAACCCCGGAACATATTCATATTGTTAAACAAGGAGAGACCCTGAGCAAGAT
CGCGAGTAAATATAACATCCCCGTCGTAGAATTAAAAAAACTTAATAAACTAAATTCGGATACTATTTTTA
CAGATCAAAGAATTCGCCTTCCGAAAAAGAAATAG

SEQ ID NO:8 - CT601 protein sequence

MLANRLFLITLLGLSSSVYGAGKAPSLQAILAEVEDTSSRLHAHHNELAMISERLDEQDTKLQQLSSTQDH
NLPRQVQRLETDQKALAKTLAILSQSVQDIRSSVQNKLQEIQQEQKKLAQNLRALRNSLQALVDGSSPENY
IDFLTGETPEHIHIVKQGETLSKIASKYNIPVVELKKLNKLNSDTIFTDQRIRLPKKK

SEQ ID NO:9 - CT279 nucleotide sequence

ATGGCATCCAAGTCTCGCCATTATCTTAATCAGCCTTGGTACATTATCTTATTCATCTTTGTTCTTAGTTT
AATTGCTGGTACCCTCCTGTCTTCTGTGTATTATGTCCTTGCACCTATCCAACAGCAAGCTGCGGAATTCG
ATCGCAATCAACAAATGCTAATGGCTGCACAAGTAATTTCTTCCGATAACACATTCCAAGTCTATGAAAAG
GGAGATTGGCACCCAGCCCTATATAATACTAAAAAGCAGTTGCTAGAGATCTCCTCTACTCCTCCTAAAGT
AACCGTGACAACTTTAAGCTCATATTTTCAAAACTTTGTTAGAGTCTTGCTTACAGATACACAAGGAAATC
TTTCTTCATTCGAAGACCATAATCTCAATCTAGAAGAATTTTTATCTCAACCAACTCCTGTAATACATGGT
CTTGCCCTTTATGTGGTCTACGCTATCCTACACAACGATGCAGCTTCCTCTAAATTATCTGCTTCCCAAGT
AGCGAAAAATCCAACAGCTATAGAATCTATAGTTCTTCCTATAGAAGGTTTTGGTTTGTGGGGACCTATCT
ATGGATTCCTTGCTCTAGAAAAAGACGGGAATACTGTTCTTGGTACTTCTTGGTATCAACATGGCGAGACT
CCTGGATTAGGAGCAAATATCGCTAACCCTCAATGGCAAAAAAATTTCAGAGGCAAAAAAGTATTTCTAGT
CTCAGCTTCTGGAGAAACAGATTTTGCTAAGACAACCCTAGGACTGGAAGTTATAAAAGGATCTGTATCTG
CAGCATTAGGAGACTCACCTAAAGCTGCTTCTTCCATCGACGGAATTTCAGGAGCTACTTTGACTTGTAAT
GGTGTTACCGAATCCTTCTCTCATTCTCTAGCTCCCTACCGCGCTTTGTTGACTTTCTTCGCCAACTCTAA
ACCTAGTGGAGAGTCTCATGACCACTAA

SEQ ID NO:10 - CT279 protein sequence

MASKSRHYLNQPWYIILFIFVLSLIAGTLLSSVYYVLAPIQQQAAEFDRNQQMLMAAQVISSDNTFQVYEK
GDWHPALYNTKKQLLEISSTPPKVTVTTLSSYFQNFVRVLLTDTQGNLSSFEDHNLNLEEFLSQPTPVIHG
LALYVVYAILHNDAASSKLSASQVAKNPTAIESIVLPIEGFGLWGPIYGFLALEKDGNTVLGTSWYQHGET
PGLGANIANPQWQKNFRGKKVFLVSASGETDFAKTTLGLEVIKGSVSAALGDSPKAASSIDGISGATLTCN
GVTESFSHSLAPYRALLTFFANSKPSGESHDH

SEQ ID NO:11 - CT443 nucleotide sequence

ATGCGAATAGGAGATCCTATGAACAAACTCATCAGACGAGCAGTGACGATCTTCGCGGTGACTAGTGTGGC
GAGTTTATTTGCTAGCGGGGTGTTAGAGACCTCTATGGCAGAGTCTCTCTCTACAAACGTTATTAGCTTAG
CTGACACCAAAGCGAAAGACAACACTTCTCATAAAAGCAAAAAAGCAAGAAAAAACCACAGCAAAGAGACT
CCCGTAGACCGTAAAGAGGTTGCTCCGGTTCATGAGTCTAAAGCTACAGGACCTAAACAGGATTCTTGCTT
TGGCAGAATGTATACAGTCAAAGTTAATGATGATCGCAATGTTGAAATCACACAAGCTGTTCCTGAATATG
CTACGGTAGGATCTCCCTATCCTATTGAAATTACTGCTACAGGTAAAAGGGATTGTGTTGATGTTATCATT
ACTCAGCAATTACCATGTGAAGCAGAGTTCGTACGCAGTGATCCAGCGACAACTCCTACTGCTGATGGTAA
GCTAGTTTGGAAAATTGACCGCTTAGGACAAGGCGAAAAGAGTAAAATTACTGTATGGGTAAAACCTCTTA
AAGAAGGTTGCTGCTTTACAGCTGCAACAGTATGCGCTTGTCCAGAGATCCGTTCGGTTACAAAATGTGGA
CAACCTGCTATCTGTGTTAAACAAGAAGGCCCAGAGAATGCTTGTTTGCGTTGCCCAGTAGTTTACAAAAT
TAATATAGTGAACCAAGGAACAGCAACAGCTCGTAACGTTGTTGTTGAAAATCCTGTTCCAGATGGTTACG

CTCATTCTTCTGGACAGCGTGTACTGACGTTTACTCTTGGAGATATGCAACCTGGAGAGCACAGAACAATT
ACTGTAGAGTTTTGTCCGCTTAAACGTGGTCGTGCTACCAATATAGCAACGGTTTCTTACTGTGGAGGACA
TAAAAATACAGCAAGCGTAACAACTGTGATCAACGAGCCTTGCGTACAAGTAAGTATTGCAGGAGCAGATT
GGTCTTATGTTTGTAAGCCTGTAGAATATGTGATCTCCGTTTCCAATCCTGGAGATCTTGTGTTGCGAGAT
GTCGTCGTTGAAGACACTCTTTCTCCCGGAGTCACAGTTCTTGAAGCTGCAGGAGCTCAAATTTCTTGTAA
TAAAGTAGTTTGGACTGTGAAAGAACTGAATCCTGGAGAGTCTCTACAGTATAAAGTTCTAGTAAGAGCAC
AAACTCCTGGACAATTCACAAATAATGTTGTTGTGAAGAGCTGCTCTGACTGTGGTACTTGTACTTCTTGC
GCAGAAGCGACAACTTACTGGAAAGGAGTTGCTGCTACTCATATGTGCGTAGTAGATACTTGTGACCCTGT
TTGTGTAGGAGAAAATACTGTTTACCGTATTTGTGTCACCAACAGAGGTTCTGCAGAAGATACAAATGTTT
CTTTAATGCTTAAATTCTCTAAAGAACTGCAACCTGTATCCTTCTCTGGACCAACTAAAGGAACGATTACA
GGCAATACAGTAGTATTCGATTCGTTACCTAGATTAGGTTCTAAAGAAACTGTAGAGTTTTCTGTAACATT
GAAAGCAGTATCAGCTGGAGATGCTCGTGGGGAAGCGATTCTTTCTTCCGATACATTGACTGTTCCAGTTT
CTGATACAGAGAATACACACATCTATTAA

SEQ ID NO:12 - CT443 protein sequence

MRIGDPMNKLIRRAVTIFAVTSVASLFASGVLETSMAESLSTNVISLADTKAKDNTSHKSKKARKNHSKET
PVDRKEVAPVHESKATGPKQDSCFGRMYTVKVNDDRNVEITQAVPEYATVGSPYPIEITATGKRDCVDVII
TQQLPCEAEFVRSDPATTPTADGKLVWKIDRLGQGEKSKITVWVKPLKEGCCFTAATVCACPEIRSVTKCG
QPAICVKQEGPENACLRCPVVYKINIVNQGTATARNVVVENPVPDGYAHSSGQRVLTFTLGDMQPGEHRTI
TVEFCPLKRGRATNIATVSYCGGHKNTASVTTVINEPCVQVSIAGADWSYVCKPVEYVISVSNPGDLVLRD
VVVEDTLSPGVTVLEAAGAQISCNKVVWTVKELNPGESLQYKVLVRAQTPGQFTNNVVVKSCSDCGTCTSC
AEATTYWKGVAATHMCVVDTCDPVCVGENTVYRICVTNRGSAEDTNVSLMLKFSKELQPVSFSGPTKGTIT
GNTVVFDSLPRLGSKETVEFSVTLKAVSAGDARGEAILSSDTLTVPVSDTENTHIY

SEQ ID NO:13 - CT372 nucleotide sequence

ATGCAGGCTGCACACCATCACTATCACCGCTACACAGATAAACTGCACAGACAAAACCATAAAAAAGATCT
CATCTCTCCCAAACCTACCGAACAAGAGGCGTGCAATACTTCTTCCCTTAGTAAGGAATTAATCCCTCTAT
CAGAACAAAGAGGCCTTTTATCCCCCATCTGTGACTTTATTTCGGAACGCCCTTGCTTACACGGAGTTTCT
GTTAGAAATCTCAAGCAAGCGCTAAAAAATTCTGCAGGAACCCAAATTGCACTGGATTGGTCTATTCTCCC
TCAATGGTTCAATCCTCGGGTCTCTCATGCCCCTAAGCTTTCTATCCGAGACTTTGGGTATAGCGCACACC
AAACTGTTACCGAAGCCACTCCTCCTTGCTGGCAAAACTGCTTTAATCCATCTGCGGCCGTTACTATCTAT
GATTCCTCATATGGGAAAGGGGTCTTTCAAATATCCTATACCCTTGTCCGCTATTGGAGAGAGAATGCTGC
GACTGCTGGCGATGCTATGATGCTCGCAGGGAGTATCAATGATTATCCCTCTCGTCAGAACATTTTCTCTC
AGTTTACTTTCTCCCAAAACTTCCCAAATGAACGGGTGAGTCTGACAATTGGTCAGTACTCACTCTATGCA
ATAGACGGAACATTATACAATAACGATCAACAACTTGGATTCATTAGTTACGCATTATCACAAAATCCAAC
AGCAACTTATTCCTCTGGAAGTCTTGGAGCTTACCTACAAGTCGCTCCTACCGCAAGCACAAGTCTTCAAA
TAGGATTTCAAGACGCTTATAATATCTCCGGATCCTCTATCAAATGGAGTAACCTTACAAAAAATAGATAC
AATTTTCACGGTTTTGCTTCCTGGGCTCCCCGCTGTTGCTTAGGATCTGGCCAGTACTCCGTGCTTCTTTA
TGTGACTAGACAAGTTCCAGAACAGATGGAACAAACAATGGGATGGTCAGTCAATGCGAGTCAACACATAT
CTTCTAAACTGTATGTGTTTGGAAGATACAGCGGTGTTACAGGACATGTGTTCCCGATTAACCGCACGTAT
TCATTCGGTATGGCCTCTGCAAATTTATTTAACCGTAACCCACAAGATTTATTTGGAATTGCTTGCGCATT
CAATAATGTACACCTCTCTGCTTCTCCAAATACTAAAAGAAAATACGAAACTGTAATCGAAGGGTTTGCAA
CTATCGGTTGCGGCCCCTATCTTTCTTTCGCTCCAGACTTCCAACTCTACCTCTACCCAGCTCTTCGTCCA
AACAAACAATCTGCCCGTGTTTATAGCGTGCGAGCTAATTTAGCTATCTAA

SEQ ID NO:14 - CT372 protein sequence

MQAAHHHYHRYTDKLHRQNHKKDLISPKPTEQEACNTSSLSKELIPLSEQRGLLSPICDFISERPCLHGVS
VRNLKQALKNSAGTQIALDWSILPQWFNPRVSHAPKLSIRDFGYSAHQTVTEATPPCWQNCFNPSAAVTIY
DSSYGKGVFQISYTLVRYWRENAATAGDAMMLAGSINDYPSRQNIFSQFTFSQNFPNERVSLTIGQYSLYA
IDGTLYNNDQQLGFISYALSQNPTATYSSGSLGAYLQVAPTASTSLQIGFQDAYNISGSSIKWSNLTKNRY
NFHGFASWAPRCCLGSGQYSVLLYVTRQVPEQMEQTMGWSVNASQHISSKLYVFGRYSGVTGHVFPINRTY
SFGMASANLFNRNPQDLFGIACAFNNVHLSASPNTKRKYETVIEGFATIGCGPYLSFAPDFQLYLYPALRP
NKQSARVYSVRANLAI

SEQ ID NO:15: CT043 nucleotide sequence

ATGTCCAGGCAGAATGCTGAGGAAAATCTAAAAAATTTTGCTAAAGAGCTTAAACTCCCCGACGTGGCCTT
CGATCAGAATAATACGTGCATTTTGTTTGTTGATGGAGAGTTTTCTCTTCACCTGACCTACGAAGAACACT
CTGATCGCCTTTATGTTTACGCACCTCTTCTTGACGGACTGCCAGACAATCCGCAAAGAAGGTTAGCTCTA
TATGAGAAGTTGTTAGAAGGCTCTATGCTCGGAGGCCAAATGGCTGGTGGAGGGGTAGGAGTCGCTACTAA
GGAACAGTTGATCTTAATGCACTGCGTGTTAGACATGAAGTATGCAGAGACCAACCTACTCAAAGCTTTTG
CACAGCTTTTTATTGAAACCGTTGTGAAATGGCGAACTGTTTGTTCTGATATCAGCGCTGGACGAGAACCC
ACTGTTGATACCATGCCACAAATGCCTCAAGGGGGTGGCGGAGGAATTCAACCTCCTCCAGCAGGAATCCG
TGCATAA

SEQ ID NO:16: CT043 protein sequence

MSRQNAEENLKNFAKELKLPDVAFDQNNTCILFVDGEFSLHLTYEEHSDRLYVYAPLLDGLPDNPQRRLAL
YEKLLEGSMLGGQMAGGGVGVATKEQLILMHCVLDMKYAETNLLKAFAQLFIETVVKWRTVCSDISAGREP
TVDTMPQMPQGGGGGIQPPPAGIRA

SEQ ID NO:17 – CT681 protein sequence

MKKLLKSVLVFAALSSASSLQALPVGNPAEPSLMIDGILWEGFGGDPCDPCATWCDAISMRVGYYGDFVFD
RVLKTDVNKEFQMGAKPTTDTGNSAAPSTLTARENPAYGRHMQDAEMFTNAACMALNIWDRFDVFCTLGAT
SGYLKGNSASFNLVGLFGDNENQKTVKAESVPNMSFDQSVVELYTDTTFAWSVGARAALWECGCATLGASF
QYAQSKPKVEELNVLCNAAEFTINKPKGYVGKEFPLDLTAGTDAATGTKDASIDYHEWQASLALSYRLNMF
TPYIGVKWSRASFDADTIRIAQPKSATAIFDTTTLNPTIAGAGDVKTGAEGQLGDTMQIVSLQLNKMKSRK
SCGIAVGTTIVDADKYAVTVETRLIDERAAHVNAQFRF

SEQ ID NO:18 – CT681 nucleotide sequence

ATGAAAAAACTCTTGAAATCGGTATTAGTATTTGCCGCTTTGAGTTCTGCTTCCTCCTTGCAAGCTCTGCC
TGTGGGGAATCCTGCTGAACCAAGCCTTATGATCGACGGAATTCTGTGGGAAGGTTTCGGCGGAGATCCTT
GCGATCCTTGCGCCACTTGGTGTGACGCTATCAGCATGCGTGTTGGTTACTACGGAGACTTTGTTTTCGAC
CGTGTTTTGAAAACAGATGTGAATAAAGAATTTCAGATGGGTGCCAAGCCTACAACTGATACAGGCAATAG
TGCAGCTCCATCCACTCTTACAGCAAGAGAGAATCCTGCTTACGGCCGACATATGCAGGATGCTGAGATGT
TTACAAATGCCGCTTGCATGGCATTGAATATTTGGGATCGTTTTGATGTATTCTGTACATTAGGAGCCACC
AGTGGATATCTTAAAGGAAACTCTGCTTCTTTCAATTTAGTTGGATTGTTTGGAGATAATGAAAATCAAAA
AACGGTCAAAGCGGAGTCTGTACCAAATATGAGCTTTGATCAATCTGTTGTTGAGTTGTATACAGATACTA
CTTTTGCGTGGAGCGTCGGCGCTCGCGCAGCTTTGTGGGAATGTGGATGTGCAACTTTAGGAGCTTCATTC
CAATATGCTCAATCTAAACCTAAAGTAGAAGAATTAAACGTTCTCTGCAATGCAGCAGAGTTTACTATTAA
TAAACCTAAAGGGTATGTAGGTAAGGAGTTTCCTCTTGATCTTACAGCAGGAACAGATGCTGCGACAGGAA
CTAAGGATGCCTCTATTGATTACCATGAATGGCAAGCAAGTTTAGCTCTCTCTTACAGACTGAATATGTTC
ACTCCCTACATTGGAGTTAAATGGTCTCGAGCAAGCTTTGATGCCGATACGATTCGTATAGCCCAGCCAAA
ATCAGCTACAGCTATTTTTGATACTACCACGCTTAACCCAACTATTGCTGGAGCTGGCGATGTGAAAACTG
GCGCAGAGGGTCAGCTCGGAGACACAATGCAAATCGTTTCCTTGCAATTGAACAAGATGAAATCTAGAAAA
TCTTGCGGTATTGCAGTAGGAACAACTATTGTGGATGCAGACAAATACGCAGTTACAGTTGAGACTCGCTT
GATCGATGAGAGAGCAGC

SEQ ID NO:19 – TC0210 protein sequence

MMKRLLCVLLSTSVFSSPMLGYSAPKKDSSTGICLAASQSDRELSQEDLLKEVSRGFSKVAAQATPGVVYI
ENFPKTGSQAIASPGNKRGFQENPFDYFNDEFFNRFFGLPSHREQPRPQQRDAVRGTGFIVSEDGYVVTNH
HVVEDAGKIHVTLHDGQKYTAKIIGLDPKTDLAVIKIQAKNLPFLTFGNSDQLQIGDWSIAIGNPFGLQAT
VTVGVISAKGRNQLHIVDFEDFIQTDAAINPGNSGGPLLNIDGQVIGVNTAIVSGSGGYIGIGFAIPSLMA
KRVIDQLISDGQVTRGFLGVTLQPIDSELAACYKLEKVYGALITDVVKGSPAEKAGLRQEDVIVAYNGKEV
ESLSALRNAISLMMPGTRVVLKVVREGKFIEIPVTVTQIPAEDGVSALQKMGVRVQNLTPEICKKLGLASD
TRGIFVVSVEAGSPAASAGVVPGQLILAVNRQRVSSVEELNQVLKNAKGENVLLMVSQGEVIRFVVLKSDE

SEQ ID NO:20 – TC0052 protein sequence

MKKLLKSVLAFAVLGSASSLHALPVGNPAEPSLMIDGILWEGFGGDPCDPCTTWCDAISLRLGYYGDFVFD
RVLKTDVNKQFEMGAAPTGDADLTTAPTPASRENPAYGKHMQDAEMFTNAAYMALNIWDRFDVFCTLGATS
GYLKGNSAAFNLVGLFGRDETAVAADDIPNVSLSQAVVELYTDTAFAWSVGARAALWECGCATLGASFQYA
QSKPKVEELNVLCNAAEFTINKPKGYVGQEFPLNIKAGTVSATDTKDASIDYHEWQASLALSYRLNMFTPY
IGVKWSRASFDADTIRIAQPKLETSILKMTTWNPTISGSGIDVDTKITDTLQIVSLQLNKMKSRKSCGLAI
GTTIVDADKYAVTVETRLIDERAAHVNAQFRF

SEQ ID NO:21 – TC0052 nucleotide sequence

ATGAAAAAACTCTTGAAATCGGTATTAGCATTTGCCGTTTTGGGTTCTGCTTCCTCCTTGCATGCTCTGCC
TGTGGGGAATCCTGCTGAACCAAGCCTTATGATTGACGGGATTCTTTGGGAAGGTTTCGGTGGAGATCCTT
GCGATCCTTGCACAACTTGGTGTGATGCCATCAGCCTACGTCTCGGCTACTATGGGGACTTCGTTTTTGAT
CGTGTTTTGAAAACAGACGTGAACAAACAGTTCGAAATGGGAGCAGCTCCTACAGGAGATGCAGACCTTAC
TACAGCACCTACTCCTGCATCAAGAGAGAATCCCGCTTATGGCAAGCATATGCAAGATGCAGAAATGTTCA
CTAATGCTGCGTACATGGCTTTAAACATTTGGGACCGTTTCGATGTATTTTGTACATTGGGAGCAACTAGC
GGATATCTTAAAGGTAATTCTGCCGCCTTTAACTTAGTTGGTCTGTTTGGAAGAGATGAAACTGCAGTTGC
AGCTGACGACATACCTAACGTCAGCTTGTCTCAAGCTGTTGTCGAACTCTACACAGACACAGCTTTCGCTT
GGAGCGTCGGTGCTAGAGCAGCTTTATGGGAGTGCGGATGTGCAACTTTAGGAGCTTCCTTCCAATATGCT
CAATCTAAGCCAAAAGTAGAGGAATTAAACGTTCTCTGTAATGCGGCAGAATTCACTATTAACAAGCCTAA
AGGATACGTTGGACAAGAGTTTCCTCTTAACATTAAAGCTGGAACAGTTAGCGCTACAGATACTAAAGATG
CTTCCATCGATTACCATGAGTGGCAAGCAAGCTTGGCTTTGTCTTACAGACTGAATATGTTCACTCCTTAC
ATTGGAGTTAAGTGGTCTAGAGCAAGCTTTGATGCCGACACTATCCGCATTGCGCAGCCTAAGCTTGAGAC
CTCTATCTTAAAAATGACCACTTGGAACCCAACGATCTCTGGATCTGGTATAGACGTTGATACAAAAATCA
CGGATACATTACAAATTGTTTCCTTGCAGCTCAACAAGATGAAATCCAGAAAATCTTGCGGTCTTGCAATT
GGAACAACAATTGTAGATGCTGATAAATATGCAGTTACTGTTGAGACACGCTTGATCGATGAAAGAGCAGC
TCACGTAAATGCTCAGTTCCGTTTCTAA

SEQ ID NO:22 – TC0106 protein sequence

MLTNFTFRNCLLFFVTLSSVPVFSAPQPRVTLPSGANKIGSEAWIEQKVRQYPELLWLVEPSPAGTSLNAP
SGMIFSPLLFQKKVPAFDIAVRSLIHLHLLIQGSRQAYAQLVQLQANESPMTFKQFLTLHKQLSLFLNSPK
EFYDSVKILETAIILRHLGCSTKAVATFKPYFSETQKEVFYTKALHVLHTFPELSPSFARLSPEQKTLFFS
LRKLANYDELLSLTNAPSLQLLSAVRSRRALLALDLYLYALDFCGEQGISSQFHMDFSPLQSMLQQYATVE
EAFSRYFTYRANRLGFAGSSRTEMALVRIATLMNLSPSEAAILTTSFKSLSLEDAESLVNSFYTNKGDSLA
LSLRGLPTLISELTRAAHGNTNAEARAQQIYATTLSLVAKSLKAHKEMQNKQILPEEVVLDFSETASSCQG
LDIFSENVAVQIHLNGSVSIHL

SEQ ID NO:23: CM homolog of CT601 = TC_0551

ATGGCATCCAAGTCTCGTCATTATCTTAACCAGCCTTGGTACATTATCTTATTCATCTTTGTTCTTAGTCT
GGTTGCTGGTACCCTTCTTTCTTCAGTTTCCTATGTTCTATCTCCAATCCAAAAACAAGCTGCAGAATTTG
ATCGTAATCAGCAAATGTTGATGGCCGCACAAATTATTTCCTATGACAATAAATTCCAAATATATGCTGAA
GGGGATTGGCAACCTGCTGTCTATAATACAAAAAAACAGATACTAGAAAAAAGCTCTTCCACTCCACCACA
AGTGACTGTGGCGACTCTATGCTCTTATTTTCAAAATTTTGTTAGAGTTTTGCTTACAGACTCCCAAGGGA
ATCTTTCTTCTTTTGAAGATCACAATCTTAACCTAGAAGAGTTCTTATCCCACCCCACATCTTCAGTACAA
GATCACTCTCTGCATGTAATTTATGCTATTCTAGCAAACGATGAATCCTCTAAAAAGTTATCATCCTCCCA
AGTAGCAAAAAATCCGGTATCCATAGAGTCTATTATTCTTCCTATAAAAGGATTTGGTTTATGGGGACCAA
TCTATGGATTTCTTGCTTTAGAAAAGGACGGTAATACGGTTCTAGGGACATGCTGGTATCAACATGGTGAG
ACTCCAGGATTAGGAGCAAATATAACTAATCCCCAATGGCAACAAAATTTCAGAGGAAAAAAAGTATTTCT
CGCTTCCTCTTCCGGAGAAACCGATTTTGCTAAAACAACTCTAGGACTAGAAGTTATAAAAGGATCTGTTT
CTGCATTATTAGGGGACTCTCCCAAAGCTAATTCCGCTGTTGATGGAATTTCAGGAGCTACACTGACCTGT
AATGGAGTTACTGAAGCTTTTGCTAATTCGCTAGCTCCTTACCGCCCCTTATTGACTTTCTTCGCCAATCT
TAACTCTAGTGGAGAATCTCATGACAACCAATAA

SEQ ID NO:24: CM homologue of CT601 protein sequence = TC_0551 protein sequence

MASKSRHYLNQPWYIILFIFVLSLVAGTLLSSVSYVLSPIQKQAAEFDRNQQMLMAAQIISYDNKFQIYAE
GDWQPAVYNTKKQILEKSSSTPPQVTVATLCSYFQNFVRVLLTDSQGNLSSFEDHNLNLEEFLSHPTSSVQ
DHSLHVIYAILANDESSKKLSSSQVAKNPVSIESIILPIKGFGLWGPIYGFLALEKDGNTVLGTCWYQHGE
TPGLGANITNPQWQQNFRGKKVFLASSSGETDFAKTTLGLEVIKGSVSALLGDSPKANSAVDGISGATLTC
NGVTEAFANSLAPYRPLLTFFANLNSSGESHDNQ

SEQ ID NO:25: CM homologue of CT372 = TC_0651 nucleotide sequence

ATGAATGGAAAAGTTCTGTGTGAGGTTTCTGTGTCCTTCCGTTCGATTCTGCTGACGGCTCTGCTTTCACT
TTCTTTTACAAACACTATGCAGGCTGCACACCATCATTATCACCGTTATGATGATAAACTACGCAGACAAT
ACCATAAAAAGGACTTGCCCACTCAAGAGAATGTTCGGAAAGAGTTTTGTAATCCCTACTCTCATAGTAGT
GATCCTATCCCTTTGTCACAACAACGAGGAGTCCTATCTCCTATCTGTGATTTAGTCTCAGAGTGCTCGTT
TTTGAACGGGATTTCCGTTAGGAGTCTTAAACAAACACTGAAAAATTCTGCTGGGACTCAAGTTGCTTTAG
ACTGGTCTATCCTTCCTCAATGGTTCAATCCTAGATCCTCTTGGGCTCCTAAGCTCTCTATTCGAGATCTT
GGATATGGTAAACCCCAGTCCCTTATTGAAGCAGATTCCCCTTGTTGTCAAACCTGCTTCAACCCATCTGC
TGCTATTACGATTTACGATTCTTCATGTGGGAAGGGTGTTGTCCAAGTGTCATACACCCTTGTTCGTTATT
GGAGAGAAACGGCTGCACTTGCAGGGCAAACTATGATGCTTGCAGGAAGTATTAATGATTATCCTGCTCGC
CAAAACATATTCTCTCAACTTACATTTTCCCAAACTTTCCCTAATGAGAGAGTAAATCTAACTGTTGGTCA
ATACTCTCTTTACTCGATAGACGGAACGCTGTACAACAATGATCAGCAGCTAGGATTTATTAGTTATGCGT
TGTCGCAAAATCCAACAGCGACTTATTCCTCTGGAAGCCTTGGCGCCTATCTACAAGTCGCTCCAACAGAA
AGCACCTGTCTTCAAGTTGGGTTCCAAGATGCCTATAATATTTCAGGTTCCTCGATCAAATGGAATAATCT
TACAAAAAATAAGTATAACTTCCATGGCTATGCATCTTGGGCTCCACACTGTTGCTTAGGACCTGGACAAT
ACTCTGTTCTTCTTTATGTAACCAGAAAGGTTCCTGAGCAAATGATGCAGACAATGGGCTGGTCTGTGAAT
GCAAGTCAATACATCTCTTCTAAACTTTATGTATTTGGAAGATACAGCGGAGTCACAGGCCAATTGTCTCC
TATTAACCGAACCTATTCATTTGGCTTAGTCTCTCCTAATTTATTGAACCGTAACCCACAAGACTTATTTG
GAGTAGCTTGCGCATTCAATAATATACACGCCTCCGCCTTTCAAAATGCTCAAAGAAAATATGAAACTGTG
ATCGAGGGATTTGCAACTATTGGTTGCGGACCTTACATCTCCTTTGCTCCAGATTTCCAACTTTACCTCTA
TCCTGCTCTGCGTCCAAATAAACAAAGCGCCCGAGTCTATAGCGTTCGCGCAAACCTAGCTATTTAG

SEQ ID NO:26: CM homologue of CT372 = TC_0651 protein sequence

MNGKVLCEVSVSFRSILLTALLSLSFTNTMQAAHHHYHRYDDKLRRQYHKKDLPTQENVRKEFCNPYSHSS
DPIPLSQQRGVLSPICDLVSECSFLNGISVRSLKQTLKNSAGTQVALDWSILPQWFNPRSSWAPKLSIRDL
GYGKPQSLIEADSPCCQTCFNPSAAITIYDSSCGKGVVQVSYTLVRYWRETAALAGQTMMLAGSINDYPAR
QNIFSQLTFSQTFPNERVNLTVGQYSLYSIDGTLYNNDQQLGFISYALSQNPTATYSSGSLGAYLQVAPTE
STCLQVGFQDAYNISGSSIKWNNLTKNKYNFHGYASWAPHCCLGPGQYSVLLYVTRKVPEQMMQTMGWSVN
ASQYISSKLYVFGRYSGVTGQLSPINRTYSFGLVSPNLLNRNPQDLFGVACAFNNIHASAFQNAQRKYETV
IEGFATIGCGPYISFAPDFQLYLYPALRPNKQSARVYSVRANLAI

SEQ ID NO:27: CM homologue of CT443 = TC_0727

ATGCGAATAGGAGATCCTATGAACAAACTCATCAGACGAGCTGTGACGATCTTCGCGGTGACTAGTGTGGC
GAGTTTATTTGCTAGCGGGGTGTTAGAGACCTCTATGGCAGAGTCTCTCTCTACCAACGTTATTAGCTTAG
CTGACACCAAAGCGAAAGAGACCACTTCTCATCAAAAAGACAGAAAAGCAAGAAAAAATCATCAAAATAGG
ACTTCCGTAGTCCGTAAAGAGGTTACTGCAGTTCGTGATACTAAAGCTGTAGAGCCTAGACAGGATTCTTG
CTTTGGCAAAATGTATACAGTCAAAGTTAATGATGATCGTAATGTAGAAATCGTGCAGTCCGTTCCTGAAT
ATGCTACGGTAGGATCTCCATATCCTATTGAGATTACTGCTATAGGGAAAAGAGACTGTGTTGATGTAATC
ATTACACAGCAATTACCATGCGAAGCAGAGTTTGTTAGCAGTGATCCAGCTACTACTCCTACTGCTGATGG
TAAGCTAGTTTGGAAAATTGATCGGTTAGGACAGGGCGAAAAGAGTAAAATTACTGTATGGGTAAAACCTC
TTAAAGAAGGTTGCTGCTTTACAGCTGCAACGGTTTGTGCTTGTCCAGAGATCCGTTCGGTTACGAAATGT
GGCCAGCCTGCTATCTGTGTTAAACAGGAAGGTCCAGAAAGCGCATGTTTGCGTTGCCCAGTAACTTATAG
AATTAATGTAGTCAACCAAGGAACAGCAACAGCACGTAATGTTGTTGTGGAAAATCCTGTTCCAGATGGCT
ATGCTCATGCATCCGGACAGCGTGTATTGACATATACTCTTGGGGATATGCAACCTGGAGAACAGAGAACA
ATCACCGTGGAGTTTTGTCCGCTTAAACGTGGTCGAGTCACAAATATTGCTACAGTTTCTTACTGTGGTGG

ACACAAAAATACTGCTAGCGTAACAACAGTGATCAATGAGCCTTGCGTGCAAGTTAACATCGAGGGAGCAG
ATTGGTCTTATGTTTGTAAGCCTGTAGAATATGTTATCTCTGTTTCTAACCCTGGTGACTTAGTTTTACGA
GACGTTGTAATTGAAGATACGCTTTCTCCTGGAATAACTGTTGTTGAAGCAGCTGGAGCTCAGATTTCTTG
TAATAAATTGGTTTGGACTTTGAAGGAACTCAATCCTGGAGAGTCTTTACAATATAAGGTTCTAGTAAGAG
CTCAAACTCCAGGGCAATTCACAAACAACGTTGTTGTGAAAAGTTGCTCTGATTGCGGTATTTGTACTTCT
TGCGCAGAAGCAACAACTTACTGGAAAGGAGTTGCTGCTACTCATATGTGCGTAGTAGATACTTGTGATCC
TATTTGCGTAGGAGAGAACACTGTTTATCGTATCTGTGTGACAAACAGAGGTTCTGCTGAAGATACAAATG
TGTCCTTAATTTTGAAATTCTCTAAAGAATTACAACCTATATCTTTCTCTGGACCAACTAAAGGAACCATT
ACAGGAAACACGGTAGTGTTTGATTCGTTACCTAGATTAGGTTCTAAAGAAACTGTAGAGTTTTCTGTAAC
GTTGAAAGCAGTATCCGCTGGAGATGCTCGTGGGGAAGCTATTCTTTCTTCCGATACATTGACAGTTCCTG
TATCTGATACGGAGAATACACATATCTATTAA

SEQ ID NO:28: CM homologue of CT443 = TC_0727

MRIGDPMNKLIRRAVTIFAVTSVASLFASGVLETSMAESLSTNVISLADTKAKETTSHQKDRKARKNHQNR
TSVVRKEVTAVRDTKAVEPRQDSCFGKMYTVKVNDDRNVEIVQSVPEYATVGSPYPIEITAIGKRDCVDVI
ITQQLPCEAEFVSSDPATTPTADGKLVWKIDRLGQGEKSKITVWVKPLKEGCCFTAATVCACPEIRSVTKC
GQPAICVKQEGPESACLRCPVTYRINVVNQGTATARNVVVENPVPDGYAHASGQRVLTYTLGDMQPGEQRT
ITVEFCPLKRGRVTNIATVSYCGGHKNTASVTTVINEPCVQVNIEGADWSYVCKPVEYVISVSNPGDLVLR
DVVIEDTLSPGITVVEAAGAQISCNKLVWTLKELNPGESLQYKVLVRAQTPGQFTNNVVVKSCSDCGICTS
CAEATTYWKGVAATHMCVVDTCDPICVGENTVYRICVTNRGSAEDTNVSLILKFSKELQPISFSGPTKGTI
TGNTVVFDSLPRLGSKETVEFSVTLKAVSAGDARGEAILSSDTLTVPVSDTENTHIY

SEQ ID NO:29: CM homologue of CT043 = TC_0313 nucleotide sequence

ATGTCCAGACAGAATGCTGAGGAAAATCTAAAAAATTTTGCTAAAGAGCTCAAGCTCCCCGACGTGGCCTT
CGATCAGAATAATACGTGCATTTTGTTTGTTGATGGAGAGTTTTCTCTTCACCTGACCTACGAAGAGCACT
CTGATCGCCTTTATGTTTACGCACCTCTCCTTGACGGACTCCCAGATAATCCGCAAAGAAAGTTGGCTCTG
TATGAGAAATTGTTGGAAGGCTCTATGCTCGGAGGCCAAATGGCTGGTGGAGGAGTAGGAGTTGCTACTAA
AGAACAGTTGATCCTAATGCATTGCGTGTTAGATATGAAATATGCAGAGACTAATCTATTGAAAGCTTTTG
CACAGCTTTTCATTGAAACTGTTGTGAAATGGCGAACGGTCTGTTCTGATATCAGCGCTGGACGAGAACCT
TCCGTTGACACTATGCCTCAAATGCCTCAAGGAGGCAGCGGAGGAATTCAACCTCCTCCAACAGGAATTCG
TGCGTAG

SEQ ID NO:30: CM homologue of CT043 = TC_0313 protein sequence

MSRQNAEENLKNFAKELKLPDVAFDQNNTCILFVDGEFSLHLTYEEHSDRLYVYAPLLDGLPDNPQRKLAL
YEKLLEGSMLGGQMAGGGVGVATKEQLILMHCVLDMKYAETNLLKAFAQLFIETVVKWRTVCSDISAGREP
SVDTMPQMPQGGSGGIQPPPTGIRA

SEQ ID NO:31 – TC0431 protein sequence

MPHSPFLYVVQPHSVFNPRLGERHPITLDFIKEKNRLADFIENLPLEIFGAPSFLENASLEASYVLSREST
KDGTLFTVLEPKLSACVATCLVDSSIPMEPDNELLEEIKHTLLKSSCDGVQYRVTRETLQNKDEAPRVSLV
ADDIELIRNVDFLGRSVDIVKLDPLNIPNTVSEENALDYSFTRETAKLSPDGRVGIPQGTKILPAPSLEVE
ISTSIFEETSSFEQNFSSSITFCVPPLTSFSPLQEPPLVGAGQQEILVTKKHLFPSYTPKLIDIVKRHKRD
AKILVNKIQFEKLWRSHAKSQILKEGSVRLDLQGFTGELFNYQLQVGSHTIAAVLIDPEIANVKSLPEQTY
AVRKIKSGFQCSLDDQHIYQVAVKKHLSLSSQPPKISPLSQSESSDLSLFEAAAFSASLTYEFVKKNTYHA
KNTVTCSTVSHSLYILKEDDGANAAEKRLDNSFRNWVENKLNANSPDSCTAFIQKFGTHYITSATFGGSGF
QVLKLSFEQVEGLRSKKISLEAAAANSLLKSSVSNSTESGYSTYDSSSSSHTVFLGGTVLPSVHDGQLDFK
DWSESVCLEPVPIHISLLPLTDLLTPLYFPETDTTELSNKRNALQQAVRVYLKDHRSAKQSERSVFTAGIN
SPSSWFTLESANSPLVVSSPYMTYWSTLPYLFPTLKERSSAAPIVFYFCVDNNEHASQKILNQTYCFIGSL
PIRQKIFGREFAENPYLSFYGRFGEAYFDGGYPERCGWIVEKLNTTKDQILRDEDEVQLKHVYSGEYLSTI
PIKDSHCTLSRTCTESNAVFIIKKPSSY

SEQ ID NO:32 CM homologue of CT279 = TC_0890 nucleotide sequence

ATGCTCGCTAATCGGTTATTTCTAATCACCCTTATAGGTTTTGGCTATTCTGCTTACGGTGCCAGCACAGG
GAAATCACCTTCTTTACAGGTTATTTTAGCTGAAGTCGAGGATACATCTTCGCGCTTACAAGCTCATCAGA
ATGAGCTTGTTATGCTCTCGGAACGTTTAGATGAGCAAGACACAAAACTTCAACAACTCTCGTCAACTCAG
GCCCGTAATCTTCCTCAACAAGTTCAACGGCTTGAGATTGATCTGAGAGCTCTGGCTAAAACAGCTGCTGT
GCTCTCGCAATCTGTTCAGGATATCCGATCATCCGTGCAAAATAAATTACAAGAAATCCAACAAGAACAAA
AAAATTTAGCTCAAAATTTACGAGCGCTTCGCAACTCCTTACAAGCACTAGTTGATGGCTCTTCCCCAGAA
AATTATATTGATTTTTTGGCCGGGGAGACACCTGAACATATTCACGTTGTTAAACAAGGAGAAACCCTGAG
TAAAATCGCTAGTAAGTACAATATCCCTGTCGCAGAATTGAAAAAACTTAATAAATTAAATTCCGATACTA
TTTTTACTGATCAAAGAATCCGACTTCCAAAAAAGAAATAA

SEQ ID NO:33: CM homologue of CT279 = TC_0890 protein sequence

MLANRLFLITLIGFGYSAYGASTGKSPSLQVILAEVEDTSSRLQAHQNELVMLSERLDEQDTKLQQLSSTQ
ARNLPQQVQRLEIDLRALAKTAAVLSQSVQDIRSSVQNKLQEIQQEQKNLAQNLRALRNSLQALVDGSSPE
NYIDFLAGETPEHIHVVKQGETLSKIASKYNIPVAELKKLNKLNSDTIFTDQRIRLPKKK

SEQ ID NO:34 – TC0660 protein sequence

MSMYIKRKKAWMTFLAIVCSFCLAGCSKESKDSVSEKFIVGTNATYPPFEFVDERGETVGFDIDLAREISK
KLGKKLEVREFAFDALVLNLKQHRIDAIMAGVSITSSRLKEILMIPYYGEEIKSLVLVFKDGDSKSLPLDQ
YNSVAVQTGTYQEEYLQSLPGVRIRSFDSTLEVLMEVLHSKSPIAVLEPSIAQVVLKDFPTLTTETIDLPE
DKWVLGYGIGVASDRPSLASDIEAAVQEIKKEGVLAELEQKWGLNG

SEQ ID NO:35 – TC0741 protein sequence

MTTPISNSPSSIPTVTVSTTTASSGSLGTSTVSSTTTSTSVAQTATTTSSASTSIIQSSGENIQSTTGTPS
PITSSVSTSAPSPKASATANKTSSAVSGKITSQETSEESETQATTSDGEVSSNYDDVDTPTNSSDSTVDSD
YQDVETQYKTISNNGENTYETIGSHGEKNTHVQESHASGTGNPINNQQEAIRQLRSSTYTTSPRNENIFSP
GPEGLPNMSLPSYSPTDKSSLLAFLSNPNTKAKMLEHSGHLVFIDTTRSSFIFVPNGNWDQVCSMKVQNGK
TKEDLGLKDLEDMCAKFCTGYNKFSSDWGNRVDPLVSSKAGIESGGHLPSSVIINNKFRTCVAYGPWNPKE
NGPNYTPSAWRRGHRVDFGKIFDGTAPFNKINWGSSPTPGDDGISFSNETIGSEPFATPPSSPSQTPVINV
NVNVGGTNVNIGDTNVSKGSGTPTSSQSVDMSTDTSDLDTSDIDTNNQTNGDINTNDNSNNVDGSLSDVDS
RVEDDDGVSDTESTNGNDSGKTTSTEENGDPSGPDILAAVRKHLDTVYPGENGGSTEGPLPANQNLGNVIH
DVEQNGSAKETIITPGDTGPTDSSSSVDADADVEDTSDTDSGIGDDDGVSDTESTNGNNSGKTTSTEENGD
PSGPDILAAVRKHLDTVYPGENGGSTEGPLPANQNLGNVIHDVEQNGAAQETIITPGDTESTDTSSSVNAN
ADLEDVSDADSGFGDDDGISDTESTNGNDSGKNTPVGDGGTPSGPDILAAVRKHLDTVYPGENGGSTERPL
PANQNLGDIIHDVEQNGSAKETVVSPYRGGGGNTSSPIGLASLLPATPSTPLMTTPRTNGKAAASSLMIKG
GETQAKLVKNGGNIPGETTLAELLPRLRGHLDKVFTSDGKFTNLNGPQLGAIIDQFRKETGSGGIIAHTDS
VPGENGTASPLTGSSGEKVSLYDAAKNVTQALTSVTNKVTLAMQGQKLEGIINNNNTPSSIGQNLFAAARA
TTQSLSSLIGTVQ

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CLAIMS:

1. A bacterium which is an ompA mutant and/or which is a mutant in one or more components of the Tol-Pal complex, which presents a heterologous antigen on its surface.
2. A bacterium according to claim 1, which does not express the OmpA protein.
3. A bacterium according to claim 2, which does not express one or more components of the Tol-Pal complex.
4. A bacterium according to claim 3, which does not express TolR.
5. A bacterium according to any one of the preceding claims, which expresses the Braun lipoprotein *lpp*.
6. A bacterium according to any one of the preceding claims, wherein the bacterium is *E. coli*.
7. A bacterium according to claim 6, wherein the *E. coli* is *E. coli* BL21(DE3).
8. A bacterium according to any one of the preceding claims, wherein the heterologous antigen is a membrane protein.
9. A bacterium according to claim 8, wherein the membrane protein is an outer membrane protein.
10. A bacterium according to any one of the preceding claims, wherein the heterologous antigen is from *Chlamydia*.
11. A bacterium according to claim 10, wherein the heterologous antigen is from *C. trachomatis*.
12. A bacterium according to claim 11, wherein the heterologous antigen is selected from the group consisting of CT823, CT681, CT372, CT443, CT043, CT733, CT279, CT601 and CT153.
13. A bacterium according to claim 12, wherein the heterologous antigen is CT823.
14. A bacterium according to claim 11, wherein the heterologous antigen is selected from a CT823 peptide comprising or consisting of one or more of the following sequences: DYFNDEFFNRFFGLP (SEQ ID NO: 56), SHREQ (SEQ ID NO: 57), ALQKMGVRVQNITPE (SEQ ID NO: 58), NQVLKNSKGENVLLM (SEQ

ID NO: 59), SPMLGYSASKKDSKADICLA (SEQ ID NO: 60), EDLLKEVSRGFSRVAAKATP (SEQ ID NO: 61), TGNQAIASPGNKRGFQENPF (SEQ ID NO: 62), IAIGNPFGLQATVTVGVISAKGRNQLHIVD (SEQ ID NO: 63) and NTAIVSGSGGYIGIGFAIPSLMAKRVIDQL (SEQ ID NO: 64).

15. A bacterium according to any one of claims 1 to 10, wherein the heterologous antigen is from *C. muridarum.*

16. A bacterium according to claim 15, wherein the heterologous antigen is selected from the group consisting of TC0210, TC0106, TC0313, TC0431, TC0551, TC0651, TC0727 and TC0890.

17. A bacterium according to claim 16, wherein the heterologous antigen is TC0210.

18. A bacterium according to claim 15, wherein the heterologous antigen is selected from a TC0210 peptide comprising or consisting of one or more of the following sequences: DYFNDEFFNRFFGLP (SEQ ID NO: 36), SHREQ (SEQ ID NO: 37), ALQKMGVRVQNLTPE (SEQ ID NO: 38), NQVLKNAKGENVLLM (SEQ ID NO: 39), SPMLGYSAPKKDSSTGICLA (SEQ ID NO: 40), EDLLKEVSRGFSKVAAQATP (SEQ ID NO: 41), TGSQAIASPGNKRGFQENPF (SEQ ID NO: 42), PRPQQRDAVR (SEQ ID NO: 43), IAIGNPFGLQATVTVGVISAKGRNQLHIVD (SEQ ID NO: 44), NTAIVSGSGGYIGIGFAIPSLMAKRVIDQL (SEQ ID NO: 45), VAAQATPGVVYIENFPK (SEQ ID NO: 46), GFQENPFDYFNDEFFNRFFGLPSHREQPRPQQR (SEQ ID NO: 47), GTGFIVSEDGYVVTNHHVVEDAGK (SEQ ID NO: 48), TDLAVIKIQAK (SEQ ID NO: 49), VIDQLISDGQVTR (SEQ ID NO: 50), AGLRQEDVIVAYNGKEVESLSALR (SEQ ID NO: 51), FIEIPVTVTQIPAEDGVSALQK (SEQ ID NO: 52), VQNLTPEICK (SEQ ID NO: 53), NAKGENVLLMVSQGEVIR (SEQ ID NO: 54) and GENVLLMVSQGEVIR (SEQ ID NO: 55).

19. An outer membrane vesicle obtained from a bacterium according to any one of claims 1 to 18, wherein the outer membrane vesicle expresses the heterologous antigen on its surface.

20. An outer membrane vesicle according to claim 19, which does not express the OmpA protein.

21. An outer membrane vesicle according to claim 19, which does not express the TolR protein.

22. An outer membrane vesicle according to any one of claims 19 to 21, which expresses the Braun lipoprotein *lpp*.

23. An immunogenic composition comprising an outer membrane vesicle according to any one of claims 19 to 22.

24. A vaccine comprising an outer membrane vesicle according to any one of claims 19 to 22.

25. An immunogenic composition according to claim 23 or a vaccine according to claim 24, which further comprises one or more additional antigens in a combined preparation for simultaneous, separate or sequential administration.

26. An immunogenic composition or vaccine according to claim 25, wherein the one or more additional antigens are also presented by the outer membrane vesicle.

27. An immunogenic composition or vaccine according to claim 25, wherein at least one of the one or more additional antigens is presented by a different outer membrane vesicle.

28. An immunogenic composition or vaccine according to any one of claims 25 to 27, wherein the heterologous antigen and the one or more additional antigens are selected from the groups consisting of: TC0106+TC0431, TC0660+TC0741, TC0551+TC0890, TC0106+TC0210+TC0741+TC0313, TC0551+TC0890+TC0106+TC0431, CT823+CT089, CT823+CT089+CT381, CT823+CT372, CT823+CT443, CT823+CT601, CT823+CT153, CT823+CT279, CT823+CT456, CT823+CT733, CT823+CT043 or CT823+CT456+CT733+CT043.

29. An outer membrane vesicle according to any one of claims 19 to 22, an immunogenic composition according to any one of claims 23 or 25 to 28 or a vaccine according to any one of claims 24 to 28 for use in raising an immune response in a mammal.

30. A method of raising an immune response in a mammal comprising administering an outer membrane vesicle according to any one of claims 19 to 22, an immunogenic composition according to claim 23 or 25 to 28 or a vaccine according to any one of claims 24 to 28.

31. An outer membrane vesicle according to any one of claims 19 to 22, an immunogenic composition according to claim 23 or 25 to 28 or a vaccine according to any one of claims 24 to 28, for use in immunising a mammal against *Chlamydia* infection, preferably against *C. trachomatis* infection.

32. A method of immunising a mammal against *Chlamydia* infection, preferably against *C. trachomatis* infection, comprising administering an outer membrane vesicle according to any one of claims 19 to 22, an immunogenic composition according to claim 23 or 25 to 28 or a vaccine according to any one of claims 24 to 28.

33. An outer membrane vesicle according to any one of claims 19 to 22, an immunogenic composition according to claim 23 or a vaccine according to claim 24 for use in raising antibodies that bind to one or more epitopes in the heterologous antigen that are not immunoaccessible and/or are not in their native conformation when the heterologous antigen is presented in a purified form.

34. A method for raising antibodies that bind to one or more epitopes in a heterologous antigen that are not immunoaccessible and/or are not in their native conformation when the heterologous antigen is presented in a purified form, comprising administering an OMV according to any one of claims 19 to 22, an immunogenic composition according to claim 23 or a vaccine according to claim 24 to a mammal.

35. An outer membrane vesicle, immunogenic composition or vaccine according to any one of claims 29, 31 and 33 or a method according to any one of claims 30, 32 and 34, wherein at least one of the epitopes is a *C. trachomatis* epitope selected from an epitope comprising or consisting of the following sequence: DYFNDEFFNRFFGLP (SEQ ID NO: 56), SHREQ (SEQ ID NO: 57), ALQKMGVRVQNITPE (SEQ ID NO: 58), NQVLKNSKGENVLLM (SEQ ID NO: 59), SPMLGYSASKKDSKADICLA (SEQ ID NO: 60), EDLLKEVSRGFSRVAAKATP (SEQ ID NO: 61),

TGNQAIASPGNKRGFQENPF (SEQ ID NO: 62), IAIGNPFGLQATVTVGVISAKGRNQLHIVD (SEQ ID NO: 63) and NTAIVSGSGGYIGIGFAIPSLMAKRVIDQL (SEQ ID NO: 64) or is a *C. muridarum* epitope selected from an epitope comprising or consisting of the following sequence: DYFNDEFFNRFFGLP (SEQ ID NO: 36), SHREQ (SEQ ID NO: 37), ALQKMGVRVQNLTPE (SEQ ID NO: 38), NQVLKNAKGENVLLM (SEQ ID NO: 39), SPMLGYSAPKKDSSTGICLA (SEQ ID NO: 40), EDLLKEVSRGFSKVAAQATP (SEQ ID NO: 41), TGSQAIASPGNKRGFQENPF (SEQ ID NO: 42), PRPQQRDAVR (SEQ ID NO: 43), IAIGNPFGLQATVTVGVISAKGRNQLHIVD (SEQ ID NO: 44), NTAIVSGSGGYIGIGFAIPSLMAKRVIDQL (SEQ ID NO: 45), VAAQATPGVVYIENFPK (SEQ ID NO: 46), GFQENPFDYFNDEFFNRFFGLPSHREQPRPQQR (SEQ ID NO: 47), GTGFIVSEDGYVVTNHHVVEDAGK (SEQ ID NO: 48), TDLAVIKIQAK (SEQ ID NO: 49), VIDQLISDGQVTR (SEQ ID NO: 50), AGLRQEDVIVAYNGKEVESLSALR (SEQ ID NO: 51), FIEIPVTVTQIPAEDGVSALQK (SEQ ID NO: 52), VQNLTPEICK (SEQ ID NO: 53), NAKGENVLLMVSQGEVIR (SEQ ID NO: 54) and GENVLLMVSQGEVIR (SEQ ID NO: 55).

36. An outer membrane vesicle, immunogenic composition or vaccine according to any one of claims 29, 31, 33 or 35 or a method according to any one of claims 30, 32, 34 or 35 for use in raising a neutralising antibody response.

FIGURE 1

FIG 1A

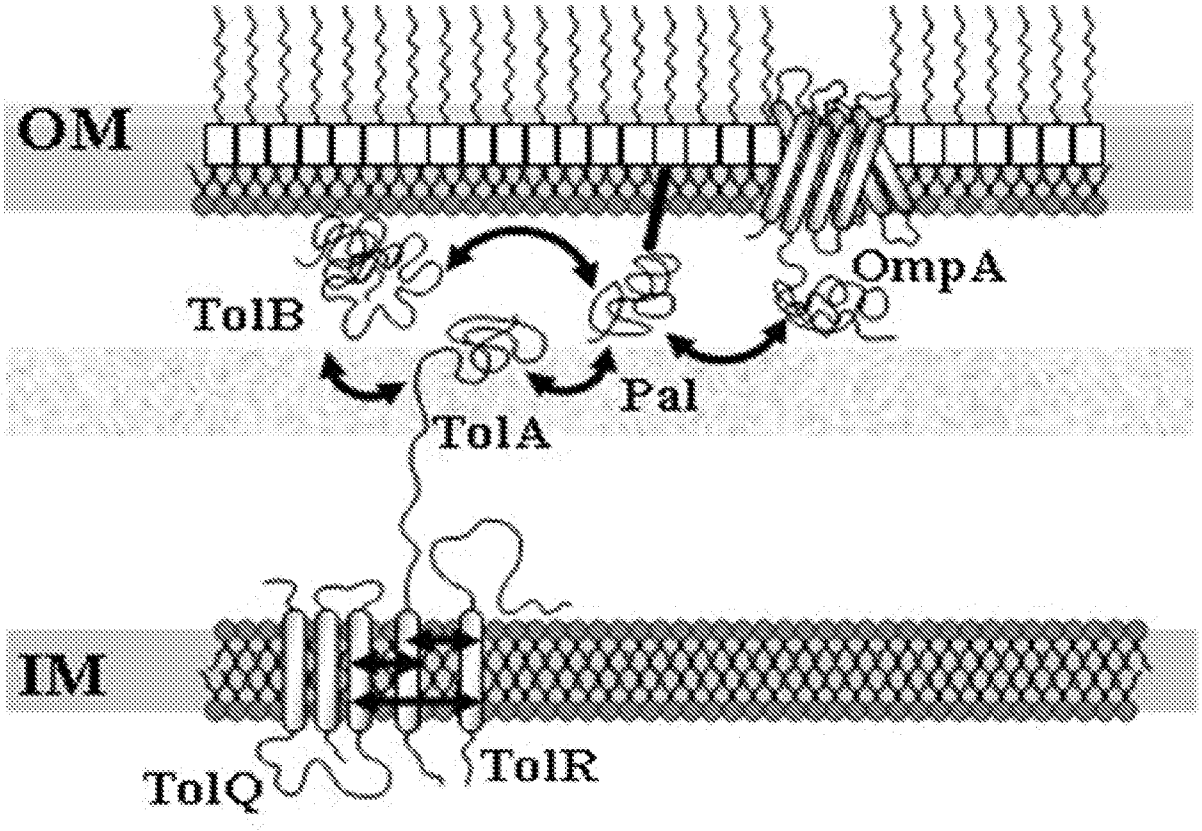


FIG. 1B

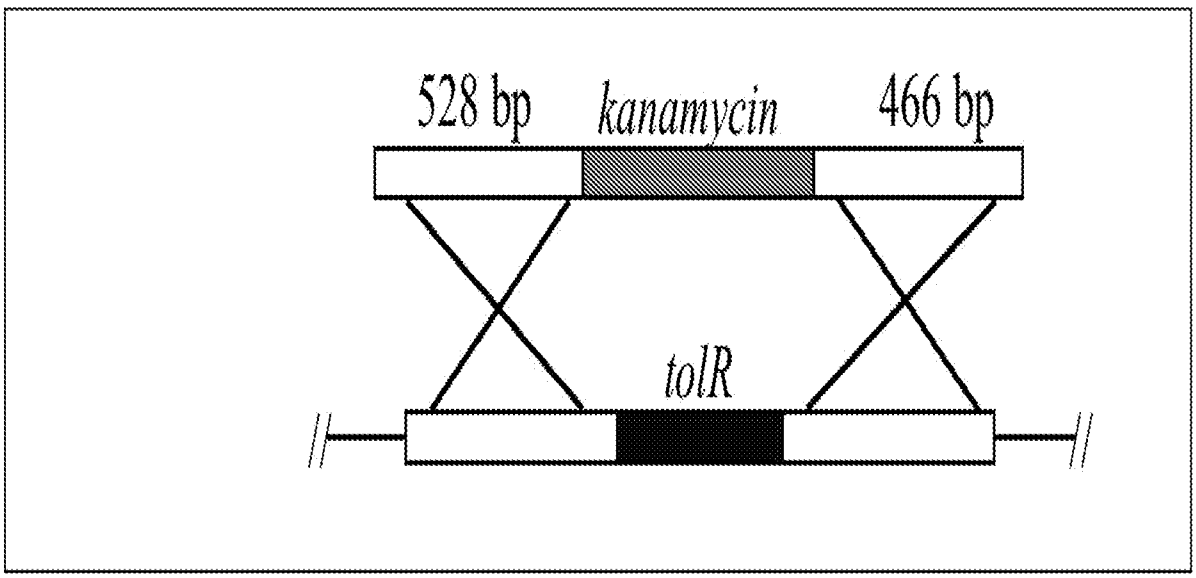


FIGURE 2

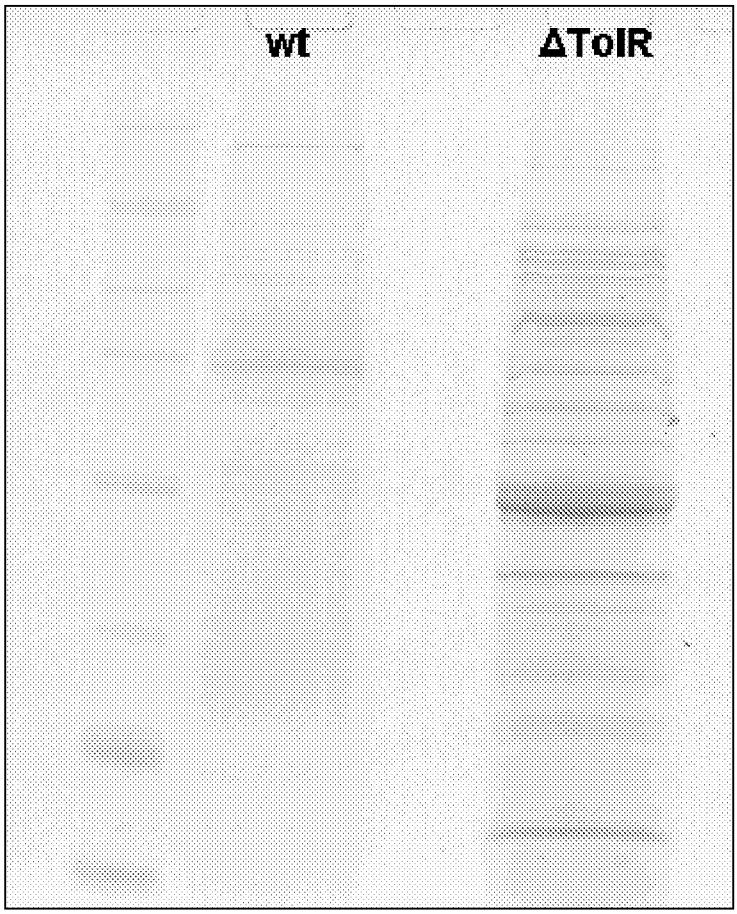


FIGURE 3

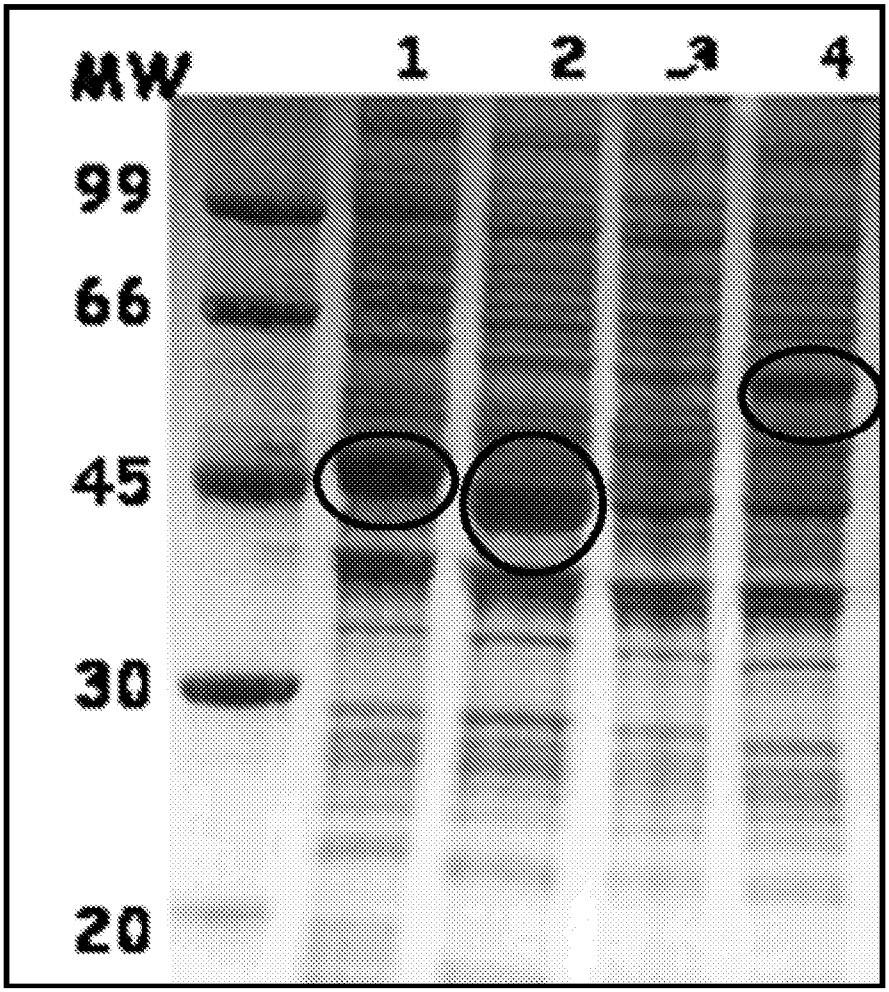


FIGURE 4

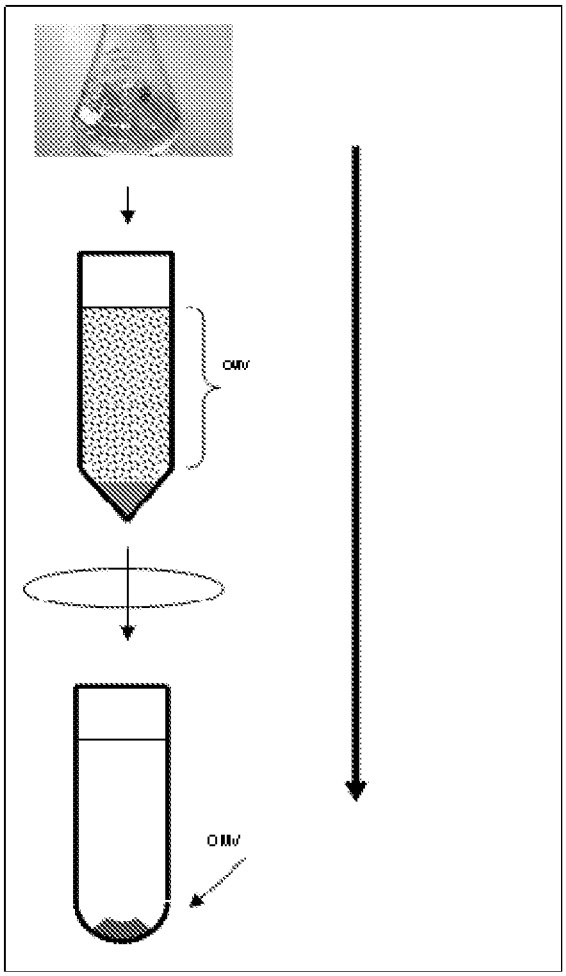


FIGURE 5

FIGURE 5A

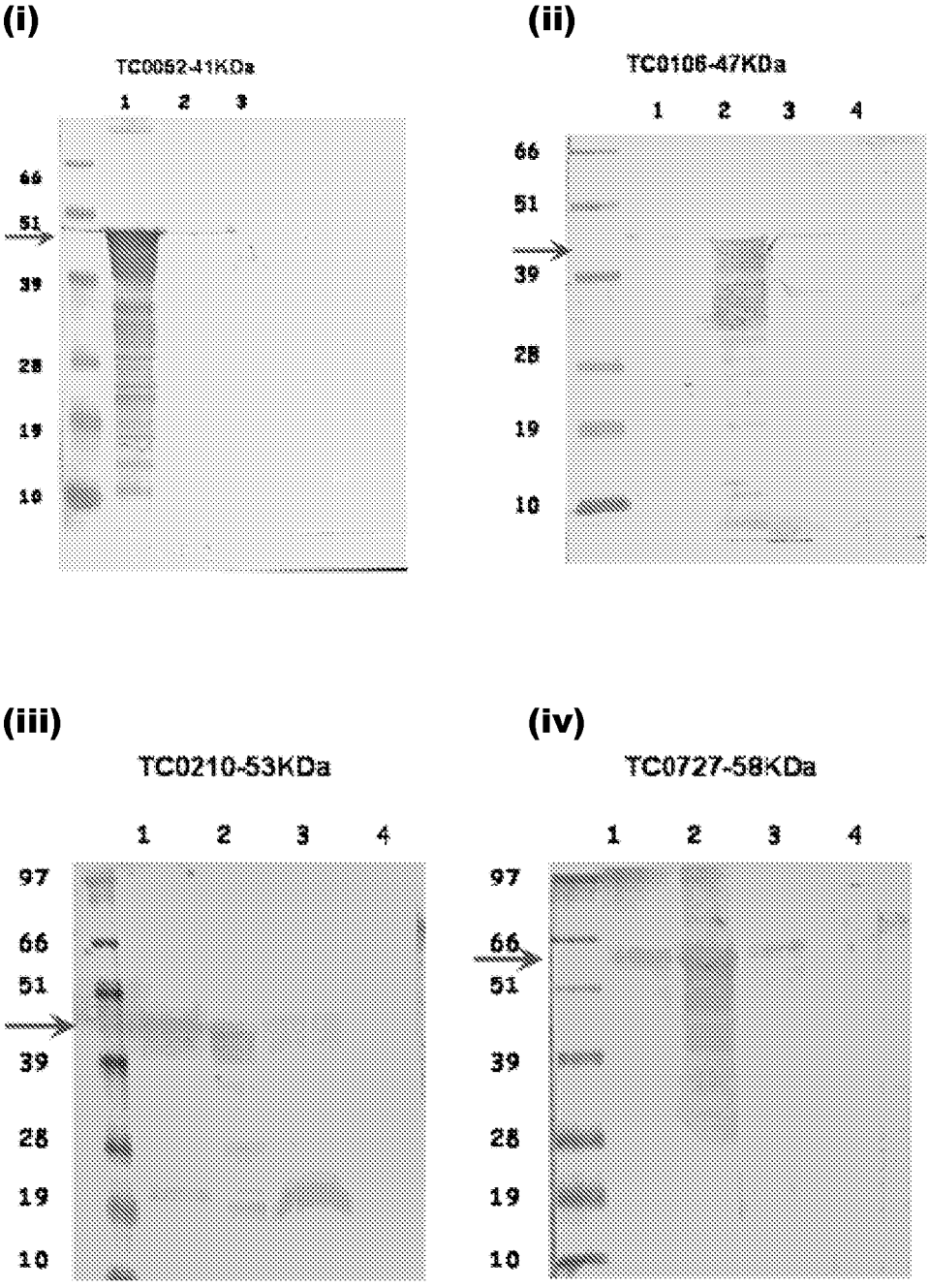


FIGURE 5B

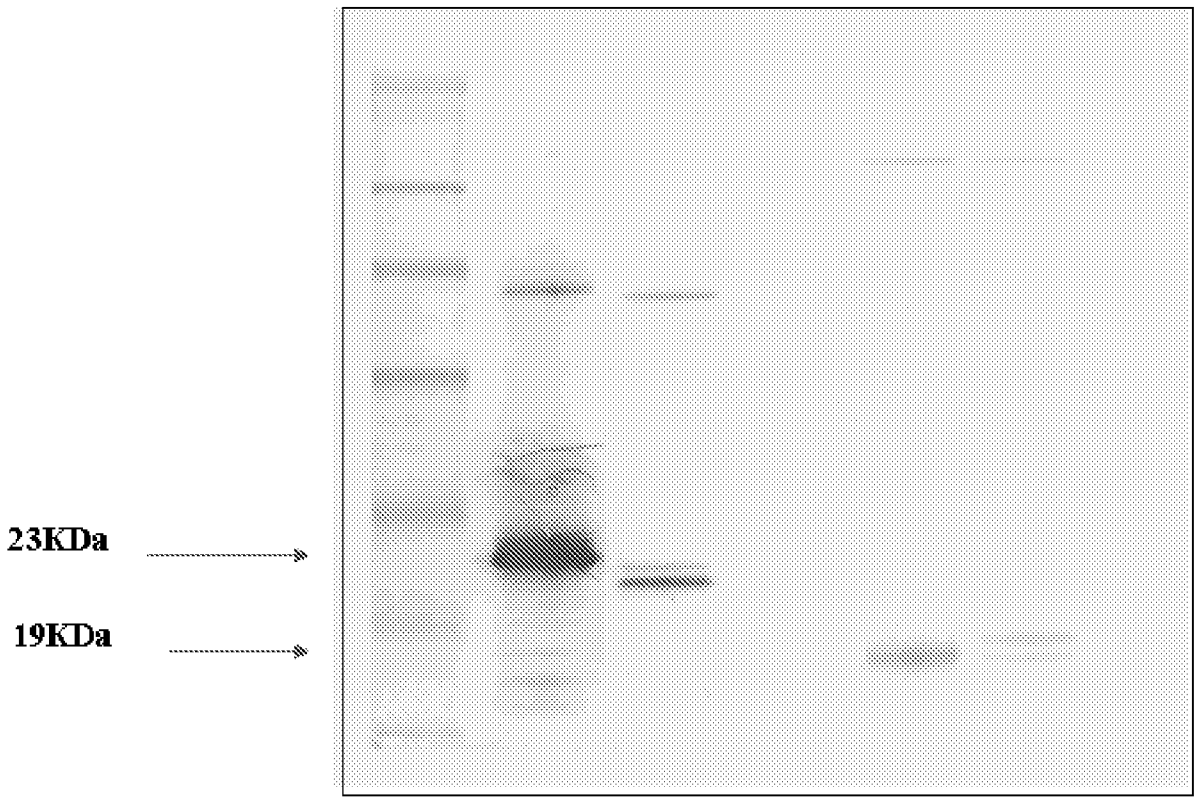


FIGURE 5C

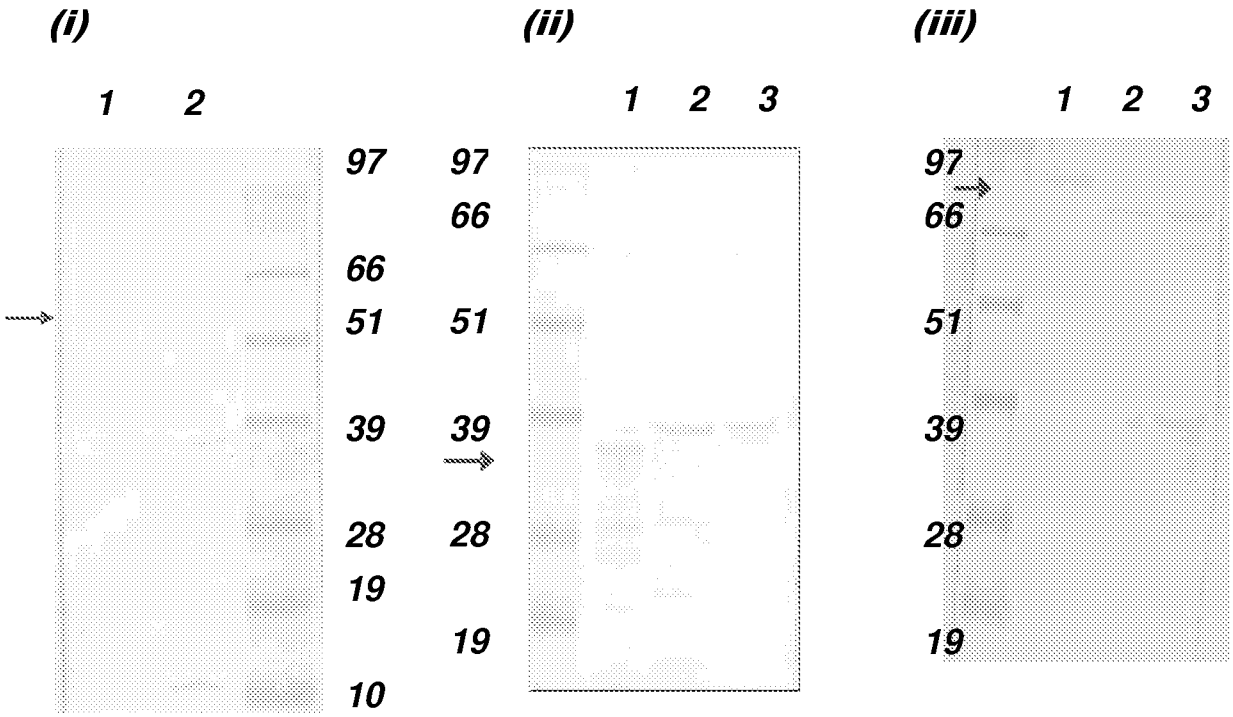
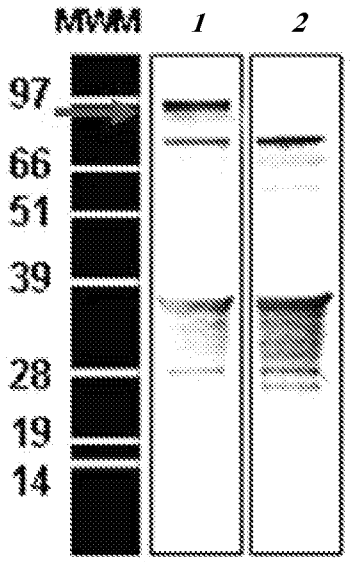

(i)
1
2
97
66
51
39
28
19
10
(ii)
1
2
3
97
66
51
39
28
19
(iii)
1
2
3
97
66
51
39
28
19

FIGURE 5D

(i)



(ii)

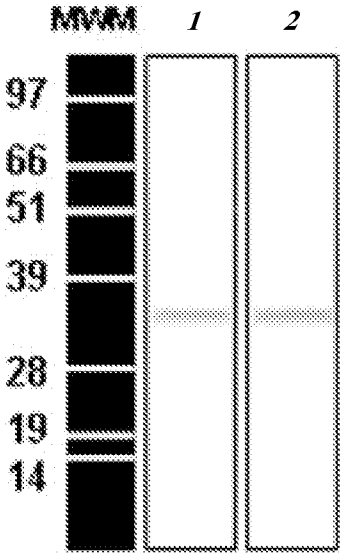


FIGURE 6

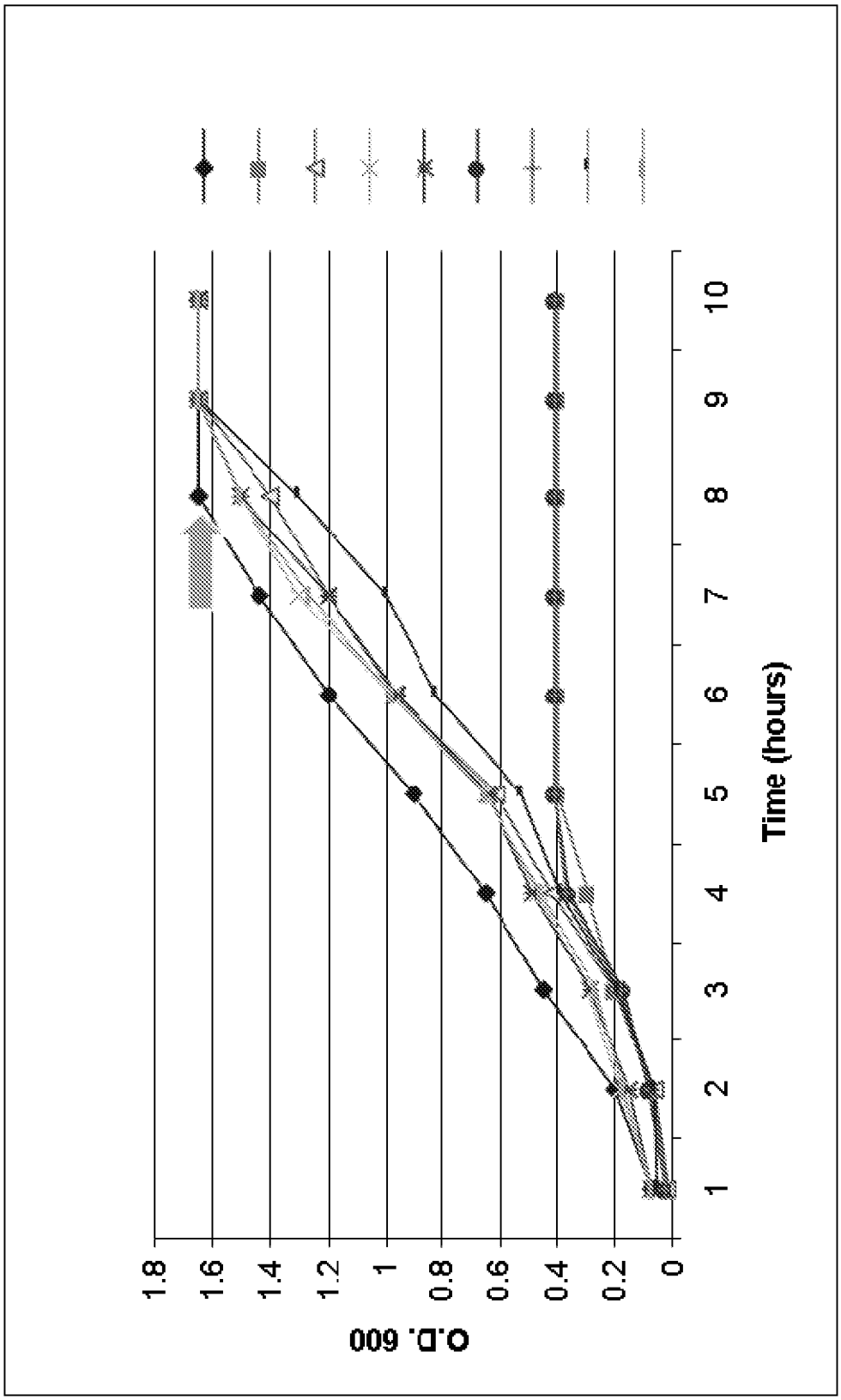


FIGURE 7

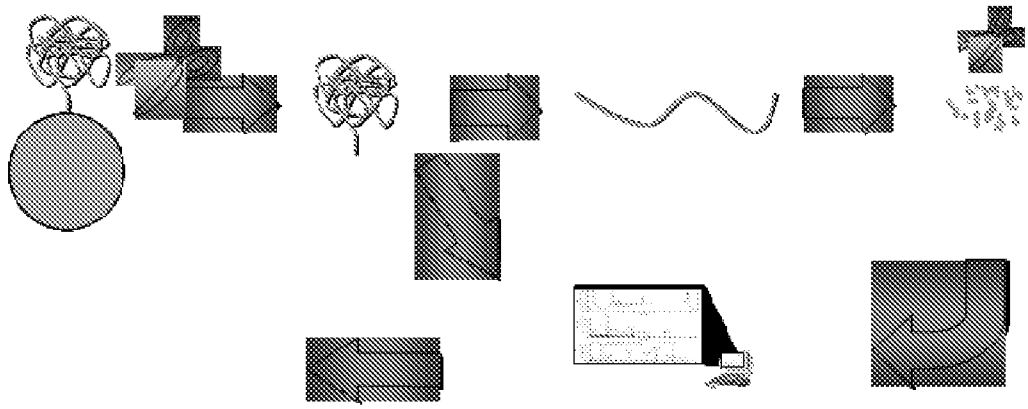


FIGURE 8

MASCOT SEARCH RESULTS

PROTEIN VIEW

Match to: **TC_0210** Score: **43**

serine protease, HtrA/DegQ/DegS family (htrA) [3.4.21.-] {Chlamydia muridarum strain Nigg}

Found in search of \\Chisie06646\MassLynx\PKL_files\Service_OMV_shaving_210_8may2009.pkl

Nominal mass (M_r): **53261**; Calculated pI value: **6.36**

NCBI BLAST search of TC_0210 against nr

Unformatted sequence string for pasting into other applications

Semi-specific cleavage, (peptide can be non-specific at one terminus only)

Cleavage by semi Trypsin: cuts C-term side of KR unless next residue is P

Sequence Coverage: **2%**

Matched peptides shown in **Bold**

```
  1 MMKRLLCVLL STSVFSSPML GYSAPKKDSS TGICLAASQS DRELSQEDLL
 51 KEVSRGFSKV AAQA<b>TPGVVY IENFPK</b>TGSQ AIASPGNKRG FQENPFDYFN
101 DEFFNRFFGL PSHREQPRPQ QRDAVRGTGF IVSEDGYVVT NHHVVEDAGK
151 IHVTLHDGQK YTAKIIGLDP KTDLAVIKIQ AKNLPFLTFG NSDQLQIGDW
201 SIAIGNPFGL QATVTVGVIS AKGRNQLHIV DFEDFIQTDA AINPGNSGGP
251 LLNIDGQVIG VNTAIVSGSG GYIGIGFAIP SLMAKRVIDQ LISDGQVTRG
301 FLGVTLQPID SELAACYKLE KVYGALITDV VKGSPAEKAG LRQEDVIVAY
351 NGKEVESLSA LRNAISLMMP GTRVVLKVVR EGKFIEIPVT VTQIPAEDGV
401 SALQKMGVRV QNLTPEICKK LGLASDTRGI FVVSVEAGSP AASAGVVPGQ
451 LILAVNRQRV SSVEELNQVL KNAKGENVLL MVSQGEVIRF VVLKSDE
```

FIGURE 9

```
>CMU:TC0210 HTRA; SERINE PROTEASE ; K01362
TOP
          LENGTH = 497

>CTR:CT823 HTRA; DO SERINE PROTEASE ; K01362
TOP
          LENGTH = 497

 SCORE =  931 BITS (2406), EXPECT = 0.0,   METHOD: COMPOSITIONAL MATRIX ADJUST.
 IDENTITIES = 464/497 (93%), POSITIVES = 483/497 (97%)

QUERY: 1   MMKRLLCVLLSTSVFSSPMLGYSAPKKDSSTGICLAASQSDRELSQEDLLKEVSRGFSKV 60
           MMKRLLCVLLSTSVFSSPMLGYSA KKDS   ICLA S  D+E+SQEDLLKEVSRGFS+V
SBJCT: 1   MMKRLLCVLLSTSVFSSPMLGYSASKKDSKADICLAVSSGDQEVSQEDLLKEVSRGFSRV 60

QUERY: 61  AAQATPGVVYIENFPKTGSQAIASPGNKRGFQENPFDYFNDEFFNRFFGLPSHREQPRPQ 120
           AA+ATPGVVYIENFPKTG+QAIASPGNKRGFQENPFDYFNDEFFNRFFGLPSHREQ RPQ
SBJCT: 61  AAKATPGVVYIENFPKTGNQAIASPGNKRGFQENPFDYFNDEFFNRFFGLPSHREQQRPQ 120

QUERY: 121 QRDAVRGTGFIVSEDGYVVTNHHVVEDAGKIHVTLHDGQKYTAKIIGLDPKTDLAVIKIQ 180
           QRDAVRGTGFIVSEDGYVVTNHHVVEDAGKIHVTLHDGQKYTAKI+GLDPKTDLAVIKIQ
SBJCT: 121 QRDAVRGTGFIVSEDGYVVTNHHVVEDAGKIHVTLHDGQKYTAKIVGLDPKTDLAVIKIQ 180

QUERY: 181 AKNLPFLTFGNSDQLQIGDWSIAIGNPFGLQATVTVGVISAKGRNQLHIVDFEDFIQTDA 240
           A+ LPFLTFGNSDQLQIGDW+IAIGNPFGLQATVTVGVISAKGRNQLHIVDFEDFIQTDA
SBJCT: 181 AEKLPFLTFGNSDQLQIGDWAIAIGNPFGLQATVTVGVISAKGRNQLHIVDFEDFIQTDA 240

QUERY: 241 AINPGNSGGPLLNIDGQVIGVNTAIVSGSGGYIGIGFAIPSLMAKRVIDQLISDGQVTRG 300
           AINPGNSGGPLLNI+GQVIGVNTAIVSGSGGYIGIGFAIPSLMAKRVIDQLISDGQVTRG
SBJCT: 241 AINPGNSGGPLLNINGQVIGVNTAIVSGSGGYIGIGFAIPSLMAKRVIDQLISDGQVTRG 300

QUERY: 301 FLGVTLQPIDSELAACYKLEKVYGALITDVVKGSPAEKAGLRQEDVIVAYNGKEVESLSA 360
           FLGVTLQPIDSELA CYKLEKVYGAL+TDVVKGSPAEKAGLRQEDVIVAYNGKEVESLSA
SBJCT: 301 FLGVTLQPIDSELATCYKLEKVYGALVTDVVKGSPAEKAGLRQEDVIVAYNGKEVESLSA 360

QUERY: 361 LRNAISLMMPGTRVVLKVVREGKFIEIPVTVTQIPAEDGVSALQKMGVRVQNLTPEICKK 420
           LRNAISLMMPGTRVVLK+VREGK IEIPVTVTQIP EDGVSALQKMGVRVQN+TPEICKK
SBJCT: 361 LRNAISLMMPGTRVVLKIVREGKTIEIPVTVTQIPTEDGVSALQKMGVRVQNITPEICKK 420

QUERY: 421 LGLASDTRGIFVVSVEAGSPAASAGVVPGQLILAVNRQRVSSVEELNQVLKNAKGENVLL 480
           LGLA+DTRGI VV+VEAGSPAASAGV PGQLILAVNRQRV+SVEELNQVLKN+KGENVLL
SBJCT: 421 LGLAADTRGILVVAVEAGSPAASAGVAPGQLILAVNRQRVASVEELNQVLKNSKGENVLL 480

QUERY: 481 MVSQGEVIRFVVLKSDE 497
           MVSQG+V+RF+VLKSDE
SBJCT: 481 MVSQGDVVRFIVLKSDE 497
```

FIGURE 10

FIGURE 10A

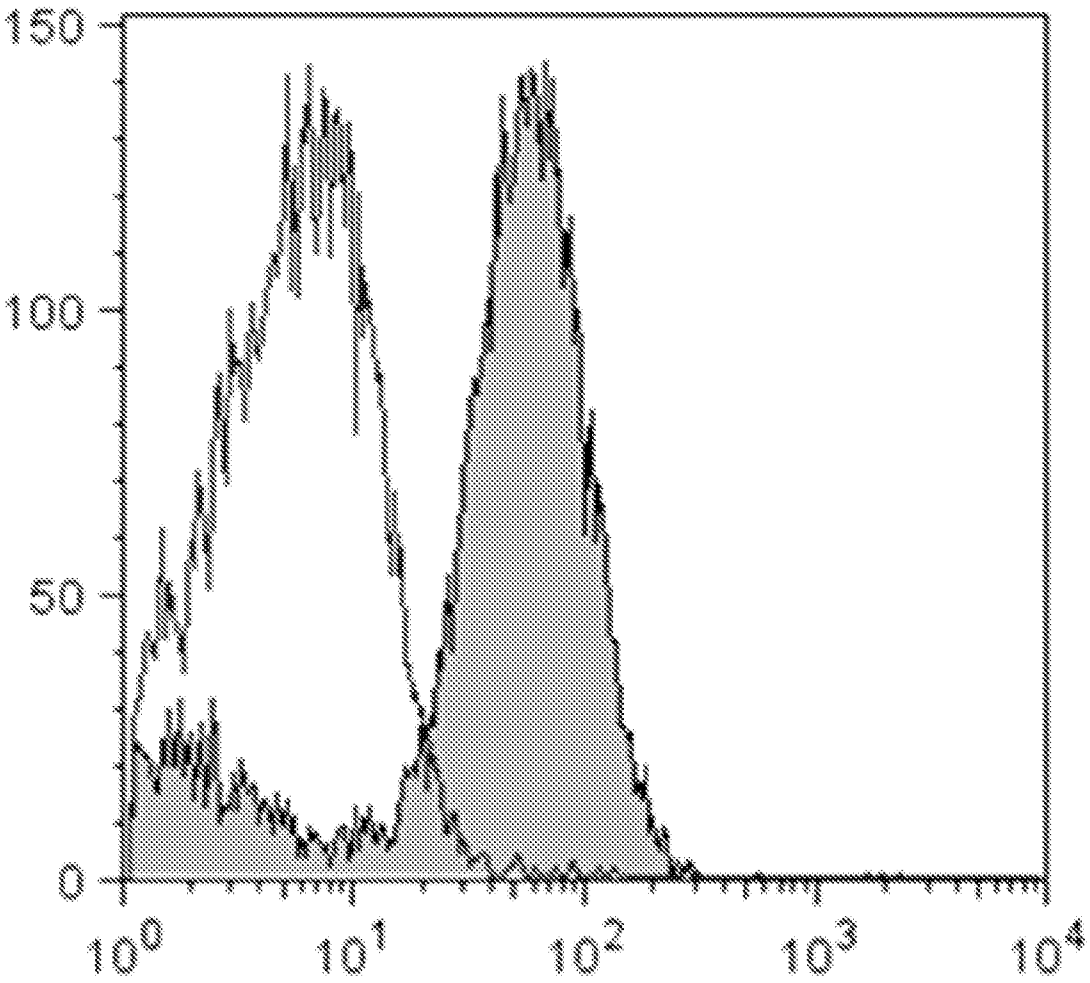


FIGURE 10B

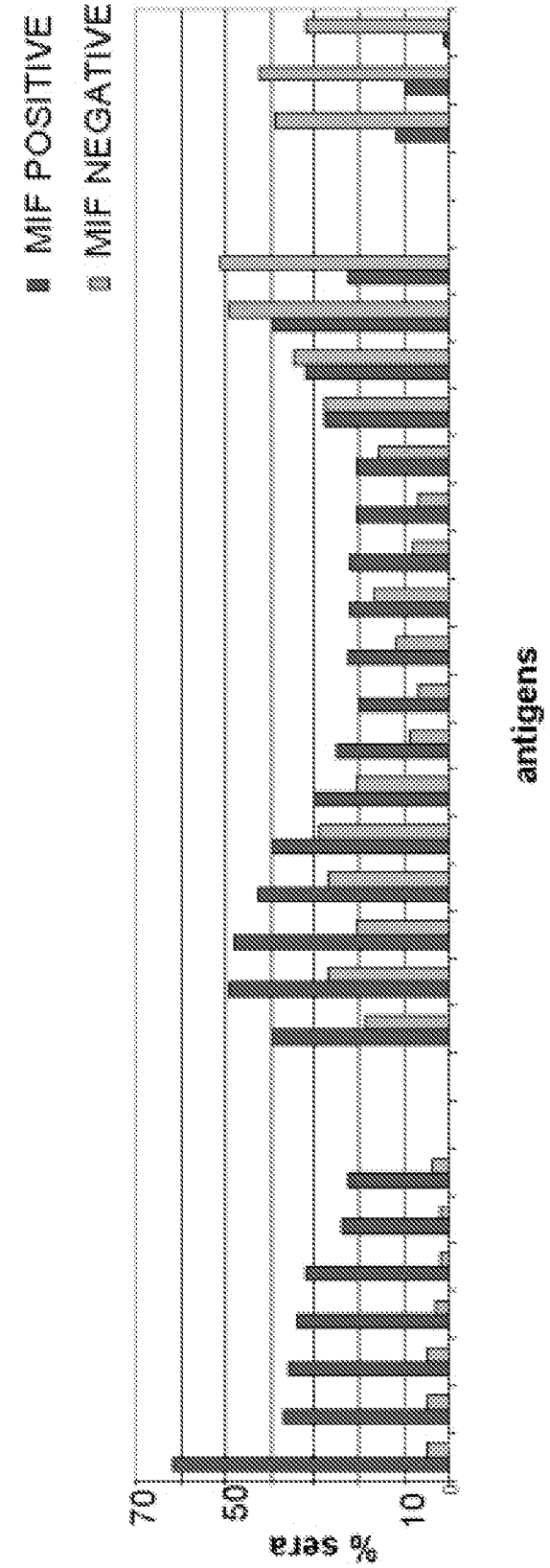
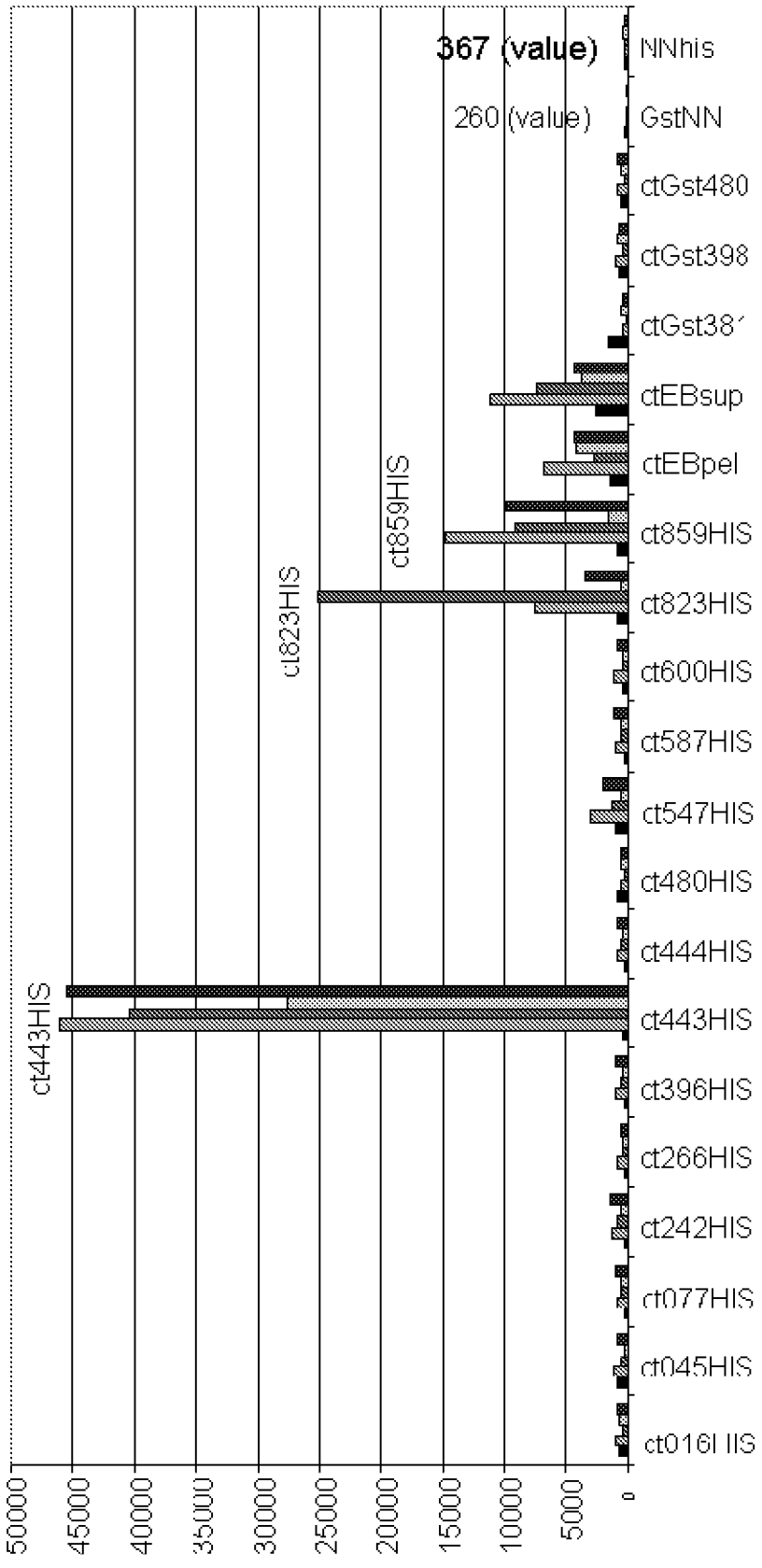


FIGURE 10C


367 (value)
260 (value)
NNhis
GstNN
ctGst480
ctGst398
ctGst38´
ctEBsup
ctEBpel
ct859HIS
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ct600HIS
ct587HIS
ct547HIS
ct480HIS
ct444HIS
ct443HIS
ct396HIS
ct266HIS
ct242HIS
ct077HIS
ct045HIS
ct016HIS
ct859HIS
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30000
25000
20000
15000
10000
5000
0

FIGURE 10D

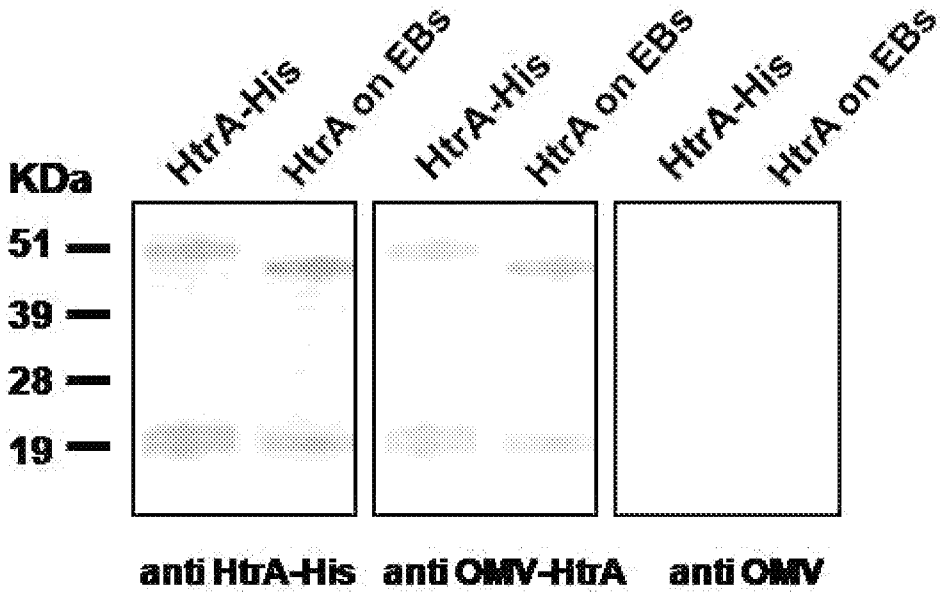

HtrA-His
HtrA on EBs
HtrA-His
HtrA on EBs
HtrA-His
HtrA on EBs
KDa
51
39
28
19
anti HtrA-His
anti OMV-HtrA
anti OMV

FIGURE 10E

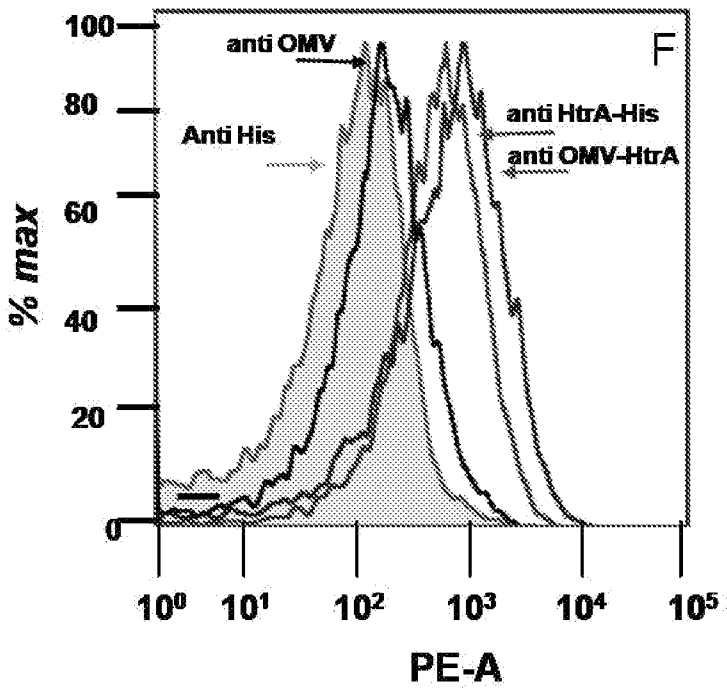

100
80
60
40
20
0
% max
anti OMV
Anti His
anti HtrA-His
anti OMV-HtrA
F
10^0
10^1
10^2
10^3
10^4
10^5
PE-A

FIGURE 11

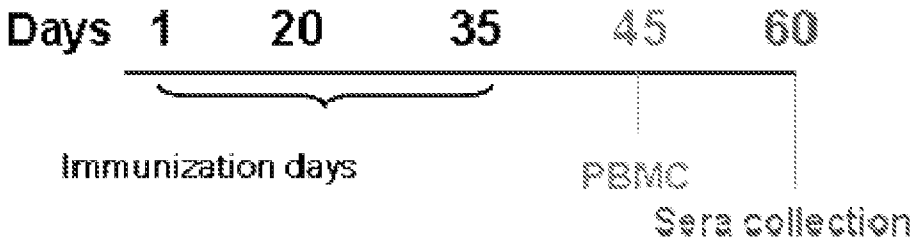


FIG. 12
neutralization assay

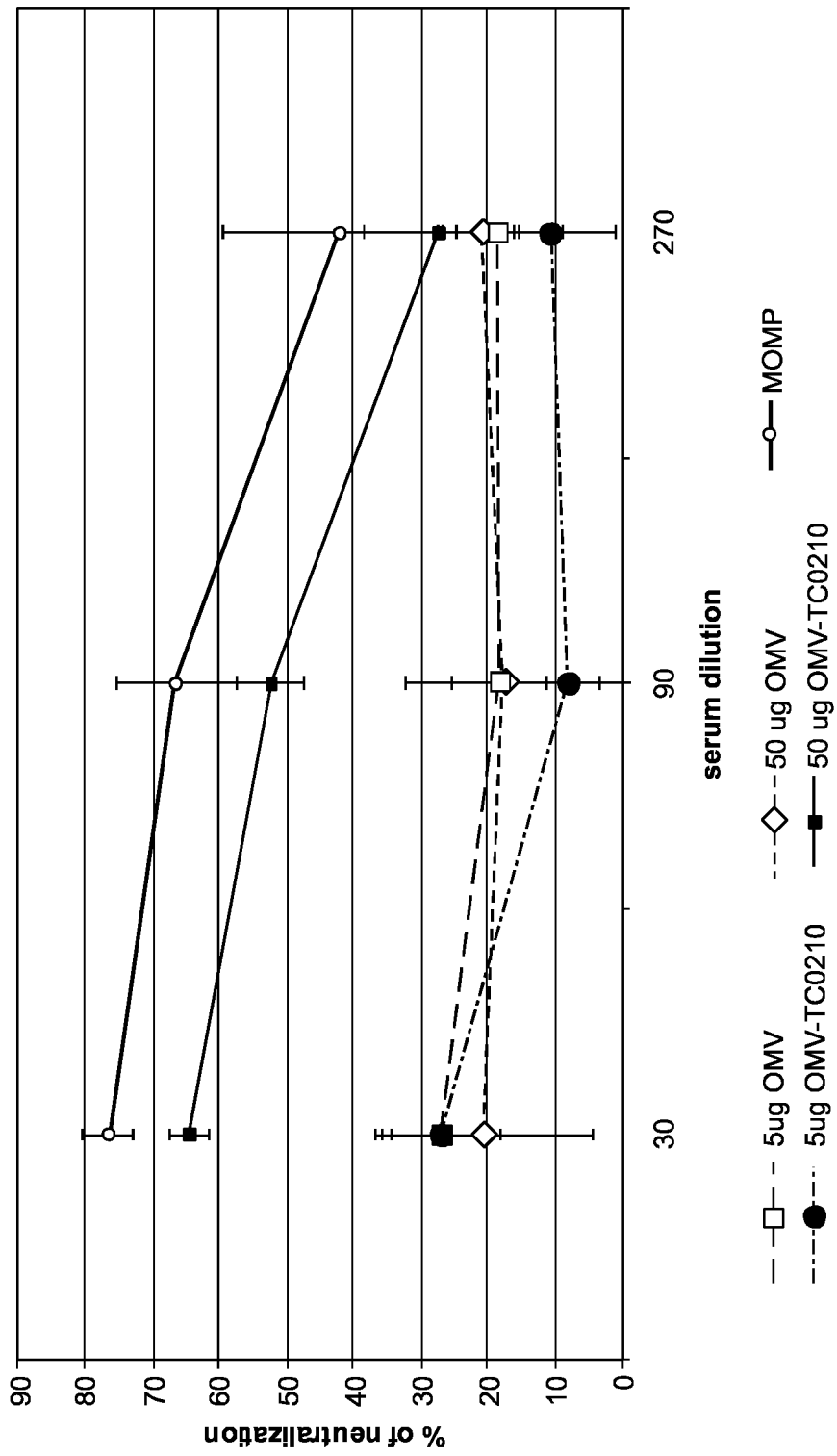


FIGURE 13

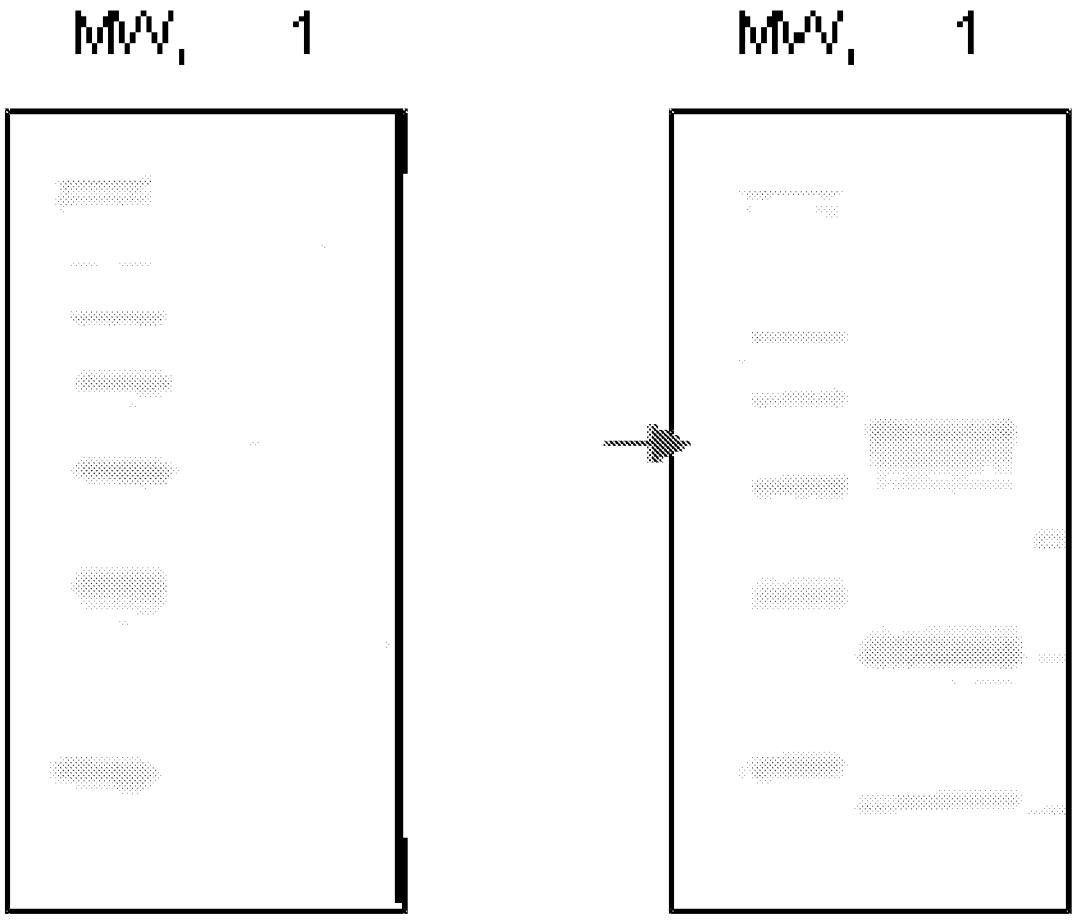


FIGURE 14

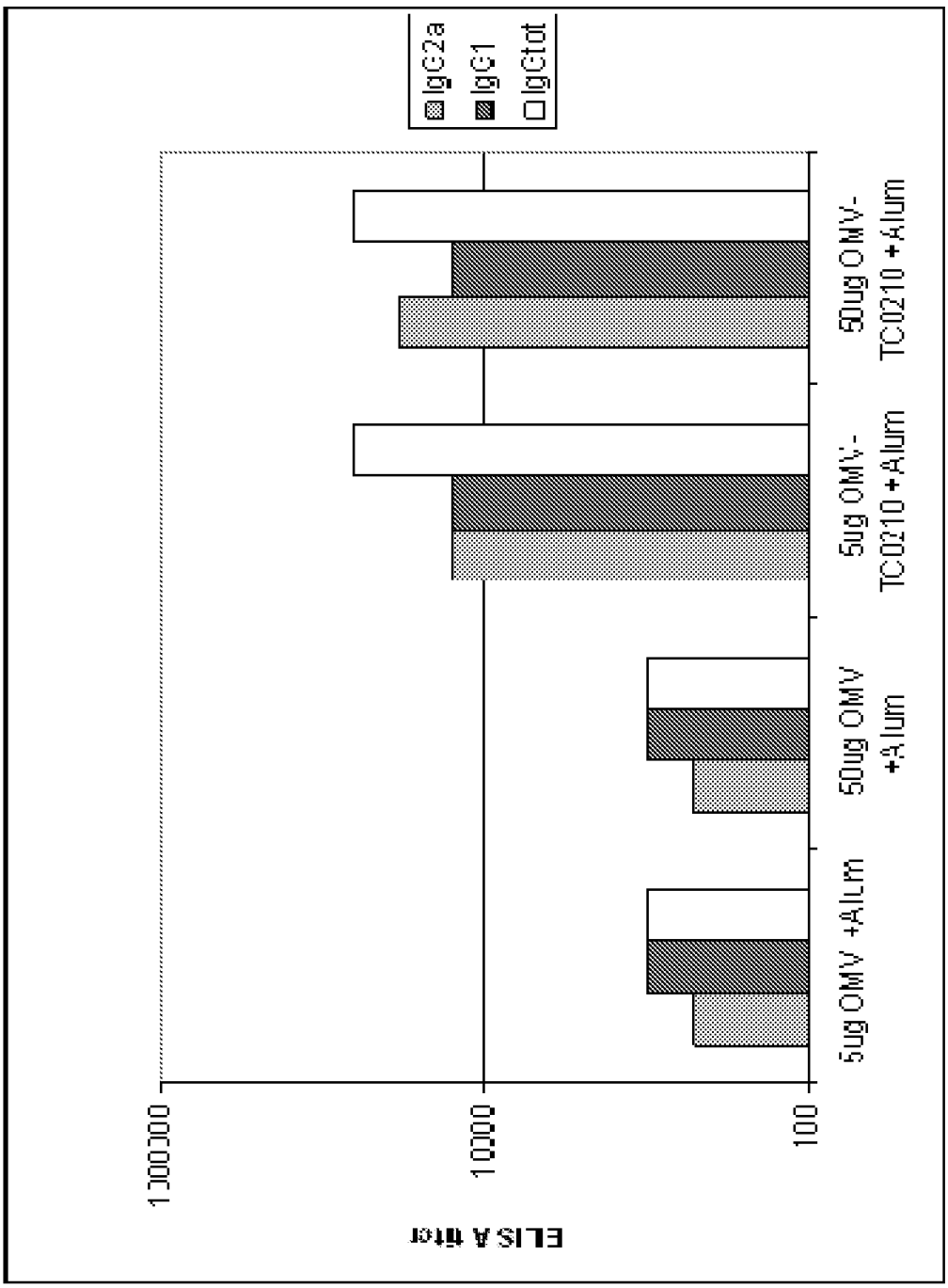

IgG2a
IgG1
IgGtot
ELISA titer
1000000
10000
100
5ug OMV +Alum
50ug OMV +Alum
5ug OMV-TC0210 +Alum
50ug OMV-TC0210 +Alum

FIGURE 15

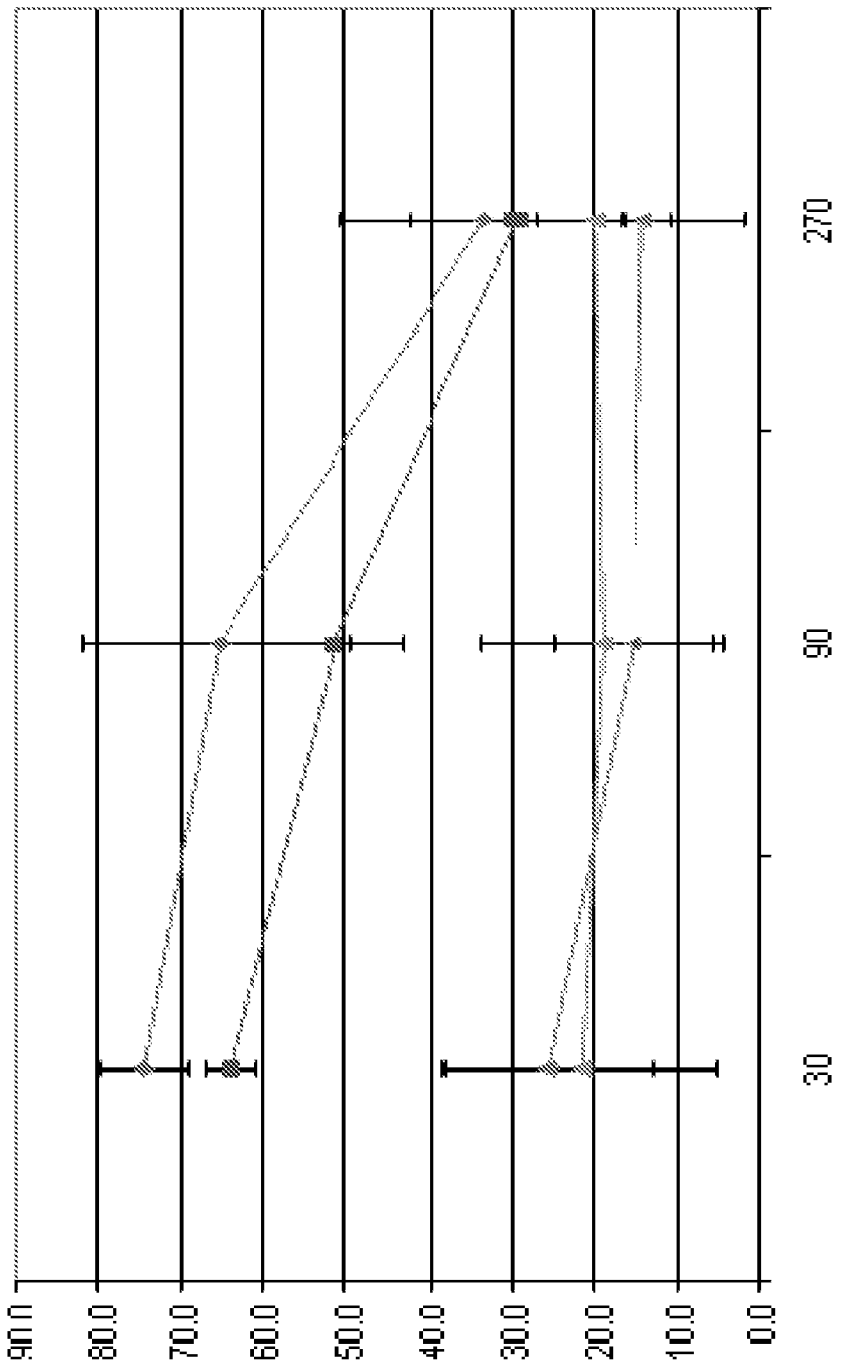


FIGURE 16

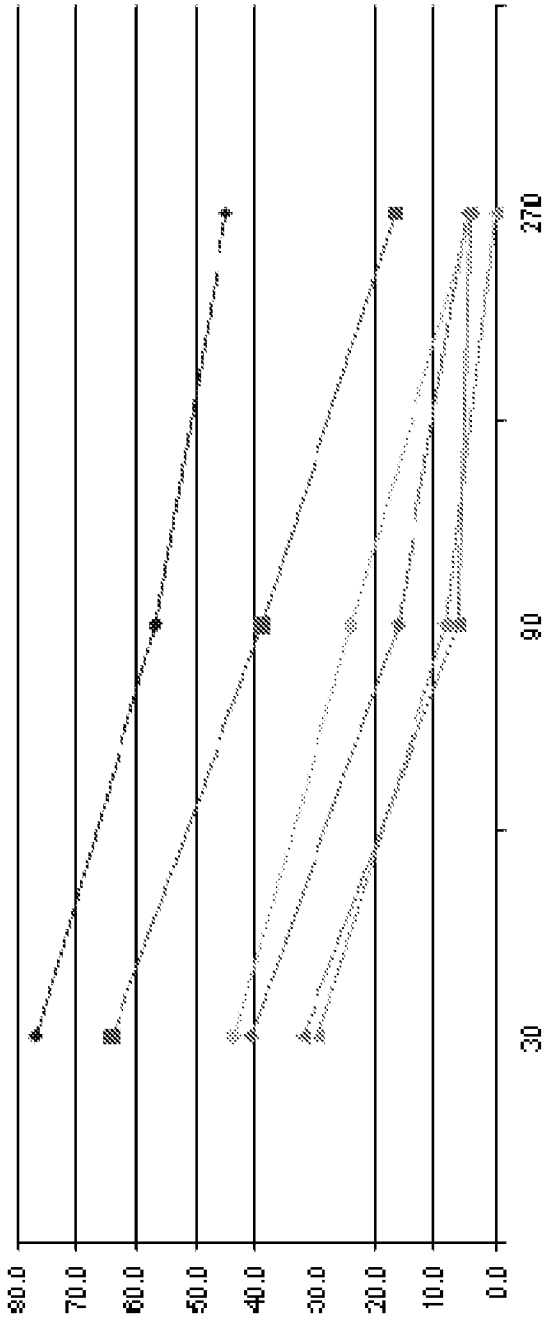


FIGURE 17A

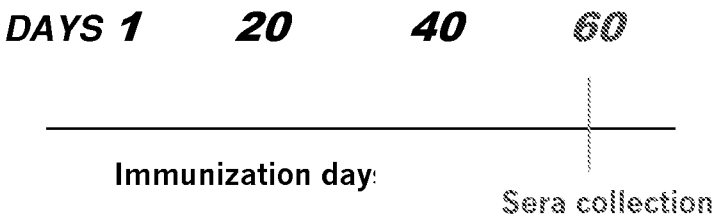


FIGURE 17B

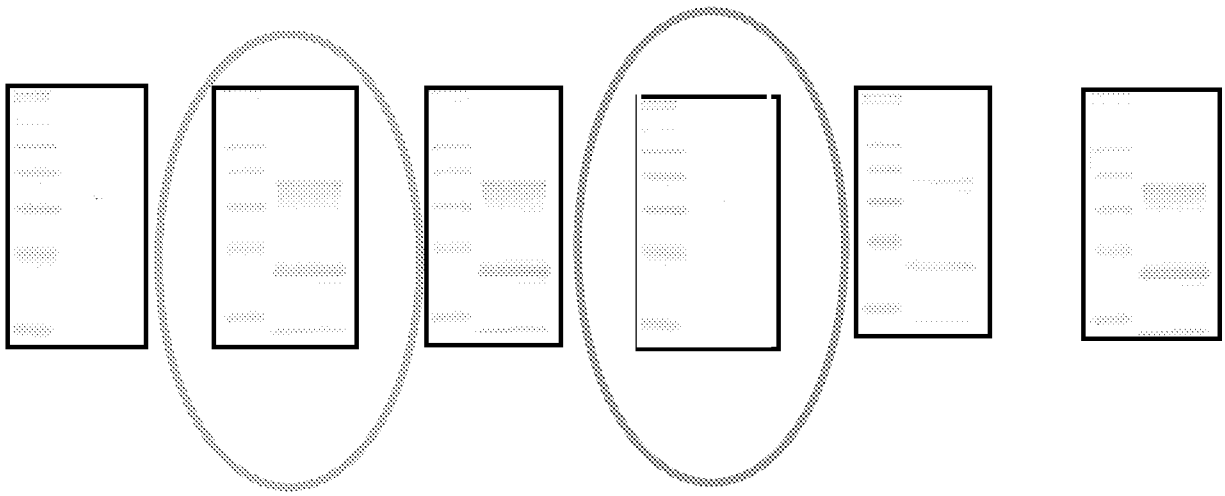


FIGURE 17C

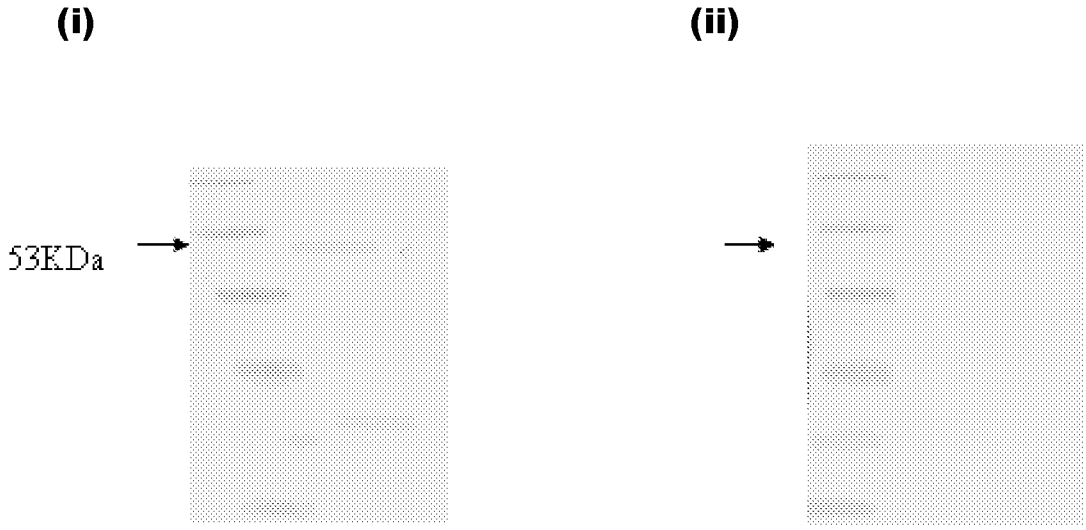


FIGURE 18

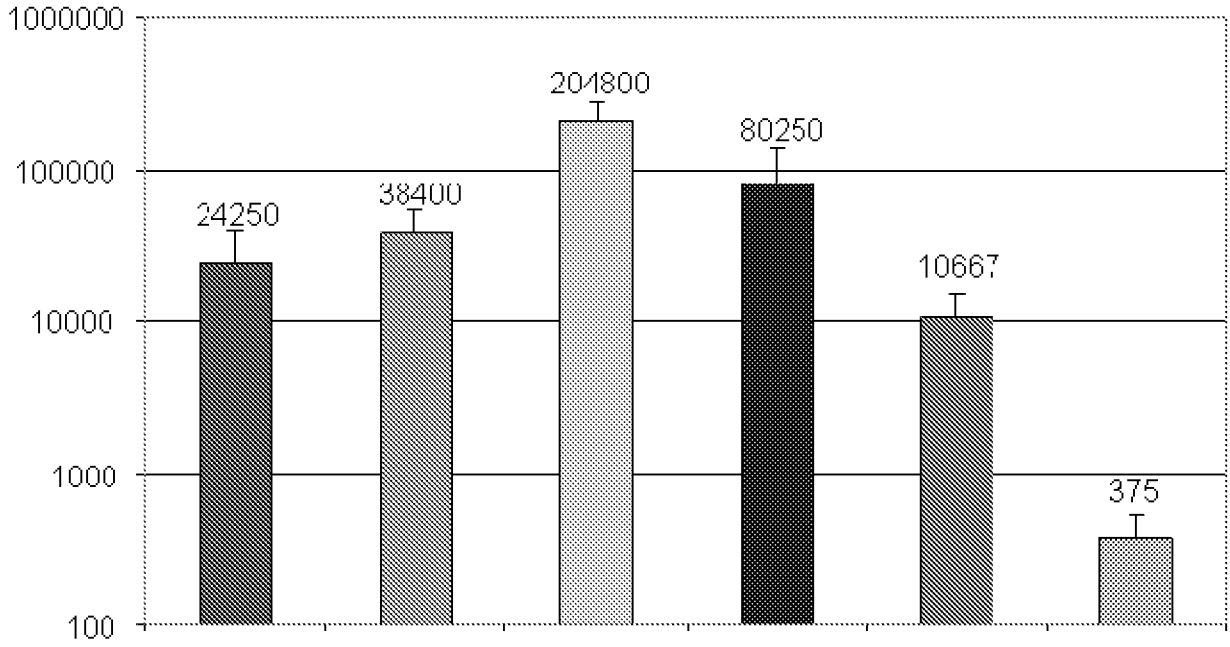

1000000
100000
10000
1000
100
24250
38400
201800
80250
10667
375

FIGURE 19

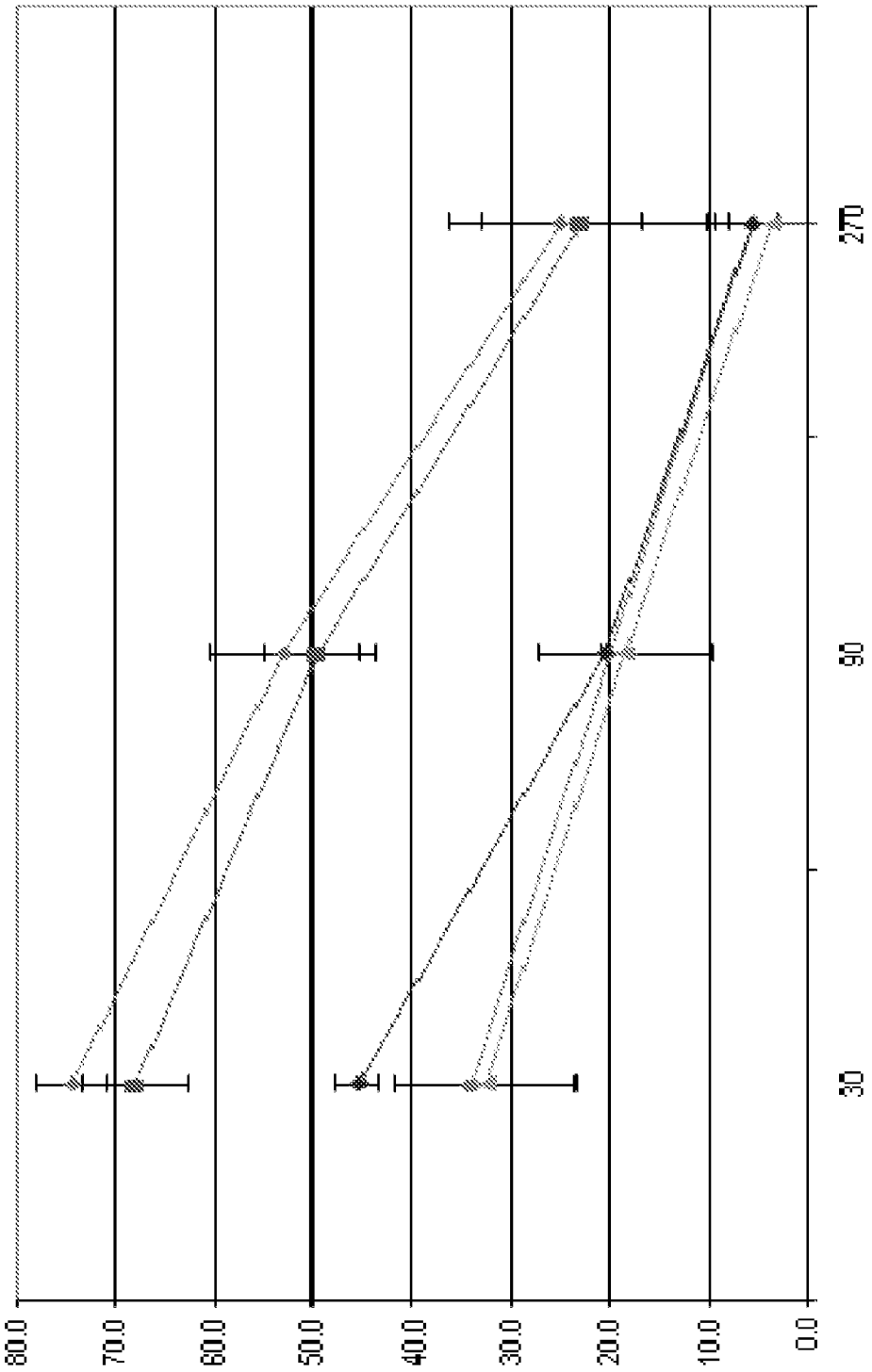


FIGURE 20

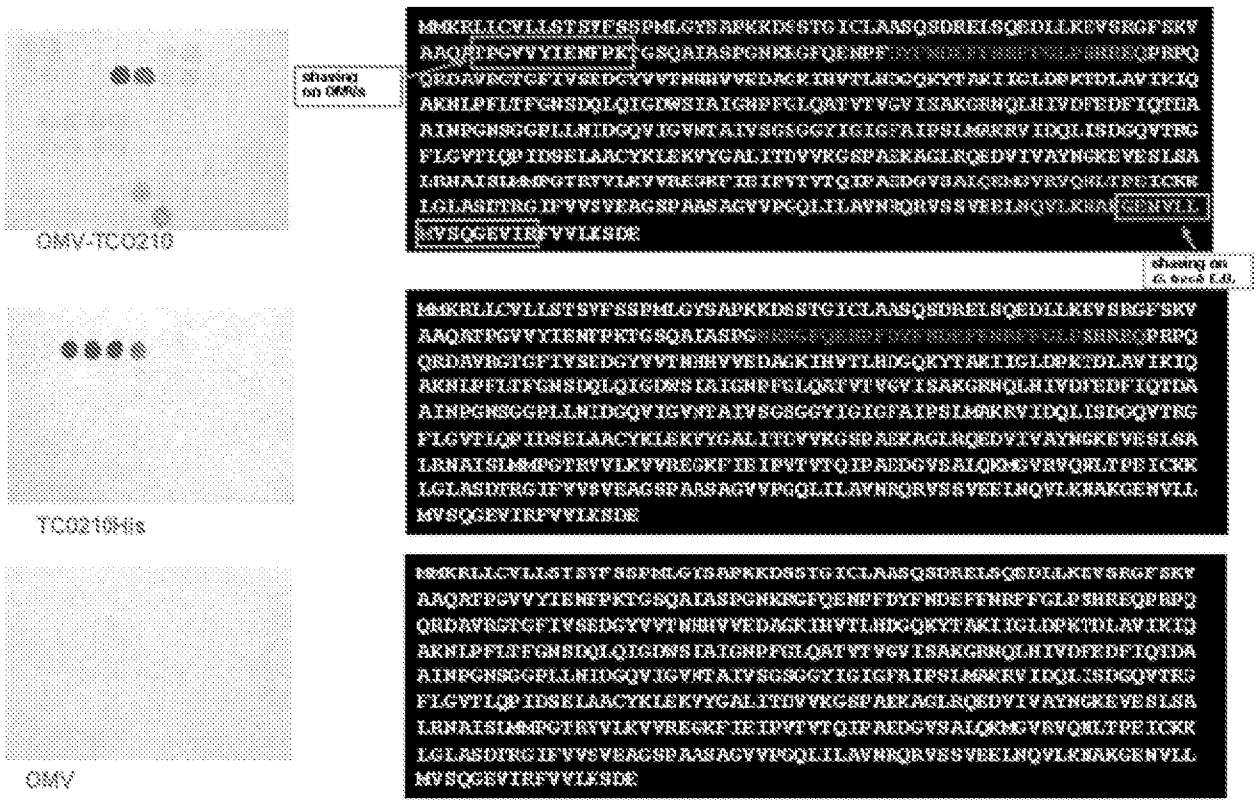
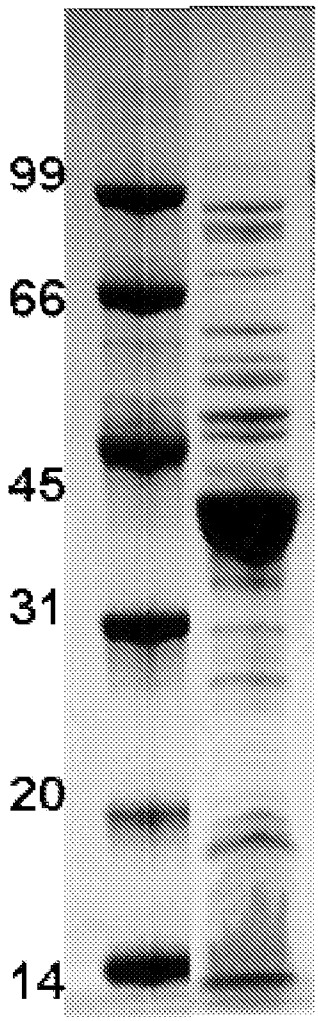


FIGURE 21

(i)



(ii)

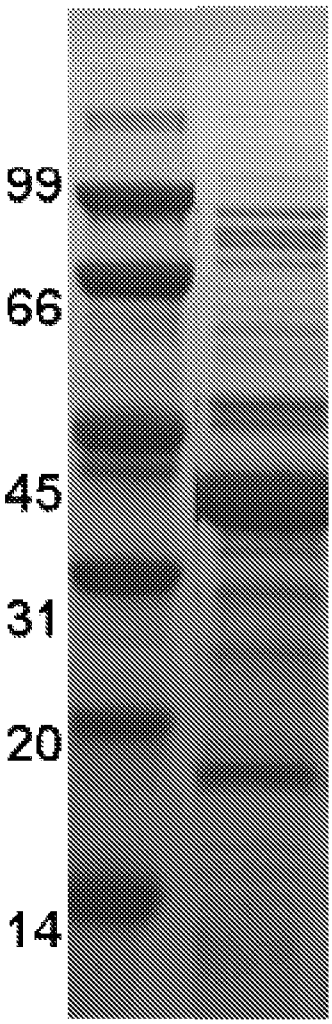


FIGURE 22

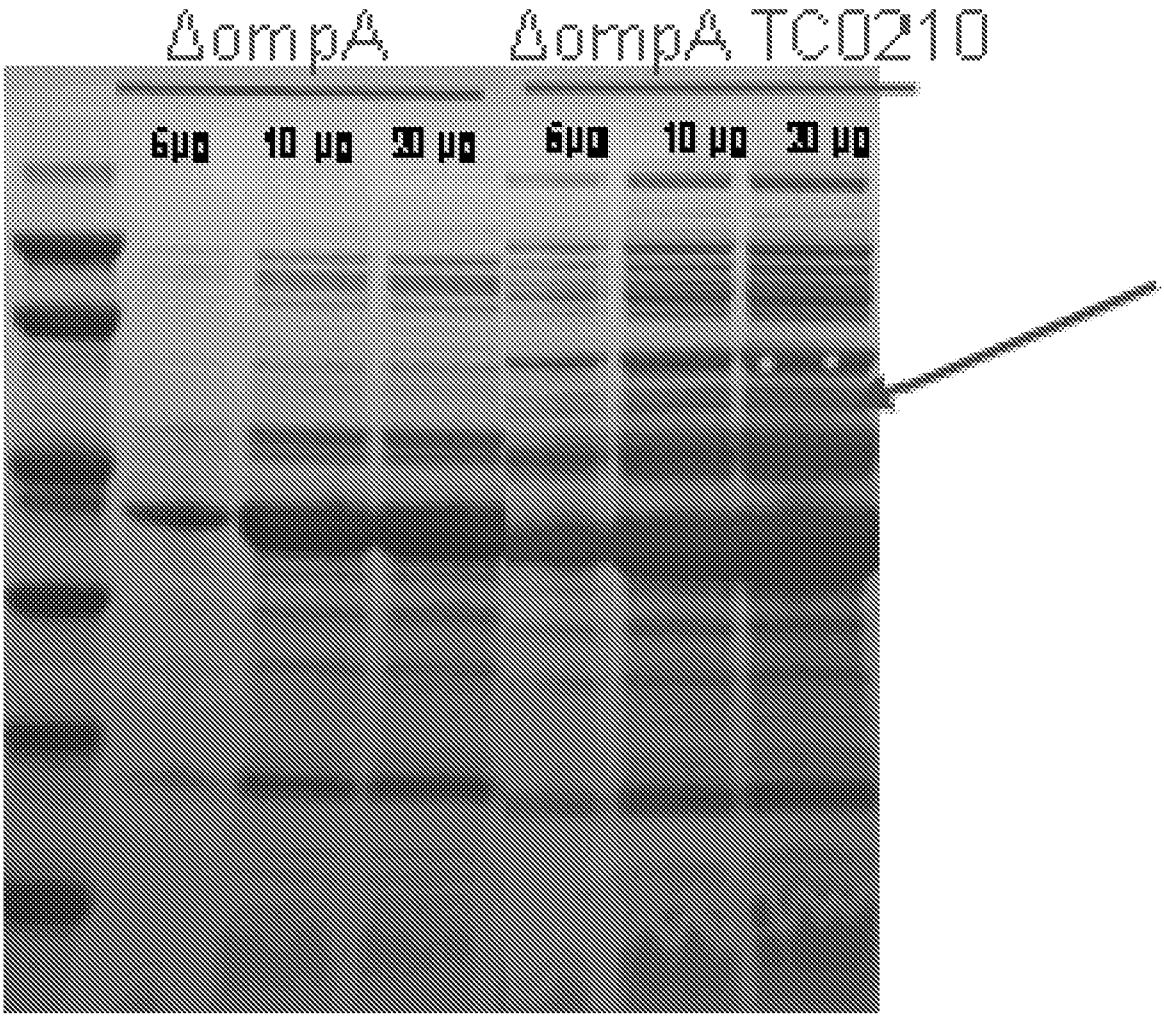


FIGURE 23

Match to: **TC_0210** Score: **102** Expect: **2.3e-006**

serine protease, HtrA/DegQ/DegS family (htrA) [3.4.21.-] {Chlamydia muridarum strain Nigg}

Nominal mass (M_r): **53261**; Calculated pI value: **6.36**
NCBI BLAST search of TC_0210 against nr

Variable modifications: Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Number of mass values searched: **57**
Number of mass values matched: **13**
Sequence Coverage: **34%**

Matched peptides shown in **Bold**

```
   1 MMKRLLCVLL STSVFSSPML GYSAPKKDSS TGICLAASQS DRELSQEDLL
  51 KEVSRGFSKV AAQATPGVVY IENFPKTGSQ AIASPGNKRG FQENPFDYFN
 101 DEFFNRFFGL PSHREQPRPQ QRDAVRGTGF IVSEDGYVVT NHHVVEDAGK
 151 IHVTLHDGQK YTAKIIGLDP KTDLAVIKIQ AKNLPFLTFG NSDQLQIGDW
 201 SIAIGNPFGL QATVTVGVIS AKGRNQLHIV DFEDFIQTDA AINPGNSGGP
 251 LLNIDGQVIG VNTAIVSGSG GYIGIGFAIP SLMAKRVIDQ LISDGQVTRG
 301 FLGVTLQPID SELAACYKLE KVYGALITDV VKGSPAEKAG LRQEDVIVAY
 351 NGKEVESLSA LRNAISLMMP GTRVVLKVVR EGKFIEIPVT VTQIPAEDGV
 401 SALQKMGVRV QNLTPEICKK LGLASDTRGI FVVSVEAGSP AASAGVVPGQ
 451 LILAVNRQRV SSVEELNQVL KNAKGENVLL MVSQGEVIRF VVLKSDE
```

Start - End	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Sequence
60 - 76	1803.89	1802.88	1802.96	-41	0	K.VAAQATPGVVYIENFPK.T
90 - 106	2185.96	2184.96	2184.92	17	0	R.GFQENPFDYFNDEFFNR.F
107 - 114	960.43	959.42	959.50	-76	0	R.FFGLPSHR.E
115 - 122	1038.46	1037.45	1037.54	-81	0	R.EQPRPQQR.D
127 - 150	2530.42	2529.42	2529.21	80	0	R.GTGFIVSEDGYVVTNHHVVEDAGK.I
172 - 182	1199.55	1198.55	1198.73	-152	1	K.TDLAVIKIQAK.N
287 - 299	1443.67	1442.66	1442.77	-78	0	R.VIDQLISDGQVTR.G
339 - 353	1632.76	1631.75	1631.86	-67	1	K.AGLRQEDVIVAYNGK.E
339 - 362	2617.63	2616.63	2616.39	91	2	K.AGLRQEDVIVAYNGKEVESLSALR.N
384 - 405	2355.39	2354.38	2354.27	45	0	K.FIEIPVTVTQIPAEDGVSALQK.M
410 - 419	1144.52	1143.51	1143.60	-73	0	R.VQNLTPEICK.K
472 - 489	1973.00	1971.99	1972.04	-26	1	K.NAKGENVLLMVSQGEVIR.F Oxidation(M)
475 - 489	1659.77	1658.77	1658.87	-61	0	K.GENVLLMVSQGEVIR.F Oxidation (M)

NO MATCH TO: 650.21, 656.18, 672.07, 697.35, 741.33, 777.37, 785.37, 861.00, 964.39, 986.43, 1002.43, 1111.49, 1155.52, 1170.52, 1243.57, 1249.46, 1287.59, 1295.51, 1331.61, 1333.59, 1367.62, 1375.63, 1388.63, 1580.64, 1717.72, 1745.71, 1763.84, 1825.89, 1847.82, 1904.86, 1932.88, 1954.02, 1974.94, 2034.92, 2149.07, 2240.07, 2275.26, 2378.32, 2553.36, 2619.60, 2645.60, 2916.71, 2970.85, 3217.98