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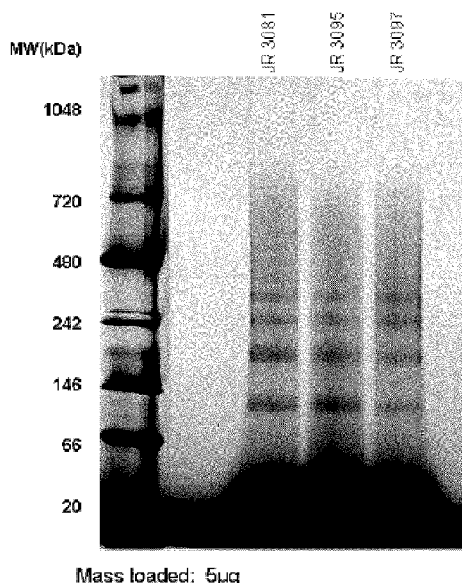
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(54) Title: EXPRESSION, PURIFICATION AND REFOLDING OF RECOMBINANT CHLAMYDIA PROTEINS, COMPOSITIONS AND RELATED METHODS

FIGURE 6A: Blue Native Gel Electrophoresis of samples of ScrE rMOMP



(57) Abstract: The present invention provides methods of obtaining a recombinant Chlamydia outer membrane protein (e.g. MOMP) that is expressed as an insoluble aggregated in a heterologous host in a soluble and immunogenic form and to proteins prepared in accordance to these methods. The invention also provides compositions comprising one or more proteins prepared in accordance to the methods of the invention. The proteins obtained in accordance to the methods of the present invention are soluble, immunogenic and in a substantially oligomeric form.



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EXPRESSION, PURIFICATION AND REFOLDING OF RECOMBINANT CHLAMYDIA PROTEINS, COMPOSITIONS AND RELATED METHODS

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims priority to U.S. Serial No. 61/413,663, filed November 15, 2010, and U.S. Serial No. 61/452,319, filed March 14, 2011, which are incorporated by reference herein in their entireties.

FIELD OF THE INVENTION

The present invention relates generally to recombinant protein purification and more particularly, to methods of purifying and folding recombinantly expressed proteins.

BACKGROUND OF THE INVENTION

Chlamydiae bacteria are obligate intracellular pathogens of eukaryotic cells. Four chlamydial species are currently known—*C. trachomatis*, *C. pneumoniae*, *C. pecorum* and *C. psittaci*—and genomic sequences for each of these are publicly available ((1999) Nature Genetics 21:385-389; (2000) Nucleic Acids Res 28:1397-1406; (2000) Nucleic Acids Res 28:2311-2314; (1998) Science 282:754-759).

C. trachomatis organisms are dimorphic, and alternate between 1) infectious “elementary bodies” (EBs) which are endocytosed by mucosal cells into vesicular inclusions; and 2) metabolically active, intracellular “reticulate bodies” (RBs). RBs replicate and redifferentiate into EBs before being released to infect neighbouring cells.

Treatment of EBs with n-lauroyl sarcosine produces “chlamydial OM complexes” (COMCs) containing three relatively detergent-resistant, cysteine-rich proteins: the Major Outer Membrane Protein (MOMP), encoded by *ompA*, and OmcB and OmcA, encoded by *omp2* and *omp3*, respectively (BMC Microbiology 2005, 5:5, and references therein). There are a number of other Chlamydial outer membrane proteins such as for example, PorB, PmpB, PmpC, PmpD, PmpG, and PmpH.

MOMP is expressed in both EBs and RBs and is situated on the outer membrane where it functions as a porin. It constitutes about 60% of the membrane protein of the infectious EB. Structural and functional analysis has shown that native MOMP exists as an oligomer; the native conformation of *C. trachomatis* MOMP is a trimer with monomers that have a β -barrel, β -sheet

secondary structure. In EB, MOMP forms trimers with disulfide bridges within and between its individual monomers (~ 40 kDa) and also between trimers (J. Bacteriol. 189:6222-6235, 2007).

There are at least 19 different *C. trachomatis* serovars capable of infecting humans (*i.e.*, A to K, Ba, Da, Ia, Ja, L1 to L3 and L2a) and these serovars have been typed based on serological differentiation of the antigenic epitopes on MOMP. The MOMPs encoded by each of these 19 different serovars share five well-conserved regions and four variable sequence segments or domains (termed VS or VD 1 to VD 4). The subspecies and serovar specific antigenic epitopes or determinants are located on the variable domains. Based on amino acid homology, the serovars have been subdivided into the following serogroups or classes: B class (B, Ba, D, Da, E, L1, L2 and L2a), C class (A, C, H, I, Ia, J, K and L3), and intermediate class (F and G). Infection with any *C. trachomatis* serovar may result in disease: serovars A, B, Ba and C cause trachoma; serovars L1-L3 are the agents of lymphogranuloma venereum; serovars D-K cause sexually transmitted infections; and serovars G, I and D have been associated with cervical cancer.

Chlamydial infection itself causes disease, and failure to clear the infection results in persistent immune stimulation which may lead to chronic infection with severe consequences (e.g., sterility and blindness). The precise immune correlates of protection remain to be determined, but cell-mediated immune responses and antibody mediated immune responses are each involved; the bacterium is an intracellular parasite, and as such, it can typically evade antibody-mediated immune responses, but antibodies to the *C. trachomatis* MOMP protein neutralize EB infectivity and B-cell-deficient mice are unable to prevent re-infection (which suggests a functional role for B cells in adaptive immunity) (Morrison *et al.*, 2005). While antibiotics may clear an infection, in many cases women are asymptomatic and therefore, are unlikely to seek treatment.

In view of these asymptomatic infections and the serious nature of the disease, there is a need for a suitable vaccine. Vaccines may be useful: (a) as a prophylactic vaccine by immunizing against chlamydial infection or against Chlamydia-induced disease; and/or (b) as a therapeutic vaccine by eradicating an established chronic chlamydial infection.

A number of the outer membrane proteins (OMPs) of the various chlamydial species have been considered as possible vaccine candidate antigens including MOMP but the study of these proteins however has been hampered by the difficulty in producing them in a purified, soluble recombinant form with the appropriate quaternary structure. A MOMP protein (either recombinantly expressed or extracted directly from the bacteria) needs to assume some degree of its native conformation such that it presents the conformational epitopes required to induce a

therapeutically effective immune response. For example, in a study using EB-MOMP extracted from *C. trachomatis mouse pneumonitis* (MoPn) (a natural pathogen of mice) under conditions to maintain its native trimeric conformation, this trimeric native-like form of EB-MOMP was able to induce in mice a protective response as effective as that elicited by live organisms against a genital challenge (Infect. Immun. 73:8153-8160, 2005).

The recombinant expression of MOMP in heterologous bacterial expression systems is commonly associated with the production of improperly folded recombinant proteins in insoluble aggregates, referred to as inclusion bodies. Solubilizing these aggregates and then refolding the proteins into their native conformation is problematic and often leads to low recovery.

MOMP is a detergent resistant, cysteine-rich protein, and as such, recombinantly expressed MOMP (rMOMP) can aggregate when oxidized or interact with, and form extensive disulfide bonds with other cysteine-rich proteins making it prone to misfolding and aggregation and difficult to purify and refold (Mol Microbiol 1992, 6:1087-1094; BMC Microbiology 2005, 5:5). Recombinant MOMP has been expressed in *E.coli* using full-length *ompA* genes that include the signal sequence to target the translated protein to the outer membrane but this approach too has proven problematic as the protein tends to misfold and aggregate (Manning et al. 1993). Others have cloned MOMP into plasmids with an in-frame histidine-tag such that the recombinantly expressed protein is his-tagged either at its N or C terminus and as such, purifiable using Ni-NTA chromatography (Coler *et al.* 2009). Such his-tagged recombinant protein preparations are not suitable however for use as commercial vaccines (e.g., due to the potential elicitation of an anti-his-tag response) and are not suitable for the large scale manufacturing required for commercial vaccines.

Therefore, a need still exists for methods that produce a soluble recombinant MOMP in a sufficiently native-like form and on a scale large enough to be commercially viable.

SUMMARY OF THE INVENTION

The present invention generally relates to methods for preparing soluble and immunogenic recombinant Chlamydial membrane outer membrane (MOMP) proteins. More particularly, the present invention relates to methods for obtaining recombinant Chlamydial MOMP that is expressed as an insoluble aggregate in a heterologous host, in a soluble and immunogenic form. The methods of the present invention are generally accomplished by:

- (a) isolating the insoluble aggregated protein;

- (b) admixing the insoluble aggregated protein from step(a) in an aqueous solution comprising a denaturing agent to denature the insoluble aggregated protein;
- (c) purifying the denatured protein from step (b) by subjecting the mixture of step (b) to at least one chromatographic purification in the presence of denaturing agent and collecting eluted solution of purified denatured protein;
- (d) admixing the purified denatured protein solution from step (c) with a reducing agent and at least one small molecule additive to enhance protein folding and/or suppress protein aggregation;
- (e) reducing concentration of denaturing agent and reducing agent to levels sufficient to allow the protein to renature into soluble and substantially oligomeric forms; and
- (f) isolating the substantially oligomeric forms.

Immunogenic compositions and methods for eliciting an immune response against Chlamydia infections (such as e.g., *C. trachomatis*) are also described. Preferred examples include immunogenic compositions comprising immunogenic recombinant MOMP proteins, methods for their production and their use. In further embodiments of the present invention, the recombinant MOMP proteins can be from any one of the known 19 different human serovars of *C. trachomatis* or any of the known serovars of *C. pneumoniae*.

In a further aspect of the invention, methods of immunizing a subject against disease caused by infection with a strain of Chlamydia are provided, which comprises administering to the subject an effective amount of a Chlamydia protein made in accordance to the methods described herein.

The invention provides several advantages. For example, the recombinant MOMP proteins obtained by the methods of the present invention are immunogenic and administration of these proteins (e.g., in the compositions of the present invention) to a subject elicits an immune response and/or an immunoprotective response against infections by *Chlamydia* (e.g., *C. trachomatis*). The methods of the present invention can be used to obtain soluble recombinant MOMP in substantially oligomeric forms. In addition, the methods of the present invention can be used to obtain recombinant MOMP suitable for use in the preparation of immunogenic compositions (e.g., vaccines).

Other features and advantages of the invention will be apparent from the following Detailed Description, the Drawings and the Claims.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will be further understood from the following description with reference to the drawings, in which:

Figure 1 shows a schematic representation of one embodiment of the purification, refolding process for recombinant proteins;

Figure 2 shows a representative SDS PAGE gel of rMOMP protein samples obtained after the various steps of the purification and refolding method;

Figures 3 shows a representative Blue Native PAGE gel analysis of rMOMP (serovar E, *C. trachomatis*) protein samples prepared in accordance to an embodiment of the invention: two pilot scale samples (left, A,B) and three lab scale samples (right, C-H). Pilot scale lot was approximately 10 fold larger than lab scale. The actual yields of the lots were as follows: pilot lot JR3182: 1.59 g, lab scale JR3081: 180 mg, JR3095: 121.9 mg, JR3097: 101.4 mg.

Figure 4 shows a representative SDS-PAGE gel of rMOMP (serovar E, *C. trachomatis*) protein samples prepared in accordance to an embodiment of the invention demonstrating the quaternary structure of the rMOMP protein at various steps (i.e., samples of the solubilized IBs, the purified denatured MOMP (Q pool), the folded protein, and the final product)

Figure 5 shows representative SDS-PAGE gels comparing two protein samples purified in accordance to an alternative embodiment. While the same starting material was used to prepare the samples run in the left panel gel and those run in the right panel gel, each panel of samples underwent a different purification process. In both gels, lanes are loaded in triplicate with 4 µg of protein. Improved purity is evident in the samples of the right panel.

Figures 6A, 6B, 6C show representative BN-PAGE gel analysis of rMOMP protein samples prepared in accordance to an embodiment of the invention.

Figure 7 shows representative SE-HPLC chromatograms for SerE rMOMP lots.

Figure 8 shows representative SE-HPLC chromatograms for SerF, Ia, and J rMOMP lots.

Figure 9 (A and B) shows a representative graphical comparison of elicited IgG titers as assessed using two different ELISA assays (the commercially available Medac™ assay and an ELISA developed using rMOMP made in accordance to the invention).

Figure 10 (A and B) shows a representative graphical correlation between sera IgG titers (as measured by IgG ELISA) and sera neutralization capacity. A strong positive correlation was

evident between IgG levels measured by the sp-IgG ELISA and neutralization titres in all participants (Figure 10A). The correlation was still evident when the 4 most visually obvious outliers were removed from the analysis (Figure 10B).

Figure 11 (A and B) shows a representative graphical correlation between sera IgG3 titers and neutralization capacity. A strong positive correlation was evident between IgG3 levels (as measured by the SP-IgG3 ELISA) and neutralization titers in all participants (Figure 11A), even when the most visually obvious outliers were excluded (Figure 11B).

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides novel methods of obtaining a recombinant Chlamydial MOMP proteins that have been recombinantly expressed in a host as an insoluble aggregate, in a soluble, immunogenic and substantially oligomeric form. Also provided are novel methods of purifying and/or refolding chlamydial outer membrane proteins that have been recombinantly expressed in a host cell as an insoluble aggregate. The present invention further provides novel recombinant proteins, particularly recombinant proteins obtained by these novel methods and immunogenic compositions comprising these proteins. These compositions are useful for eliciting an immune response (e.g., a neutralizing immune response) against a Chlamydia infection and/or for treating and/or preventing Chlamydial infections.

The methods for preparing such proteins are generally accomplished by: a) isolating the insoluble aggregated protein (e.g., by isolating inclusion body fractions enriched in MOMP protein); b) admixing the insoluble aggregated protein in an aqueous solution comprising a denaturing agent to denature (i.e., solubilize) the insoluble aggregated protein to provide a solution containing denatured MOMP protein; c) purifying the denatured MOMP protein by subjecting the mixture of step (b) to at least one chromatographic purification in the presence of denaturing agent and collecting eluted solution of purified denatured protein; d) renaturing (refolding) the purified denatured MOMP protein by admixing a reducing agent and at least one small molecule additive (and in preferred examples of the invention, using n-lauroyl sarcosine and in other preferred examples of the invention, using DTT, l-arginine and n-lauroyl sarcosine) and then reducing the concentration of both the denaturing and reducing agents to levels sufficient to allow the protein to renature into soluble and substantially oligomeric forms.

DEFINITIONS

The following includes definitions of selected terms that may be used throughout the disclosure. The definitions include various examples and/or forms of components that fall within the scope of a term and that may be used for implementation. The examples are not intended to be limiting. Both singular and plural forms of the terms fall within the definitions.

"Adjuvant", as used herein, refers to agents or substances that modulate the immunogenicity of an antigen. "Modulate the immunogenicity" includes enhancing the magnitude, duration and/or specificity or type of an immune response stimulated by an antigen.

A "heterologous host", as used herein, refers to a host cell (*e.g.*, prokaryotic, eukaryotic) that is transfected with a construct encoding a target protein not normally found in said host.

As used herein, "immunogenicity" refers to the ability of a substance to induce an immune response when administered to a subject (*e.g.*, a cellular immunogen-specific immune response and/or a humoral antibody response). As used herein and defined in the art, "antigenicity" is the ability of an antibody to recognize and bind to a protein (*e.g.*, an antigen).

An "immunoprotective response", as used herein, is meant to encompass humoral and/or cellular immune responses that are sufficient to: 1) inhibit or prevent infection by a microbial organism, particularly a pathogenic microbial organism; and/or 2) prevent onset of disease, reduce the risk of onset of disease, or reduce the severity of disease symptoms caused by infection by a microbial organism, particularly a pathogenic microbial organism.

As used herein, "inclusion bodies" are insoluble protein aggregates. The terms "insoluble aggregates" and "inclusion bodies" are used herein interchangeably.

"Isolated", as used herein, is meant to describe a compound of interest that is in an environment different from that in which the compound naturally occurs. "Isolated" is meant to include compounds that are within samples that are substantially enriched for the compound of interest and/or in which the compound of interest is partially or substantially purified.

Oligomeric proteins contain a number of polypeptide chains or subunits (*e.g.*, monomer units). Dimers, trimers and tetramers are examples of oligomers.

As used herein, the term "substantially purified" refers to a compound that is removed from its natural environment and is at least 60% free, preferably 75% free, more preferably 80% free and most preferably 90% free from other components with which it is naturally associated. The "% purity" of a compound (*e.g.*, protein sample) refers to % by which the compound is free

from detectable quantities of contaminants. Preferably a protein made in accordance to the method described herein is greater than 80% pure. Purity may be assessed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and scanning densitometry as described herein.

5 As used herein, the term “subject”, is meant any mammalian subject, particularly humans. Other subjects may include cattle, sheep (e.g. in detection of sheep at risk of abortion due to chlamydial infection), dogs, cats (e.g. in detection of cats having eye and/or respiratory infections), birds (e.g. chickens or other poultry), pigs, rabbits, rats, mice, horses, and so on. Of particular interest are subjects having or susceptible to Chlamydia infection, particularly to
10 infection by *C. trachomatis*, *C. psittaci* and/or *C. pneumoniae*.

As used herein, the term “effective amount” or “therapeutically effective amount” means a dosage sufficient to provide for treatment for the disease state being treated or to otherwise provide the desired effect (e.g. induction of an effective immune response or reduction of bacterial load). The precise dosage will vary according to a variety of factors such as subject-
15 dependent variables (e.g., age, immune system health, etc), the disease (e.g., the species of the infecting pathogen), and the treatment being effected. In the case of an intracellular pathogen infection, an “effective amount” is that amount necessary to substantially improve the likelihood of treating the infection, in particular that amount which improves the likelihood of successfully preventing infection or eliminating infection when it has occurred.

20 As used herein, non-reducing conditions for gel electrophoresis such as blue native polyacrylamide gel electrophoresis (BN-PAGE) means the preparation of samples without boiling and without the addition of reducing agents such as dithiothreitol (DTT) or 2-mercaptoethanol or beta-mercaptoethanol (BME).

“Treatment” or “treating” as used herein means any therapeutic intervention in a subject,
25 usually a mammalian subject, generally a human subject, including: (i) prevention, that is, causing the clinical symptoms not to develop, e.g. preventing infection and/or preventing progression to a harmful state; (ii) inhibition, that is, arresting the development or further development clinical, symptoms, e.g. mitigating or completely inhibiting an active (ongoing) infection so that bacterial load is decreased to the degree that it is no longer seriously harmful,
30 which decrease can include complete elimination of an infectious dose of Chlamydial bacteria from the subject; and/or (iii) relief, that is, causing the regression of clinical symptoms, e.g., causing a relief of fever, inflammation, and/or other symptoms and pathologies caused by an infection.

A polypeptide used with the invention may comprise an amino acid sequence that:
is identical (i.e. 100% identical) to a sequence disclosed in the sequence listing;
shares sequence identity (e.g. 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%,
99%, 99.5% or more) with a sequence disclosed in the sequence listing;
5 has 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 (or more) single amino acid alterations (deletions, insertions,
substitutions), which may be at separate locations or may be contiguous, as compared to the
reference sequence; and
when aligned with a particular sequence from the sequence listing using a pair-wise alignment
algorithm, such as the Needleman-Wunsch global alignment algorithm, using default parameters
10 (e.g. with Gap opening penalty = 10.0, and with Gap extension penalty = 0.5, using the
EBLOSUM62 scoring matrix). This algorithm is implemented in the needle tool in the EMBOSS
package.

Variants of MOMP polypeptides may comprise an amino acid sequence having 80% or
more sequence identity (e.g. 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%,
15 99.5% or more) to the serovar sequences disclosed herein (e.g. SEQ IDs 3, 8, 11, 13, 15, and 17)
or any other known or existing in the art.

Immunogenic compositions of the invention may be useful as vaccines. Vaccines
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.

20 The term "vaccine", as used herein, refers to a pharmaceutically acceptable formulation
of at least one antigen. Such pharmaceutical acceptable formulations of an antigen may also
include for example, adjuvants, excipients, diluents, and/or other similar substances that enhance
the activity, stability, and/or other properties of a formulation or administration.

Methods of Purifying and Refolding

25 In one aspect, the present invention provides a method of obtaining an outer membrane
protein (e.g., rMOMP) that is recombinantly expressed as an insoluble aggregate in a
heterologous host, in a soluble and immunogenic form.

The isolation, purification and refolding of recombinantly expressed MOMP proteins into
a form having a degree of fidelity to the native oligomeric form, is desirable.

30 Recombinant proteins (such as for example, rMOMP) often form aggregates or inclusion
bodies when over expressed in heterologous expression systems usually in an insoluble form.
This usually occurs because the expressed protein is not correctly folded. In order to obtain

proteins in a correctly refolded form, various steps are required (e.g., disruption of the host cells, isolation of the inclusion bodies, and then dissolving (i.e., denaturing) them in a chaotropic agent). The denatured protein is then transferred to an environment that favours a return to its native conformation; during the process the protein undergoes a transition through various semi-stable intermediates, any of which can lead to aggregation as opposed to the desired native conformation.

The present invention provides an efficient method for purifying and refolding a recombinant Chlamydia outer membrane protein from a denatured state, which overcomes the shortcomings of current methods.

In these methods a suitable heterologous host cell (e.g., *E. coli*) is transfected with a construct encoding the target protein (e.g., MOMP of any serovar of a species of Chlamydia). Suitable host cells can be prokaryotic or eukaryotic. Examples of suitable hosts cells that can be used to express recombinant proteins include bacteria, yeast, insect and mammalian cells. Bacteria cells are particularly useful, especially *E. coli*. Methods of expressing a heterologous protein in a host cell are well known in the art and examples are provided herein.

The present invention encompasses a wide variety of recombinant proteins. These proteins include outer membrane proteins (such as for example, MOMP, OmcA, OmcB and PorB) of each Chlamydial species (*C. trachomatis*, *C. pneumoniae*, *C. pecorum*, *C. psittaci*). The present methods also can enhance the expression recovery and purification of these recombinant outer membrane proteins.

MOMP expressed recombinantly typically is produced intracellularly in an insoluble aggregated form (i.e., as inclusion bodies). The cells expressing the target protein in an insoluble aggregated form are collected and lysed, to isolate the insoluble aggregates. Cell lysis can occur prior to, or coincident with, the solubilization procedures described below. Cell lysis can be accomplished by, for example, mechanical shear such as a French pressure cell, enzymatic digestion, sonification, homogenization, glass bead vortexing, detergent treatment, organic solvents, freeze thaw, grinding with alumina or sand, treatment with a denaturing agent as defined below, and the like.

The insoluble aggregated material (inclusion bodies) can be separated from soluble proteins by various methods such as centrifugation, filtration (including ultrafiltration), precipitation or settling. Generally, the inclusion bodies are separated from cell debris using low-speed centrifugation after cell lysis as they are denser than most of the cellular components.

These recovered inclusion bodies may be contaminated with *E. coli* cell wall and outer membrane components. The later may largely be removed by selective extraction with detergents and low concentration of either urea or guanidine-HCl to produce wash pellets. Alternatively, the application of mild detergents to cell lyates will promote dissolution of most cellular structures, while leaving the inclusion bodies intact, which can then be collected by low speed centrifugation.

Next, the insoluble aggregated material is solubilized (i.e., rendered soluble, denatured or monomeric) by exposing the insoluble aggregated material (or whole cells without prior lysis) to a denaturing agent. Useful denaturing agents include chaptropic reagents such as urea, guanidine (guanidine hydrochloride), agents such as arginine, and sodium thiocyanate, extremes in pH (dilute acids or bases), detergents (e.g., SDS, N-lauroyl sarcosine), salts (e.g., chlorides, nitrates, thiocyanates, cetylmethylammonium salts, trichloroacetates), chemical derivatization (e.g., sulfitolysis, reaction with citraconic anhydride), solvents (e.g., 2-amino-2-methyl-1-propanol or other alcohols, DMSO, DMF). In preferred examples, the denaturing agent is guanidine and/or urea. Useful concentrations of either guanidine or urea are 1-8M with 6-8M being preferred concentrations. Chelating agents such as EDTA may be used in this step to prevent metal catalyzed air oxidation of cysteines during the removal of chaptrotic reagents (such as for e.g., guanidine, urea).

Preferably, a disulfide reducing agent is also used in conjunction with the denaturing agent. Useful disulfide reducing agents include thiol compounds such as dithiothreitol (DTT) or beta-mercaptoethanol. These compounds can be used in the range of 1-100 mM with ~ 10 mM being a typical concentration.

In one example, inclusion bodies are solubilized in guanidine (e.g., 6M). Prior to purification, the guanidine is exchanged with urea (e.g., 8M urea) for example, by tangential flow filtration. In a further example, inclusion bodies are recovered by centrifugation and the inclusion body pellet is solubilized in 8M urea (at about pH 12.5). Preferably, the solubilization solution also includes a reducing agent such as for example, DTT (e.g., 10mM).

In one embodiment, isolated inclusion bodies are solubilized by adding (20-40 mL/g) in 8M Urea, pH 12.5 and incubating mixture for about 30 minutes. Following incubation, DTT (10mM) is added and the pH of the solution is reduced to about pH 6 with citrate/phosphate buffer. The solubilization supernatant is then diluted with Urea (e.g., 8M with 10mM DTT) to reach a conductivity of preferably about 2.0-2.5 mS/cm. The resulting supernatant is then filtered.

Following solubilization, the soluble and denatured protein is recovered and isolated from other proteins in the soluble fraction (mixture). Such recovery and purification methods are known or readily determined by those skilled in the art, including for example, centrifugation, filtration, dialysis, chromatography, including size exclusion, ion-exchange, hydrophobic interaction and affinity chromatography procedures and the like. The denatured protein solution subjected to at least one chromatographic purification (such as for example, ion-exchange, hydrophobic interaction and affinity chromatography). Preferably the solution is subjected to at least one anion exchange chromatography. In preferred examples, the solution is subjected first to cation exchange chromatography (to capture impurities) and then to anion exchange chromatography. With anion exchange chromatography, the column is preferably washed with a solution comprising Urea and a salt (e.g., 8M Urea, 15 mM NaCl) and the rMOMP protein may be eluted from the column using a solution of Urea with a higher salt concentration (e.g., (8M Urea, 30-90 mM NaCl). Throughout the purification process the solubilized protein is maintained in a denatured state (for example, by ensuring the constant presence of urea).

The next step in the process is to refold the solubilized and purified protein to obtain the desired conformation. In the achieved refolded conformation, the protein is soluble, immunogenic and in form that is similar to the native oligomeric form. In the achieved refolded conformation, the protein is capable of eliciting neutralizing antibodies to the corresponding species (and/or serovar) of Chlamydia from which it was derived. In preferred examples, the protein is in a substantially oligomeric form which preferably includes at least a trimeric form and preferably includes less than 6% of the monomeric form. Preferably, less than 5% is in the monomeric form.

The purification of a denatured protein before subjecting it to refolding conditions is unconventional; chromatographic approaches for purification are generally not well suited to protein mixtures containing the high levels of strong denaturants (e.g., 8 M urea or 6 M guanidine hydrochloride) required to solubilize inclusion body preparations.

The aqueous solution of purified protein is mixed with a reducing agent (e.g., DTT, DTE, or 2-mercaptoethanol) and at least one small molecule additive. Refolding is achieved by reducing the concentrations of the denaturing agent and the reducing agent to levels sufficient to allow the protein to re-nature into a soluble, conformationally native-like form (i.e., oligomeric). This can be achieved by dialysis, dilution, gel filtration, precipitation of the protein or by immobilization on a resin followed by buffer washes. In preferred examples, concentrations are reduced using at least one tangential flow filtration.

Conditions for this step are chosen to allow for regeneration of the protein's native disulfide bond(s) and therefore consider the redox environment. A reducing agent such as DTT, DTE, or 2-mercaptoethanol is used to reduce disulfide bonds to the sulfhydryl state when the protein is denatured, ensuring complete unfolding. Re-establishment of these disulfide bonds, which confers appropriate secondary structure, occurs when the buffer environment is allowed to promote oxidation, when the protein is newly and correctly re-folded. Alternatively, this can be accomplished through the addition of an oxidizing agent and a reducing agent, to catalyze a disulfide exchange reaction. Preferably, a reagent or combination of reagents are chosen that result in native disulfide bond formation (e.g., oxygen, cysteine/oxygen, cysteine/cystine, cysteine/cystamine, cysteamine/cystamine, reduced glutathione/oxidized glutathione, and the like).

Small molecule additives (also known as chemical chaperones) may be added to guide the protein towards the correct conformation. Examples of small molecule additives for use in the disclosed methods, include those which enhance protein folding and/or suppress protein aggregation. Examples of small molecule additives for use in the disclosed methods include l-arginine, N-lauroyl sarcosine, sucrose and ammonium sulfate. Preferred small molecule additives are l-arginine and N-lauroyl sarcosine.

The purified protein is refolded by removing or reducing the concentration of the denaturant and any excess reducing agent. Preferably, the concentration of the denaturant (e.g., Urea or guanidine hydrochloride) is reduced over a few minutes, to provide a stable, folded protein. There are intermediate folding states of the protein, from which insoluble misfolded aggregates can form and these intermediate folding states are sensitive to concentration as they follow second order kinetics. Reducing the concentration of the denaturant (and reducing agent if applicable) in a minimal amount of time reduces the opportunity for mis-folding.

In the preferred practice of the present invention, as exemplified herein, the refolding process involves a diafiltration of a volume of purified denatured recombinant protein (e.g., rMOMP) in urea (e.g., 8M) mixed with equal volumes of arginine (e.g., 1M – 3M) and NLS (e.g., 2-30% v/v) and DTT (e.g., 10 mM). Preferred embodiments of the present invention are depicted in Figure 1 and are described in the examples herein.

The recombinant proteins obtained according to these methods can be further processed if desired. For example, residual contaminants can be removed. The isolated rMOMP protein is preferably in a buffered solution having a pH of 7.0 to 8.5, and preferably 7.5 to 8.5. The protein

solution preferably has a residual concentration of NLS of 0.5% (w/v) or less, of DTT of 24 µg/ml or less, and of urea of 10 mg/ml or less.

The purified recombinant MOMP proteins obtained by the methods of the present invention are identifiable via their known physical, chemical, immunological or biological properties (e.g., by means of SDS-PAGE, BN-PAGE, isoelectric focusing, specific monoclonal antibodies, and immunological parameters such as, antibody ELISA, neutralizing antibody titres, cytokine levels, lymphoproliferative responses.

The yield of rMOMP obtained by the methods of the present invention is 75-95% or higher.

The protein preparations produced by the present methods can be used for a variety of *in vitro* and *in vivo* applications. The proteins and their derivatives of the present invention can be used for research, diagnostic, prophylactic or therapeutic purposes. *In vitro* uses include, for example the use of the protein for screening, detecting and/or purifying other proteins.

Compositions, Formulations and Administration

The present invention provides immunogenic compositions useful for treating and/or preventing Chlamydial infections. In another aspect, the present invention provides methods of inducing anti-chlamydial immunity by administering the immunogenic compositions provided, either alone or in a prime boost protocol. An anti-chlamydial immune response can be defined as a reduction in bacterial load in the immunized host upon challenge with live Chlamydia, and/or the stimulation of protective levels of IFN-γ in the host cells (immunoprotective response).

The data presented herein and described in detail below demonstrates that immunization with compositions comprising recombinantly expressed outer membrane proteins (e.g., rMOMP) made in accordance to the methods described herein elicits both cellular and humoral immune responses.

Compositions (e.g., vaccine compositions) of the present invention may be administered in the presence or absence of an adjuvant. Adjuvants generally are substances that can enhance the immunogenicity of antigens. Adjuvants may play a role in both acquired and innate immunity (e.g., toll-like receptors) and may function in a variety of ways, not all of which are understood. Preferably the antigen induces a Th1 biased response or a balanced Th1/Th2 response.

Many substances, both natural and synthetic, have been shown to function as adjuvants. For example, adjuvants may include, but are not limited to, mineral salts, squalene mixtures,

muramyl peptide, saponin derivatives, mycobacterium cell wall preparations, certain emulsions, monophosphoryl lipid A, mycolic acid derivatives, nonionic block copolymer surfactants, Quil A, cholera toxin B subunit, polyphosphazene and derivatives, immunostimulating complexes (ISCOMs), cytokine adjuvants, MF59 adjuvant, lipid adjuvants, mucosal adjuvants, certain
5 bacterial exotoxins and other components, certain oligonucleotides, PLG, and others. These adjuvants may be used in the compositions and methods described herein.

In preferred examples, the composition comprises at least one Chlamydial MOMP protein (produced in accordance to the methods of the invention), and an adjuvant, characterized in that the adjuvant comprises at least:

- 10 - an oil-in-water emulsion comprising at least squalene, an aqueous solvent, a polyoxyethylene alkyl ether hydrophilic nonionic surfactant, a hydrophobic nonionic surfactant, wherein said oil-in-water emulsion is obtainable by a phase inversion temperature process and wherein 90% of the population by volume of the oil drops has a size less than 200 nm, and optionally less than 150 nm; and
- 15 - a TLR agonist (e.g., TLR4 agonist such as for example, the product E6020 having CAS Number 287180-63-6).

Such an adjuvant is described in WO2007006939 (Vaccine Composition Comprising a Thermoinversable Emulsion) which is incorporated herein in its entirety. The product E6020 is described in US2007/0082875 (which is incorporated herein by reference in its entirety).

- 20 In certain embodiments, the immunogenic composition includes at least one Chlamydia outer membrane protein made in accordance to the methods described herein with or without an adjuvant. For example, the immunogenic composition may include MOMP from at least one serovar of a Chlamydia species (e.g., *C. trachomatis*) and preferably, it includes MOMP from at least two or at least three or more serovars of a Chlamydia species. Preferably, the immunogenic
25 composition prevents infection in a subject by inducing functional antibodies and appropriate CD4 and CD8 T-cell responses. Ideally, the composition elicits appropriate functional antibodies and IFN- γ producing CD4 T-cells (analogous to a Th1 type response in mice).

- 30 The immunogenic compositions of the present invention are preferably in liquid form, but they may be lyophilized (as per standard methods) or foam dried (as described in WO2009012601, Antigen-Adjuvant Compositions and Methods). A composition according to one embodiment of the invention is in a liquid form. An immunization dose may be formulated

in a volume of between 0.5 and 1.0 ml. Liquid formulations may be in any form suitable for administration including for example, a solution, or suspension.

The pH of the formulation (and composition) is preferably between about 6.4 and about 9. More preferably, the pH is about 7.4. The pH may be maintained by the use of a buffer.

5 The pharmaceutical formulations of the immunogenic compositions of the present invention may also optionally include one or more excipients (e.g., diluents, buffers, preservatives, detergents and/or immunostimulants) which are well known in the art. Suitable excipients are compatible with the antigen and with the adjuvant as is known in the art. Examples of detergents include a Tween (polysorbate) such as Tween 80.

10 The immunogenic compositions of the invention find use in methods of preventing or treating a disease, disorder condition or symptoms associated with Chlamydia. The terms disease disorder and condition will be used interchangeably herein. Specifically the prophylactic and therapeutic methods comprise administration of a therapeutically effective amount of a pharmaceutical composition to a subject. In particular embodiments, methods for preventing or
15 treating Chlamydia are provided.

As used herein, preventing a disease or disorder is intended to mean administration of a therapeutically effective amount of a pharmaceutical composition of the invention to a subject in order to protect the subject from the development of the particular disease or disorder associated with Chlamydia.

20 By treating a disease or disorder is intended administration of a therapeutically effective amount of a pharmaceutical composition of the invention to a subject that is afflicted with a disease caused by Chlamydia or that has been exposed to Chlamydia where the purpose is to cure, heal alleviate relive alter remedy ameliorate improve or affect the condition or the symptoms of the disease.

25 A therapeutically effective amount refers to an amount that provides a therapeutic effect for a given condition and administration regimen. A therapeutically effective amount can be determined by the ordinary skilled medical worker based on patient characteristics (e.g., age, weight, gender, condition, complications other diseases). The therapeutically effective amount will be further influenced by the route of administration of the composition.

30 For prophylactic uses, one skilled in the art can readily determine the appropriate dose, frequency of dosing and route of administration. Factors in making such determinations include, without limitation, the nature of the protein to be administered, the condition to be treated,

potential patient compliance, the age and weight of the patient and the like. The immunogenic preparations and vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective (i.e., protective against Chlamydial infection). The quantity to be administered is subject dependent, including for example the capacity of the individual's immune system to synthesize antibodies to the composition and produce a cell-mediated immune response. Suitable dosage ranges are readily determinable by one skilled in the art and may be of the order of about 1 μ g to about 1mg of the soluble, immunogenic recombinant protein (e.g., rMOMP). Suitable regimes for initial administration and booster doses are also variable but may include an initial administration followed by subsequent administration. The dosage may also depend on the route of administration and will vary according to the size of the subject. The invention also provides compositions including antigenic material of several pathogens (combined vaccines). Such combined vaccines contain for example material from various pathogens or from various strains of the same pathogen, or from combinations of various pathogens.

In another aspect, the present invention provides methods of inducing anti-chlamydial immunity by administering the immunogenic compositions provided, either alone or in a prime boost protocol. An anti-chlamydial immune response can be defined as a reduction in bacterial load in the immunized host upon challenge with live chlamydia, and/or administration of protective levels of IFN- γ in the host cells.

Immunogenic compositions may be presented in a kit form comprising the immunogenic composition and an adjuvant or a reconstitution solution comprising one or more pharmaceutically acceptable diluents to facilitate reconstitution of the composition for administration to a mammal using conventional or other devices. Such a kit would optionally include the device for administration of the liquid form of the composition (e.g. hypodermic syringe, microneedle array) and/or instructions for use.

The present disclosure also provides methods of eliciting an immune response in a subject by administering the immunogenic compositions, or formulations thereof, to subjects. This may be achieved by the administration of a pharmaceutically acceptable formulation of the compositions to the subject to effect exposure of the immunogenic polypeptide and/or adjuvant to the immune system of the subject. The administrations may occur once or may occur multiple times. In one example, the one or more administrations may occur as part of a so-called "prime-boost" protocol.

Compositions of the invention can be administered by an appropriate route such as for example, percutaneous (*e.g.*, intramuscular, intravenous, intraperitoneal or subcutaneous), transdermal, or mucosal (*e.g.*, intranasal), in amounts and in regimes determined to be appropriate by those skilled in the art. Exposure of the subject to the compositions disclosed herein may result in establishment of a temporary or permanent immune response in the subject. The immune response may protect the subject from subsequent exposure to the antigen, often by subsequent exposure to an infectious agent from which the antigen was derived. Therapeutic effects may also be possible.

The composition may be administered in dosage unit formulations containing conventional pharmaceutically acceptable carriers and vehicles. The term "pharmaceutically acceptable carrier" as used herein refers to one or more formulation materials suitable for accomplishing or enhancing the delivery of a protein or polypeptide as a pharmaceutical composition. In certain embodiments, a pharmaceutical composition is a composition comprising a therapeutically effective amount of a polypeptide or protein. The terms "effective amount" and "therapeutically effective amount" each refer to the amount of a polypeptide or protein used to induce or enhance an effective immune response. It is preferred that compositions of the present invention provide for the induction or enhancement of an immune response in a host which protects the host from the development of an infection or allows the host to eliminate an existing infection from the body.

The compositions and vaccines disclosed herein may also be incorporated into various delivery systems. In one example, the compositions may be applied to a "microneedle array" or "microneedle patch" delivery system for administration. These microneedle arrays or patches generally comprise a plurality of needle-like projections attached to a backing material and coated with a dried form of a vaccine. When applied to the skin of a subject, the needle-like projections pierce the skin and achieve delivery of the vaccine, effecting immunization of the subject.

While the compositions of the invention can be administered as the sole active pharmaceutical agent, they can also be used in combination with one or more other compositions or agents (*i.e.*, other chlamydial antigens, co-stimulatory molecules, adjuvants). When administered as a combination, the individual components can be formulated as separate compositions administered at the same time or different times, or the components can be combined as a single composition.

All references cited within this disclosure are hereby incorporated by reference in their entirety.

Examples

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific Examples. These Examples are described solely for purposes of illustration and are not intended to limit the scope of the invention. Changes in form and substitution of equivalents are contemplated as circumstances may suggest or render expedient. Although specific terms have been employed herein, such terms are intended in a descriptive sense and not for purposes of limitations.

Methods of molecular genetics, protein biochemistry, immunology and fermentation technology used, but not explicitly described in this disclosure and these Examples, are amply reported in the scientific literatures and are well within the ability of those skilled in the art.

Example 1A. Recombinant Cloning of Mouse MOMP Protein (MoPn)

This Example describes the cloning of the MOMP gene (and MOMP gene fragments) from the *C. trachomatis* mouse pneumonitis strain (MoPn), a natural murine pathogen.

MOMP DNA sequence (full-length but lacking the signal peptide) was amplified from genomic DNA of Chlamydia MoPn with primers containing NdeI and XhoI sites (SEQ ID NOs. 1 and 2). The resulting PCR product was digested with NdeI and XhoI and ligated into NdeI and XhoI digested and dephosphorylated pET24b(+). The ligation mixture was transformed into chemically competent *E. Coli* DH5 α and positive clones were selected by plating on Luria agar containing 50 μ g/ml kanamycin. Single colonies were cultured overnight in Luria Broth containing 50 μ g/ml kanamycin and plasmid DNA was isolated using a QIAprep Spin Miniprep Kit. NdeI/ XhoI digests were performed to determine which clones had the correct fragment. Clone pKNE38 had the correct size fragment and an aliquot was sent for DNA sequence analysis, confirming the correct MOMP DNA sequence (and the predicted amino acid sequence of inserted coding sequence is set out as SEQ ID NO:3). Plasmid DNA from pKNE38 was transformed into chemically competent *E. Coli* BL21(DE3) cells using heat shock. Protein expression was assessed by culturing a single colony and inducing expression with 1 mM IPTG. Glycerol stocks were made from the overnight culture of this material for subsequent use. SDS-PAGE and Western analysis were used to identify protein expressed.

Sequence of Primers (5' $\rightarrow$ 3')

Primer No.1A: GAA TCA GTC ACA TAT GCT GCC TGT GGG GAA TCC TGC (SEQ ID NO:1)
 Primer No. 1B: CTG CAG TCA CTC GAG TCA TTA GAA ACG GAA CTG AGC ATT TAC (SEQ ID NO:2)

Predicted Amino Acid Sequence of Inserted Coding Sequence (SEQ ID NO:3)

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1      MLPVGNPAEP SLMIDGILWE GFGGDPCDPC TTWCDAISLR LGYYGDFVFD
51     RVLKTDVNKQ FEMGAAPTGD ADLTTAPTPA SRENPAYGKH MQDAEMFTNA
101    AYMALNIWDR FDFVCTLGAT SGYLKGNsAA FNLVGLFGRD ETAVAADDIP
5      151     NVSLSQAVVE LYTDTAFAWS VGARAALWEC GCATLGASFQ YAQSKPKVEE
201    LNVLCNAAEF TINKPKGYVG QEFPLNIKAG TVSATDTKDA SIDYHEWQAS
251    LALSRLNMF  TPYIGVKWSR ASFDADTIRI AQPKLETSIL KMTTWNPTIS
301    GSGIDVDTKI TDTLQIVSLQ LNKMKSRKSC GLAIGTTIVD ADKYAVTVET
351    RLIDERAAHV NAQFRF

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10 **Example 1B. Recombinant Cloning of MOMP from human afflicting serovars of *C. trachomatis***

This Example describes the cloning of MOMP from *C. trachomatis* serovars that afflict humans. The rMOMP cloned as described below were used in various studies, as described in the examples that follow.

15 **MOMP Serovar D**

The nucleotide sequence of full-length MOMP (but lacking signal sequence) was amplified from genomic DNA of *C. trachomatis* serovar D strain UW-3/Cx , and the resulting PCR product was digested and ligated into pET24b(+). The ligation mixture was transformed into chemically competent *E. Coli* DH5 α and positive clones were selected and single colonies were cultured overnight. Plasmid DNA was isolated using a QIAprep Spin Miniprep Kit. A single clone was selected and DNA sequence analysis, confirmed the presence of the correct MOMP DNA sequence (SEQ ID NO: 9). The predicted amino acid sequence of the insert is SEQ ID NO: 8. Plasmid DNA from the clone was transformed into chemically competent *E. Coli* BL21(DE3) cells and MOMP protein expression was induced with 1mM IPTG, confirmed by SDS-PAGE and Western analysis (and was considered sufficient for downstream protein purification).

MOMP was cloned in a substantially similar manner from serovars E, F, J and Ia. Corresponding DNA and amino acid sequences for cloned serovar E, F, J and Ia rMOMP proteins are set out below. For serovars J and Ia, Primer Nos. 2A and 2B were utilizing for cloning. The primers utilized for cloning of MOMP from serovars E and F are provided below. Sequencing of cloned proteins from all serovars was done using the T7 and 7294.BB primers.

Sequence of Primers used for sequencing (5' $\rightarrow$ 3')

T7: TAA TAC GAC TCA CTA TAG GG (SEQ ID NO: 4)

7294.BB: TAT GCT AGT TAT TGC TCA GCG GTG (SEQ ID NO: 5)

Sequence of Primers used for Cloning (5' → 3')

Primer No.2A: GCA TGA CAT ATG CCT GTG GGG AAT CCT GC
(SEQ ID NO: 6)

5 Primer No. 2B: CGA TCG GGA TCC TTA GAA GCG GAA TTG TGC ATT TAC
(SEQ ID NO:7)

Predicted Amino Acid Sequence of Inserted Coding Sequence (SEQ ID NO: 8)

1 MPVGNPAEPS LMIDGILWEG FGGDPCDPCA TWCDASMRV GYYGDFVFDR
51 VLKTDVNKEF QMGAKPTTDT GNSAAPSTLT ARENPAYGRH MQDAEMFTNA
101 ACMALNIWDR FDFVCTLGAT SGYLKGNAS FNLVGLFGDN ENQKTVKAES
10 151 VPNMSFDQSV VELYTDTTFA WSVGARAALW ECGCATLGAS FQYAQSKPKV
201 EELNVLNAA EFTINKPKGY VGKEFPLDLT AGTDAATGTK DASIDYHEWQ
251 ASLALSRYLN MFTPYIGVKW SRASFDADTI RIAQPKSATA IFDTTTTLNPT
301 IAGAGDVKTG AEGQLGDTMQ IVSLQLNKM SRKSCGIAVG TTIVDADKYA
351 VTVETRLIDE RAAHVNAQFR F

15 MOMP Serovar E

atgcctgtgggaatcctgctgaaccaagccttatgatcgacggaattctgtgggaaggtttcggcggagat
ccttgcgatccttgaccacttggtgtgacgctatcagcatgcgtatgggttactatgggtgactttgttttc
gaccgtgttttgaaaacagatgtgaataaagaattccaaatgggtgacaagcctacaagtactacaggcaat
gctacagctccaaccactcttacagcaagagagaatcctgcttacggccgacatatgcaggatgctgagatg
20 tttacaaatgccgcttgcatggcattgaatatttgggatcgctttgatgtattctgtacactaggagcctct
agcggataccttaaaggaaactctgcttctttcaatttagttggattgtttggagataatgaaaatcaaagc
acggtcaaaacgaattctgtaccaaatatgagccttagatcaatctgttggtaactttacacagatactgcc
ttctcttgagcgtgggcgctcgagcagctttgtgggagtgccgatgtgacgactttaggggcttctttccaa
tacgctcaatctaaacctaagtcgaagaattaaacgttctctgtaacgcagctgagtttactatcaataag
25 cctaaaggatatgtagggcaagaattccctcttgcaactcatagcaggaactgatgcagcgacgggcactaaa
gatgcctctattgattaccatgagtggaagcaagtttagctctctcttacagattgaatatgttactccc
tacattggagttaaatggtctcgagcaagttttgatgccgatacgattcgtatagcccagccaaaatcagct
acagctatctttgatactaccacgcttaacccaactattgctggagctggcgatgtgaaagctagcgcagag
ggtcagctcggagataccatgcaaactcgtctccttgcaattgaacaagatgaaatctagaaaatcttgcggt
30 attgcagtaggaacgactattgtagatgcagacaaatacgcagttacagttgagactcgcttgatcgatgag
agagctgctcacgtaaatgcacaattccgcttctaa (SEQ ID NO:10)

MPVGNPAEPS LMIDGILWEG FGGDPCDPCA TWCDASMRM GYYGDFVFDR VLKTDVNKEF
QMGDKPTSTT GNATAPTTLT ARENPAYGRH MQDAEMFTNA ACMALNIWDR FDFVCTLGAS
35 SGYLKGNAS FNLVGLFGDN ENQSTVKTNS VPNMSLDQSV VELYTDTAFS WSVGARAALW
ECGCATLGAS FQYAQSKPKV EELNVLNAA EFTINKPKGY VGQEFPLALI AGTDAATGTK
DASIDYHEWQ ASLALSRYLN MFTPYIGVKW SRASFDADTI RIAQPKSATA IFDTTTTLNPT
IAGAGDVKTG AEGQLGDTMQ IVSLQLNKM SRKSCGIAVG TTIVDADKYA VTVETRLIDE
RAAHVNAQFR F (SEQ ID NO:11)

40 *Sequence of Primers used for Cloning (5' → 3')*

9494.JL: CCTGTGGGGAATCCTGCTGAA (SEQ ID NO:18)

9502.JL: TTTGGGTCGACCTATTAGAAGCGGAATTGTGCATTTACGTGA (SEQ ID NO:19)

MOMP Serovar F

45 atgcctgtgggaatcctgctgaaccaagccttatgatcgacggaattctgtgggaaggtttcggcggagat
ccttgcgatccttgaccacttggtgtgacgctatcagcatgcgtatgggttactatgggtgactttgttttc
gaccgtgttttgaaaacagatgtgaataaagagtttgaaatgggcgaggcttttagccggagcttctgggaat

- acgacctctactctttcaaaattggtagaacgaacgaaccctgcatatggcaagcatatgcaagacgcagag
atgtttaccaatgccgcttgcatgacattgaatatttgggatcgtttgatgtattctgtacattaggagcc
accagtggatatcttaaaggaaattcagcatctttcaacttagttgggttattcggcgatggtgtaaacgcc
acgaaacctgctgcagatagtattcctaactgagtgcaagtaaatcagtcgtggtggaactgtatacagatact
5 acttttgcttgagtggttgagctcgtgcagcttctggtggaatgtggatgtgcaactttaggagcttctttc
caatatgctcaatctaaacctaaaatcgaagaattaaacgcttctctgtaacgcagcagaggttactattaat
aaacctaaagggtatgtaggtaggaggttctcttgatcttacagcaggaacagatgcagcgcaggggact
aaagatgcctctattgattaccatgagtggaagcaagtttatctcttcttacagactcaatatgttcaact
10 cctacattggagttaaatggtctcgtgcaagcttctgattctgatacaattcgtatagccagccgaggttg
gtaacacctgttgtagatattacaaccttaacccaactattgcaggatgcggcagtgtagctggagctaac
acggaaggacagatatctgatacaatgcaaactcgtctccttgcaattgaacaagatgaaatctagaaaatct
tgcggtattgcagtaggaactattgtggatgcagacaaatacgcagttacagttgagactcgttgatc
gatgagagagctgctcacgtaaatgcacaattccgcttctaa (SEQ ID NO:12)
- 15 MPVGNPAEPSLMIDGILWEGFGGDPCTTWCDAI SMRMGYYGDFVDFRVLKTDVNKEFEMGEALAGASGN
TTSTLSKLVERTNPAYGKHMQDAEMFTNAACMTLNIWDRFDVFTLGATSGYLKGNSSASFNLVGLFGDGVNA
TKPAADSI PNVLQNSVVELYTDTTFAWSVGARAALWECGCATLGASFQYAQSKPKIEELNVLNAAEFTIN
KPKGYVGKEFLDLTAGTDAATGTKDASIDYHEWQASLSYRLNMFTPYIGVKWSRASFDSDTIRIAQPR
VTPVVDITTLNPTIAGCGSVAGANTEGQISDTMQIVSLQLNKMKSRSKSCGIAVGTTIVDADKYAVTVETRLI
20 DERAHVNAQFRF (SEQ ID NO:13)

Sequence of Primers used for Cloning (5' → 3')

9494.JL: CCTGTGGGAATCCTGCTGAA (SEQ ID NO:20)

MOMP-RK: GGGGTACCCTATTAGAAGCGGAATTGTGCATTTACGTGA (SEQ ID NO:21)

MOMP Serovar J

- 25 atgcctgtgggaatcctgctgaaccaagccttatgatcgacggaattctgtgggaagggttctcggtggagat
ccttgcatccttgaccacttgggtgtgacgctatcagcatgcgtatgggttactatgggtgactttgttttc
gaccgtgttttgaaaacagatgtgaataaagaatttcagatgggagcggcgccactaccagcgatgtagca
ggcttacaaaacgatccaacaacaaatgttgctcgtccaaatcccgttatggcaaacacatgcaagatgct
30 gaaatgtttacgaacgctgcttacatggcattaaatatctgggatcgttttgatgtattttgtacattggga
gcaactaccggttatttaaaaggaaactccgcttccttcaacttagttggattattcggaaacaaaaacacaa
gcttctagctttaatacagcgaatcttttccaaactgctttgaatcaagctgtgggttgagctttataca
gacactacctttgcttgagcgttaggtgctcgtgcagctctctgggaatgtgggtgtgcaacgttaggagct
tctttccaatatgctcaatctaaacctaaagttagaagagttaaatgttctttgtaatgcatccgaatttact
attaataagccgaaaggatatgttggggcggaatttccacttgatattaccgcaggaacagaagctgcgaca
35 gggactaaggatgcctctattgactaccatgagtggaagcaagtttagcccttcttacagattaaatatg
ttcactccttacattggagttaaatggtctagagtaagttttgatgccgacacgatccgtatcgctcagcct
aaattggctgaagcaatcttgatgtcactactctaaccgccaccatcgctggttaaaggaaactgtggtcgct
tccggaagcgaacacgacctggctgatacaatgcaaatcgtttcttgagttgaacaagatgaaatctaga
aaatcttgcggtattgcagtaggaacgactattgtagatgcagacaaatacgcagttacagttgagactcgc
40 ttgatcgatgagagagcagctcacgtaaatgcacaattccgcttctaa (SEQ ID NO:14)
- MPVGNPAEPSLMIDGILWEGFGGDPCTTWCDAI SMRMGYYGDFVDFRVLKTDVNKEFQMGAAPTTSDVA
GLQNDPTTNVARPNPAYGKHMQDAEMFTNAAYMALNIWDRFDVFTLGATTGYLKGNSSASFNLVGLFGTKTQ
ASSFNTANLFPNTALNQAVVELYTDTTFAWSVGARAALWECGCATLGASFQYAQSKPKVEELNVLNASEFT
45 INKPKGYVGAEFPLDITAGTEAATGTKDASIDYHEWQASLALSRLNMFTPYIGVKWSRVSFADTIRIAQP
KLAEAILDVTTLNPTIAGKGTVVASGSENDLADTMQIVSLQLNKMKSRSKSCGIAVGTTIVDADKYAVTVETR
LIDERAHVNAQFRF (SEQ ID NO:15)

MOMP Serovar Ia

- 50 atgcctgtgggaatcctgctgaaccaagccttatgatcgacggaattctgtgggaagggttctcgccggagat
ccttgcatccttgaccacttgggtgtgacgctatcagcatgcgtatgggttactacggagactttgttttc

gaccgtgttttgaaaacagatgtgaataaagaatttcagatgggagcggcgccactactaccaaggatatagca
 ggcttagaaaacgatccaacaacaaatgttgctcgtccaaatcccgcttatggcaaacacatgcaagatgct
 gaaatgtttacgaacgctgcttacatggcattaaatatctgggatcggtttgatgtattttgtacattggga
 gcaactaccggttattttaaaaggaaactccgcttccttcaacttagttggattattcgggaacaaaaacacaa
 5 tcttctaactttaatacagcgaagcttattcctaacgctgctttgaatcaagctgtggttgagctttataca
 gacactacctttgcttgagcgtaggtgctcgtgcagctctctgggaatgtgggtgtgcaacgtaggagct
 tctttccaatatgctcaatctaaacctaaagtagaagagttaaatgttctttgtaatgcatccgaatttact
 attaataagccgaaaggatatgttggggcggaatttccacttgatattaccgcaggaacagaagctgcgaca
 10 gggactaaggatgcctctattgactaccatgagtggaagcaagtttagccctgtcttacagattaaatatg
 ttcactccttacattggagttaaatgggtctagagtaagttttgatgccgacacgatccgtatcgctcagcct
 aaattggctgaagcaatcttgatgtcactactctaaacccgaccatcgctggtaaaggaactgtggtcgct
 tccggaagcgataacgacctggctgatacaatgcaaactcgtttccttgagttgaacaagatgaaatctaga
 aaatcttgcggtattgcagtaggaacgactattgtagatgcagacaaatacgcagttacagttgagactcgc
 15 ttgatcgatgagagagcagctcacgtaaatgcacaattccgcttctaa (SEQ ID NO:16)
 MPVGNPAEPLMIDGILWEGFGGDPDCTTWCDAISMRMGYYGDFVDFRVLKTDVNKEFQMGAAPTTKDIA
 GLENDPTTNVARPNPAYGKHMQDAEMFTNAAYMALNIWDRFDVFCTLGATTGYLKGNSASFNLVGLFGTKTQ
 SSNFNTAKLIPNAALNQAVVELYDITFAWSVGARAALWECGCATLGASFQYAQSKPKVEELNVLCNASEFT
 20 INKPKGYVGAEFPLDITAGTEAATGTKDASIDYHEWQASLALSYRLNMFTPYIGVKWSRVSFADTIRIAQP
 KLAELILDVTTLNPTIAGKGTVASGSDNDLADTMQIVSLQLNKMKSRSKCGIAVGTTIVDADKYAVTVETR
 LIDERAHVNAQFRF (SEQ ID NO:17)

EXAMPLE 2

This example illustrates one embodiment of the process for purification and refolding of
 25 recombinant MOMP for immunization.

MOMP protein derived from the mouse pneumonitis strain (MoPn) and two human
 serovars (serovars D and E) of *C. trachomatis* were each independently expressed in *E. coli*,
 grown in fermenters, and subjected to a purification and folding procedure. A schematic diagram
 of the method is set out in Figure 1 and is described further below. This embodiment of the
 30 purification and refolding process of the present invention uses, for illustrative purposes, MOMP
 cloned from serovar D of *C. trachomatis*.

Briefly, *E. coli* cells expressing recombinant serovar D of *C. trachomatis* MOMP (cloned
 in substantial accordance with the teaching of Example 1B) were grown in a shake flask at 37°C
 overnight, after which the culture is used to inoculate a 20L fermenter with an initial OD of 0.5.
 35 The fermentation was carried out under the following conditions: 37 °C, pH 6.8, 30% dissolved
 oxygen, 700-1200 rpm agitation. Fermentation was carried out in a fed-batch mode using a pH-
 stat method. Glucose was fed into the fermenter to maintain a glucose concentration between 0 to
 2 g/L. The culture was induced with 1mM IPTG, and then grown for 4-6 hours. The final
 fermenter OD reached approximately 30-40.

40 Primary Recovery step (Preparation of Inclusion Body Fraction):

The fermenter broth was harvested and concentrated using 0.2 μ m tangential flow filtration (TFF) unit. Cell disruption was performed at 13000 psi using a homogenizer. Typically, a centrifugation step was followed by homogenization to remove most impurities and to reduce viscosity of the homogenate. Alternatively, lysozyme was added to the thawed broth following centrifugation. The inclusion bodies (IBs; sedimentable detergent insoluble MOMP of a purity of about 50 %) were then washed with buffer containing Triton X-100.

Purification step:

The IB pellets were solubilized in 6M guanidine hydrochloride (a charged denaturant) and following solubilization, this denaturant was exchanged with 8M urea (a non-charged denaturant) using tangential flow filtration (TFF). The urea solution was applied to a chromatography column containing a Q-type anion exchange material column (Q Ceramic HyperD® 20 Chromatography Sorbent, Pall Corporation). In this chromatography step, the running, washing, and elution buffers used also contained about 8 M urea at a pH of about 8.5 (pH range 7.5 to 9.0) to maintain the protein denatured and soluble throughout the purification procedure. The recombinant MOMP protein was eluted from the column with 50-90 mM sodium chloride. The purity of the resulting protein was evaluated by SDS-PAGE (see Figure 2). Protein gel densitometry following Coomassie staining indicated a purity of approximately 90% or greater.

Folding step:

A TFF approach was used to exchange buffer solutions in the rMOMP sample. Briefly, the pooled eluate from the chromatography step was mixed with equal volumes of about 10% v/v of the detergent sodium N-lauroyl sarcosine (NLS) (range for NLS is about 6 to 10% v/v), and 1 M l-arginine; DTT was added to a final concentration of 10 mM. This mixture was then subjected to TFF diafiltration using a 10 kDa MWCO membrane filter, in which the replacement (diafiltration) buffer was about 10 mM Tris-HCl pH 8, 0.06% NLS. Filtration continued until approximately 10 sample volumes of the diafiltration buffer passed through the filter. The retentate, designated TFF1, optionally underwent a second TFF procedure with diafiltration buffer of 50 mM Tris-HCl, pH 8.0, resulting in a fraction designated TFF2.

Samples of the rMOMP protein were taken throughout the process at various stages and were assessed by SDS-PAGE. A representative SDS-PAGE gel is set out in Figure 2.

The above-described purification and folding method was also applied to rMOMP derived from MoPn and to rMOMP derived from *C. trachomatis* serovar E, with similar results.

This method may also be applied to rMOMP derived from the other *C. trachomatis* serovars (e.g. F, Ia, and J) and from serovars of *C. pneumoniae*.

The rMOMP derived from MoPn demonstrated immunological protection in at least one animal study using a mouse protection model, and recognition of at least one conformational epitope by a monoclonal antibody that specifically recognizes native MOMP.

These data indicate that the method resulted in a purified yet denatured target protein and that renaturation (and a return to aqueous solubility) was achieved by the subsequent protein folding process. The diafiltration of a volume of purified denatured rMOMP in urea (8M) mixed with equal volumes of small molecule additives such as for example, arginine (1M) and NLS (30% v/v) can be used to refold the denatured protein.

EXAMPLE 3

This example describes an alternative process for preparing rMOMP from *C. trachomatis* serovars D, E, F, J and Ia using Urea and pH shock (as a denaturing agent rather than guanidine hydrochloride) and a reduced concentration of NLS.

A number of lab scale lots of rMOMP from *C. trachomatis* serovars D (3 lots), E (3 lots), J (2 lots) and Ia (1 lot) were prepared. First, rMOMP was cloned from each of serovar in substantial accordance with the teaching set out in Example 1B. IB fractions/pellets were prepared in substantial accordance with the teaching set out in Example 2 and were then subjected to the purification and folding process described further below.

Purification: The IB fraction/pellet was mixed at a ratio of 20 mL/g wet weight with a solubilization buffer containing about 25 mM KCl, 25 mM NaOH, 8 M urea at a pH of approximately 12.5. The pH of the mixture was adjusted following re-suspension of the pellet to about 12.5. Following incubation at room temperature, the mixture was subjected to centrifugation (10397 g, 4°C). The supernatant, containing solubilized rMOMP, was mixed with an equal volume of buffer (50 mM Tris-HCl pH 7.0 and 8M urea), and the pH was adjusted with 6 N HCl to pH 8.5. DTT was added to achieve a concentration of 10 mM. Conductivity was reduced to 2.0-2.5 mS by dilution with a buffer of 50 mM Tris-HCL (pH 8.5), 8 M urea, 1 mM EDTA and the solution was then filtered.

Chromatographic operations were carried out using an AKTA Explorer 100 (G.E. Healthcare) chromatography unit with a column packed with Pall BioSeptra Q Ceramic HyperD F anion exchange sorbent.

Following column equilibration (using an equilibration buffer of 50 mM Tris-HCl, pH 8.5; 8 M urea; 1 mM EDTA), 120-240 mL of the solubilized rMOMP mixture was loaded onto the column. Following collection of the flow-through and chase fractions, a wash step of 3-5 CV ensued with the equilibration buffer (to which had been added 20-40 mM NaCl). Elution was performed with equilibration buffer (containing 40-90 mM NaCl), and fractions were collected. The concentration of sodium chloride (NaCl) in the wash and elution buffers was adjusted for each serovar based on empirical experimentation; suitable NaCl concentrations in wash buffer ranged from 20-40 mM NaCl, and in elution buffers ranged from of NaCl in suitable wash buffers ranged from 40-90 mM NaCl.

In subsequent studies, to increase the purity of the resultant rMOMP protein an additional purification step using CEX chromatography in the flow-through mode was added upstream of the AEX step. The CEX step was conducted at a pH of 5.5-6 after which the solution pH was adjusted to 8.5 for the AEX step. However, since the CEX step is conducted at a pH of approximately 5.5 which is close to the pI of rMOMP (~ 4.8), a second pH shock step may be added before the AEX step to help avoid the development of soluble aggregates.

Refolding procedure: The refolding procedure involved the reduction of rMOMP's disulfide bonds with DTT (e.g., by adding about 10mM DTT), the addition of the small molecule additive l-arginine, (e.g. about 333 mM), and the addition of the small molecule additive (and detergent) n-lauroyl sarcosine (NLS), (e.g. about 2% NLS). The mixture was then subjected to diafiltration, which is briefly described below.

1. Following the addition of the arginine and NLS solutions, the mixture was concentrated by TFF (using TFF filtration apparatus, Sartocoon 2Plus unit with Sartorius Hydrosart cassettes) until it reached the original rMOMP sample volume (i.e., approximately a threefold reduction).

2. TFF was operated in the diafiltration mode for approximately five volumes of Tris buffer.

3. The mixture was diluted 1:3 with Tris buffer, and subjected to a further (approximately) 5 volumes of diafiltration with Tris buffer, while maintaining the diluted volume.

4. The mixture was concentrated (approximately) threefold by TFF recovered from the TFF device, and EDTA was added to achieve a concentration of 1 mM.

Protein concentration was estimated by micro BCA assay. Endotoxin was assessed (Endo safe; Charles River Labs) and NLS content was estimated spectrophotometrically using a wavelength of 210 nm. SDS-PAGE gel protein analysis with scanning densitometry was used to assess for protein purity, and protein conformation was assessed by Blue Native PAGE (representative gels are provided in Figures 3,6A,B,C).

The procedure described above was executed in a similar manner for all five MOMP serovars, with satisfactory results. The display of characteristic ladder patterns was evident with BN-PAGE gels. Residual NLS content following folding was in the range 0.175-0.30 %. The reduction of NLS concentration in the final product was significant as earlier studies in mouse models indicated that the concentration of residual NLS in the final product is preferably $\leq 0.5\%$.

The purity of the final protein product was assessed by SDS-PAGE with scanning densitometry. Table 3 presents a summary of the purity (percent of total protein) and yield (expressed as mg bulk rMOMP product per gram wet weight of IB) obtained in preparing lots. In the case of serovars D and E, the mean of three lots is provided.

Table 3

Purity and Yield results for rMOMP from 4 *C. trachomatis* serovars

Serovar rMOMP (from which derived)	Purity (approx.)	Yield(approx.) (mg/g IB)
D	91.22%	24.4
E	91.17%	8.45 – 28.8
J	92%	39.5
Ia	93%	40.7

In all cases a characteristic ladder pattern on blue native PAGE gels was noted; as such, oligomeric forms of rMOMP were present in sample.

These data indicate that outer membrane proteins (*e.g.*, MOMP) may be prepared in accordance to the process described (*i.e.*, using pH shock). More particularly, these data indicate that reducing the NLS concentration in the refolding process to a final concentration of 2% does not adversely effect the refolding process and results in a final protein product with a reduced concentration of residual NLS (*e.g.*, ≤ 0.5).

EXAMPLE 4

This example describes a process utilized for preparing pilot scale lots of rMOMP from *C. trachomatis* serovar E for immunization.

Preceding examples have related to lab scale lot processes. Such processes are at a scale sufficient to provide a protein preparation sufficient for characterization and animal studies and typically, start with 6-12 g of MOMP IB material. For pilot scale lots, the MOMP production process is sufficiently scaled up such that 50% of the product of a 20 L fermenter run can be processed in one series of unit operations.

C. trachomatis Serovar E rMOMP was cloned in substantial accordance to the procedure set out in Example 1B. An inclusion body fraction of the protein was prepared substantially in accordance with the procedure set out in Example 2 except that fermentation was conducted using a 20L fermenter.

Purification: The IB fraction/pellet was mixed in a solubilization buffer (of 25 mM KCl, 25 mM NaOH and 8 M urea at pH 12.5) at a ratio of approximately 20 mL/g wet weight of IB pellet. The mass of Ser E MOMP IB pellet used was about 118.5 g. The pH was adjusted with NaOH following re-suspension of the pellet to about 12.5, and the mixture was incubated at room temperature with gentle agitation for 40 min. Following incubation, the mixture was subjected to centrifugation (10397 g, 20 min, 4°C). The supernatant, containing solubilized rMOMP, was mixed with an equal volume of buffer containing 50 mM Tris-HCl pH 7.0, and 8M urea, and the pH was adjusted with 6 N HCl to pH 8.5. DTT was added to the solution to achieve a concentration of 10 mM. The conductivity of the solution was checked with a conductivity meter, and was reduced to 2.0-2.5 mS by dilution with 50 mM Tris-HCL pH 8.5, 8 M urea, 1 mM EDTA. The solution was filtered through a dead end 0.22 um filter; the final volume was 5.5 L.

Chromatographic operations were carried out using an AKTA Pilot (G.E. Healthcare) chromatography unit with a column (BPG100, G.E. Healthcare) packed with Pall BioSeptra Q Ceramic HyperD F anion exchange sorbant. The column was washed, regenerated, and equilibrated as previously described at the lab scale (as described in Example 3). The flow rate for all operations, unless noted otherwise, was 160 mL/min. Following column equilibration (using an equilibration buffer of 50 mM Tris-HCl, pH 8.5; 8 M urea; 1 mM EDTA), 5 L of the starting material was loaded onto the column. The chase volume (with equilibration buffer) was about 4 CV, and this was followed by a wash step at about 5 CV with equilibration buffer to

which was added NaCl to 25 mM. A suitable range of NaCl concentration in wash buffer is 20 mM – 40 mM and a preferred concentration is 20 mM NaCl. Elution was performed with 4.5 CVs equilibration buffer containing 75 mM NaCl. A suitable range of NaCl concentration in elution buffer is 40 mM – 90 mM and a preferred concentration is 40 mM NaCl.

5 In subsequent studies, to increase the purity of the resultant rMOMP protein an additional purification step using CEX chromatography in the flow-through mode was added upstream of the AEX chromatographic step. The CEX step was conducted at a pH of 5.5-6 after which the solution pH was adjusted to 8.5 for the AEX step. However, since the CEX step is conducted at a pH of approximately 5.5 which is close to the pI of rMOMP (~ 4.8), a second pH shock step
10 before the AEX step may also be included to avoid the development of soluble aggregates.

Prior to diafiltration, 3.9 g DTT was added to the purified rMOMP pool, resulting in a concentration of 10 mM. To this was added 2.5 L of 1 M l-arginine, followed by 2.5 L 6% v/v NLS; each of these solutions having been prepared in a buffer containing 50 mM Tris-HCl, pH 8. The rMOMP-arginine-NLS mixture was then mixed at room temperature.

15 MOMP folding procedure: The purified rMOMP was treated with DTT, arginine, and NLS, followed by a two part TFF operation which induces folding and reduces residual NLS detergent. The refolding procedure involved the reduction of rMOMP's disulfide bonds with DTT (e.g., about 10mM DTT), the addition of the molecular chaperone, l-arginine, (e.g. about 333 mM), and the addition of the detergent n-lauroyl sarcosine (NLS), (e.g. about 2% NLS).

20 The MOMP-arginine-NLS mixture (7.5L) was then subjected to the two-part TFF operation, substantially as described in Example 3. Volume reduced 3 fold to 2.5L with filtrations. Diafiltration ensued for 7 volumes, using a diafiltration buffer consisting of 50 mM Tris-HCL pH 8. The solution was then diluted 1:3 (to 7.5L) with 50 mM Tris-HCL pH 8, then another 7 volume diafiltration step with the diafiltration buffer containing 0.1% v/v NLS.
25 Following the second diafiltration, the material was concentrated to 1.2L and EDTA (1mM) was added.

The TFF operations had a total elapsed time of ~ 2.5 h, similar to the target time established for lab scale (2-4 h). Previous investigations found that prolonged TFF folding, originally adopted to reduce shear forces and provide a gentler environment, was in fact
30 deleterious to the folding attempt, in which the development of aggregated protein as indicated by BN-PAGE gel analysis occurred.

The folding process was considered successful if the resulting protein product was soluble in aqueous buffers at $> 1\text{mg/mL}$, and the characteristic ladder pattern in BN-PAGE gels was evident. The presence of discrete bands of MOMP protein over a range of molecular weights is indicative of multimeric units (somewhat analogous to the putative trimeric state found with the native protein). Figure 3 illustrates BN-PAGE gel patterns with the pilot scale lot and three lots of lab scale Ser E rMOMP protein. Patterns are similar, with the presence of a ladder consisting of at least four bands of similar apparent molecular weight. Additionally, there is little evidence of monomeric rMOMP, normally seen just below the 66 kDa marker (not shown).

The purity of the final protein product was assessed by scanning densitometry of SDS-PAGE gels. Figure 4 shows an image of an SDS-PAGE purity gel for the pilot Ser E lot. Protein purity was assessed as approximately 88% by scanning densitometry, with a low molecular weight band accounting for 6% of the total protein. The percentage assessed may be an underestimate of MOMP purity as putative MOMP related bands (fragments, complexes) on the gel were not identified.

The concentration of residual components was assessed and considered well within appropriate limits: endotoxin content was $0.005\text{ EU}/\mu\text{g}$ protein, and residual NLS was 0.49% (i.e., approximately 0.24% at 1 mg/mL).

MOMP Serovar F

Similar results were obtained when a pilot scale lot of Serovar F rMOMP was prepared in substantial accordance to the process used for Serovar E rMOMP: total yield of purified/folded MOMP was 6.15 g; 89.8-90.5 % protein purity; $0.007\text{ EU}/\mu\text{g}$ endotoxin content; 0.31% v/v residual NLS.

These data indicate that the purification and folding process is adaptable for use as a large scale process to manufacture recombinant protein in commercially significant amounts.

EXAMPLE 5

This example illustrates the evaluation of the effects of process modifications on protein purity.

C. trachomatis rMOMP was cloned from serovar E in substantial accordance with the teaching set out in Example 1B. IB fraction/pellet of the recombinant protein was prepared in substantial accordance with the teaching set out in Example 2.

A wash buffer was prepared consisting of 50 mM Tris-HCl pH 8.0, 2 M Urea, 0.5% v/v NLS. Thawed IB material was mixed with NLS buffer at 20 mL per IB gram for approximately 30 minutes. The IB mixture was divided equally into centrifuge bottles and then centrifuged. The supernatant was decanted and the pellet (with the target protein) was retained and was subjected to a purification and folding process in substantial accordance to the teaching in Example 3. Protein concentration was estimated by BCA assay and purity was assessed by SDS-PAGE with scanning densitometry. A number of lab scale lots were similarly prepared using an IB fraction wash buffer with either 0.5% NLS or 0.5% Triton X-100, and 2 or 4 M urea.

The effect of NaCl concentration in the purification process on protein purity was also evaluated. An IB fraction prepared in substantial accordance with the teaching of Example 2 was divided into multiple samples. One sample of the IB fraction was subjected to a purification and folding process in substantial accordance to the method described in Example 3. A second sample of the IB fraction was first washed (in substantial accordance to the method described above in this Example) and was then subjected to substantially the same purification and folding process as was the first sample of the IB fraction except that the NaCl concentration in the wash buffer and the elution buffer was increased from 15 mM to 20 mM and 30 mM to 40 mM, respectively.

Protein concentration and purity of the resulting purified and folded protein product from each of samples 1 and 2 was estimated by BCA assay and SDS-PAGE with scanning densitometry, respectively. The resulting SDS-PAGE gels are illustrated in Figure 5. The left panel illustrates a gel with samples (run in three lanes) from the first preparation and the right panel illustrates a gel with three lanes loaded with samples from the second preparation. In both gels, lanes are loaded in triplicate with 4 µg of protein. With the first preparation, the purity of the resulting material was approximately 75% whereas with the second preparation, protein purity was approximately 98%. A number of lab scale and pilot scale lots have been prepared using the additional IB buffer wash and increased NaCl concentration and similar increases in protein purity have been noted (with an average increase in protein purity was about 10%, and providing protein with a purity as high as 100%).

These data indicate that the washing the IB pellet with a suitable buffer before subjecting it to the purification and folding process increases protein purity without adversely affecting protein yield. Subjecting the IB fraction/pellet to an NLS buffer wash before the purification process and increasing the concentration of NaCl in the wash buffer and the elution buffer of the purification process increases protein purity (without adversely affecting protein yield, folding,

and solubility). These results were surprising since repeated washing of IB fractions in buffers of various urea stringencies (concentrations) had previously resulted in decreased yield.

EXAMPLE 6

This example describes the evaluation of the immunogenicity of the rMOMP (purified and refolded in accordance to the process of the present invention) in an animal model.

Recombinant MOMP derived from *C. trachomatis* serovar E was prepared in substantial accordance with the teaching of Example 4. Each dose of immunization contained 50µg of rMOMP protein and adjuvant [i.e., ADJ.A, ADJ.SQ, aluminum hydroxide (Alhydrogel), Montanide/CpG] in a volume of 50µl. Blood samples were taken periodically following immunization in order to conduct a number of tests, including detection of anti-Chlamydia antibodies by ELISA, quantification of total IgG and Th1/Th2 sub-typing.

Groups of female BALB/c mice (15 per group) (Charles River) were immunized intramuscularly three times, at approximately 3 week intervals with 50µl of the applicable composition (as noted in Table 4). The mice were approximately 7-8 weeks of age at the time of the 1st immunization. As a control, 4 groups were administered compositions of adjuvanted Ovalbumin. Pre-bleed samples were obtained approximately 4 days before the first immunization.

Table 4: Vaccine component and volume to inoculate per mouse

Grp.	No. mice	Vol. injected/mouse	Composition (antigen + adjuvant)
A	15	50 µl	OVA + ADJ.A
B	15	50 µl	OVA + ADJ.SQ
C	15	50 µl	OVA + aluminum hydroxide (Alhydrogel)
D	14	50 µl	OVA + Montanide/CpG
E	15	50 µl	rMOMP alone
F	15	50 µl	rMOMP + ADJ.A
G	15	50 µl	rMOMP + ADJ.SQ
H	15	50 µl	rMOMP + aluminum hydroxide (Alhydrogel) (50 µg/dose)
I	14	50 µl	rMOMP + Montanide/CpG (10 µg/dose); PBS

Formulations were freshly prepared before each immunization. Antigens, buffers and adjuvants were stored at 4°C. First, antigen (i.e., Ovalbumin or rMOMP) 15 µg/dose was diluted in buffer

50 mM Tris, pH 8.0, 0.1% NLS (30µl/dose). To this mixture the applicable adjuvant was added, plus buffer when necessary to obtain an immunization dose of 50µl.

Aluminum adjuvant used is the one called Alhydrogel which is aluminum oxyhydroxyde or AlOOH, at a concentration of 9.9 mg/ml. The final quantity of aluminum in the immunization doses is 50µg/dose

Adjuvant A (ADJ.A) was prepared as follows: In a 1st container, the following ingredients were mixed, under agitation and at 40°C: 39.37g of Phosphate Buffer, (Eurobio); 4.68g of mannitol, (Roquette); 4.822g of EumulginTM B1, (Cognis); and 20.3 mg of E6020 (Eisai). This aqueous phase had a weight of 48.91g. In a 2nd container, 30.48g of squalene were mixed with 4.52g of MontaneTM80 under agitation at ambient temperature. This oily phase had a weight of 35g.

When both phases were homogeneous, 29.11g of the oily phase was incorporated into the aqueous phase. The mixture was then gently agitated, and put in an oily bath at 80-90°C, under still gentle agitation (300rpm). When the mixture reached 75°C, the emulsion was taken out of the oily bath and put on ice, while agitation was maintained at about 250 rpm. When the temperature of the mixture returned to ambient temperature, a homogeneous thermoreversible oil-in-water emulsion was obtained, in which more than 90% of the population by volume of the oil droplets had a size $\leq 200\text{nm}$ and in which the composition by weight was as follows: 32.5% of squalene, 6.18% of cetareth-12 (EumulginTMB1), 4.82% of sorbitan monooleate (MontaneTM80), 6% of mannitol, 0.026% of E6020 and 50.5% of PBS. This stock solution was then diluted at 1/5 with phosphate buffer (1X) to obtain an emulsion having 6.5% squalene.

About 5 minutes to 1 hour in advance of the applicable immunization, antigen samples (10 µg of antigen suspended in 25 µl buffer) was gently mixed with 25 µl of adjuvant. Volume of composition inoculated was 50 µl.

The second Adjuvant, ADJ.SQ, was prepared from a stock solution prepared as ADJ.A's with the exception that no E6020 was included. As such, the stock solution of ADJ.SQ was diluted at 1/6.5 with PBS to get an emulsion comprising 5% squalene.

As the concentration of squalene and surfactants were higher in ADJ.A than in ADJ.SQ, the preparation of the formulations utilized for the immunizations were different for both adjuvants to obtain the same final concentration of 2.5% squalene in the immunization doses (although, inadvertently, for the 1st immunization, the final concentration of squalene in the ADJ.SQ group was 2%).

One mouse from group B was found dead 4 days before the 3rd immunization (and was bled out). Sera was collected from immunized animals about 2 weeks post-immunization and was pooled for each group to assess antibody response by ELISA.

Mice were euthanized and their spleens removed aseptically. Single cell suspensions were prepared. Splenocytes from mice belonging to the same group were pooled and were pelleted by centrifugation. Erythrocytes in the suspension were lysed. The cell suspension was transferred to another tube and centrifuged to pellet the cells. Process was repeated to ensure that most of the erythrocytes were lysed. The cell pellet was resuspended, cells were counted and plated. Cells were stimulated with Ovalbumin, rMOMP, UV-inactivated *C. trachomatis* MoPn EB MOMP, or PMA.

Isotyping analysis of the antibody profile generated was done using ELISA based reagents. Induction of Th1/Th2 responses was analyzed by quantifying the antigen-specific IgG2a (Th1) and IgG1 (Th2) antibody response and by measuring levels of cytokines (e.g., IFN- γ (Th1), interleukin-10 (Th2) in antigen-stimulated splenocyte culture (*i.e.*, by assaying in vitro cytokine production by splenic T cells).

A summary of the ELISA results and the cytokine profile by MSD analysis are set out in Tables 5 and 6.

Table 5

	ELISA Titre						% Neutralizing Mice 0	Average Titre	Range Titre (positive mice)
	Total IgG Avg	sd	IgG1 Avg	Sd	IgG2a Avg	sd			
OVA+ADJ.A	50	0	50	0	50	0	0	NA	NA
OVA+ADJ.SQ	50	0	50	0	50	0	0	NA	NA
OVA+Alum (AIOOH)	50	0	50	0	50	0	0	NA	NA
OVA+CpG/Montanide	106	160	50	0	50	0	0	NA	NA
rMOMP alone	1437	3316	317	820	50	0	0	NA	NA
rMOMP+ADJ.A	1621333	1482613	45070	102076	17767	28802	33	65	25-100
rMOMP+ADJ.SQ	421547	815814	11067	17389	50	0	0	NA	NA
rMOMP+Alum	2427	3104	93	100	50	0	0	NA	NA
rMOMP+CpG/Montanide	310613	419299	7397	8790	23680	26795	40	38	25-100

Table 6

	MSD Analysis					
	INF γ	IL-10	IL-4	IL-5	IL-2	IL-12(p70)
OVA+ADJ.A	845.5	2890.1	295.0	3472.5	1656.6	99.7
OVA+ADJ.SQ	323.1	244.4	70.2	1475.7	1116.7	49.7
OVA+Alum (AlOOH)	816.0	267.8	79.8	194.1	1729.9	101.6
OVA+CpG/Montanide	316.8	95.6	38.7	68.8	1119.3	24.4
rMOMP alone	930.5	9343.5	392.2	8975.5	1000.0	146.6
rMOMP+ADJ.A	14747.1	18601.1	1365.4	13504.6	4231.4	857.0
rMOMP+ADJ.SQ	1364.1	1113095.5	1189.8	40230.4	830.6	896.6
rMOMP+Alum	1440.9	5222.7	501.9	8194.6	1897.6	301.9
rMOMP+CpG/Montanide	3818.3	6349.9	810.6	266.1	1147.3	258.4

In accordance to the EB-ELISA (antibody) results, the immunogenic composition with unadjuvanted rMOMP was weakly immunogenic and of the IgG classes tested, solely IgG1 was detectable. The carrier Aluminum hydroxide was not an effective adjuvant for rMOMP. Adjuvanting rMOMP with the other carrier, ADJ.SQ, elicited strong immune responses (i.e., total IgG) but the response was pre-dominantly a Th2 type, not a Th1/Th2 balanced response (e.g., no detectable IgG2a was elicited). Adjuvanting rMOMP with ADJ.A (an adjuvant comprising E6020) or CpG/Montanide elicited strong immune responses with balanced Th1/Th2 subclasses, at comparable levels. In addition, ADJ.A switched the immune profile induced by un-adjuvanted rMOMP from an IgG1 only, to a balanced Th1/Th2 antibody response.

The capacity of the sera from immunized mice to neutralize *C. trachomatis* serovar E was assessed with an *in vitro* neutralization assay against serovar E EBs. The *in vitro* neutralization assay was performed substantially as follows: The assay utilized 96 well round bottom plates. Dilutions of each serum sample were prepared using PBS and 5% baby rabbit complement (Sigma). Into each dilution sample well, 1500 IFU/50 μ l of EBs from *C. trachomatis* (diluted in PBS + 5% baby rabbit complement just prior to use), was added and the mixtures were incubated at 37°C for 45 minutes with gentle rocking. A 96 well plate containing a HeLa cell monolayer (of HeLa cells seeded at 5 x 10⁴ cells/well, about 24 hours earlier) was prepared and 50 μ l of each dilution sample was transferred to sample wells in the HeLa cell monolayer plate. The plate was centrifuged for 60 minutes at room temperature at 1800 rpm. DMEM with L-glutamine and sodium pyruvate (Invitrogen) supplemented with 1 μ g/ml cyclohexamide, 10% FBS, gentamicin was added to each sample well and plates were incubated at 35°C with 5% CO₂ for 44-48 hours.

As controls, dilutions of EBs alone and dilutions of PBS + 5% baby rabbit complement sera alone were also prepared and added to specific wells of the HeLa cell plate.

Inclusion bodies were stained and the 50% neutralization titre was determined by calculating the value of percent neutralization for each of the sample dilutions by applying the formula, (IFU prebleed – IFU bleed)/IFU prebleed x 100. Alternatively, the value could be determined by taking the average of the control samples with EBs alone in place of prebleed IFU. For each sample, the 50% neutralization titre was the lowest dilution with a value greater or equal to 50% (e.g., if a 1:400 dilution had 71% neutralization and a 1:800 had 34% neutralization, the 50% neutralization titre of that sample was 400). A summary of the results obtained are set out in Table 5.

These data show that neutralizing antibodies were detected in the group immunized with compositions comprising rMOMP and ADJ.A (comprising E6020). Although strong antibody responses (of predominantly IgG1 subclass) were stimulated in mice immunized with compositions comprising rMOMP and ADJ.SQ, the sera from these mice had no detectable neutralizing capacity. Of the compositions administered, those including rMOMP adjuvanted with ADJ.A or CpG/Montanide stimulated neutralizing antibodies to serovar E.

EXAMPLE 7

Immunogenic compositions comprising rMOMP (2 different doses) adjuvanted with Adjuvant No.1 (ADJ.A) with varying concentrations of a TLR4 agonist (E6020), were evaluated in an animal model.

Recombinant MOMP derived from *C. trachomatis* serovar E was prepared in substantial accordance with the teaching of Example 4. Groups of female CDI mice (6 to 12 per group) (Charles River) were immunized intramuscularly on three separate occasions (at about 3 week intervals) with 50µl of the applicable composition (as noted in Table 7). Two doses of antigen (rMOMP) were used, 10µg and 25µg. E6020 were tested at 3 doses, 0.25µg, 0.5µg and 1µg. A group of mice was also tested with one carrier alone, this being the group with ADJ.SQ considered as 0µg of E6020. CD1 is an outbred strain, in contrast to Balb/C which is an inbred strain. This outbred strain provides a more robust model and is more akin to humans (e.g., more diversified). The mice were approximately 7-8 weeks of age at the time of the 1st immunization. As a negative control, groups E and F were administered compositions of adjuvanted Ovalbumin. Pre-bleed samples were obtained a few days before the first immunization.

Table 7: Vaccine component and volume to inoculate per mouse

Grp	No. mice	Vol. injected/mouse	Composition (antigen + adjuvant)	Ratio of antigen: E6020
A	12	50 μ l	JR3182 10 μ g + ADJ.SQ	NA
B	12	50 μ l	JR3182 10 μ g + ADJ.A (E6020 1 μ g)	10:1
C	12	50 μ l	JR3182 10 μ g + ADJ.A (E6020 0.5 μ g)	20:1
D	12	50 μ l	JR3182 10 μ g + ADJ.A (E6020 0.25 μ g)	40:1
E	6	50 μ l	OVA 25 μ g + ADJ.SQ	NA
F	6	50 μ l	OVA 25 μ g ADJ.A (E6020 1 μ g)	NA
G	12	50 μ l	JR3182 25 μ g + ADJ.SQ	NA
H	12	50 μ l	JR3182 25 μ g + ADJ.A (E6020 1 μ g)	25:1
I	12	50 μ l	JR3182 25 μ g + ADJ.A (E6020 0.5 μ g)	50:1
J	12	50 μ l	JR3182 25 μ g + ADJ.A (E6020 0.25 μ g)	100:1

Formulations were freshly prepared before each immunization. Proteins, buffers and adjuvant were stored at 4°C. Mixtures were prepared by diluting protein (Ovalbumin or rMOMP) in buffer (50mM Tris, pH 8.0, 0.1% NLS), 25 μ l/dose and then adding to this mixture the applicable adjuvant. Mixtures including E6020 were vortexed on high for about 1min. Prepared formulations were placed on ice until required.

The adjuvants used in the present example were prepared in the following manner; ADJ.SQ was prepared as previously described in Example 6. ADJ.A was prepared as described in Example 6, and diluted by ADJ.SQ to reach the requisite concentration of E6020. This means that for Groups B, F and H, the ADJ.A used was the same as the one described in Example 6 having 5% squalene and 40 μ g/ml of E6020. For Groups C and I, the ADJ.A used is the same as the one used for the preceding group which has been diluted once at $\frac{1}{2}$ with ADJ.SQ to have a concentration of E6020 of 20 μ g/ml. And for Groups D and J, the ADJ.A used has been diluted once more at $\frac{1}{2}$ by ADJ.SQ to have a concentration of E6020 which is 10 μ g/ml.

One mouse from group F3 was found dead 2 days following the 2nd immunization (and was bled out). Sera was collected from immunized animals about 2 weeks post-immunization and was pooled for each group to assess antibody response by ELISA.

Mice were euthanized and their spleens removed aseptically. Single cell suspensions were prepared. Splenocytes from mice belonging to the same group were pooled. The splenocytes were restimulated *in vitro* with rMOMP (or as a control, with Ovalbumin, rMOMP, UV-inactivated *C. trachomatis* MoPn EB-MOMP, or PMA) for 3 days. The culture supernatants were collected and the cytokine production was measured for IFN- γ , IL-4, IL-5, and IL-10 by MSD.

Isotyping analysis of the antibody profile generated was done using ELISA based reagents. Induction of Th1/Th2 responses was analyzed by quantifying the antigen-specific IgG2a (Th1) and IgG1 (Th2) antibody response and by measuring levels of cytokines (e.g., IFN- γ (Th1), interleukin-10 (Th2) in antigen-stimulated splenocyte culture (*i.e.*, by assaying in vitro cytokine production by splenic T cells).

The ability of the elicited antibodies to neutralize *C. trachomatis* serovar E was assessed by neutralization assay which was conducted substantially in accordance to the assay described in Example 6.

A summary of the ELISA titres, neutralizing titres and cytokine profile by MSD analysis are set out in Table 8.

Table 8

Adjuvant		Antigen		Results								
E6020 + ADJ.SQ				Neutralizing		ELISA & MSD						
Grp.	Dose E6020 (μ g)	lot	Ag Dose (μ g)	Positive mice	Avg Titre	Total IgG	IgG1	IgG2a	INF-g	IL-10	IL-4	IL-5
B	1	JR31 82	10	11/12	451	623467	3796	770742	14495	3836	84	1450
C	0.5		10	8/12	200	558967	19975	320300	19241	4788	86	2351
D	0.25		10	7/12	134	159733	5675	46283	17149	5728	94	3479
H	1		25	10/12	420	441600	7667	377096	14931	3739	80	1009
I	0.5		25	11/12	372	401033	8675	652433	14518	5196	123	2027
J	0.25		25	11/12	422	844600	12413	378200	10750	4458	91	2075
F	1	ova	25	NA	12.5	50	50	50	148	159	6	55
ADJ.SQ												
A			10	7/12	122	340267	3896	10992	2215	7726	87	4841
G			25	8/12	200	298667	22938	48854	4451	4343	96	3646
F		ova	25	NA	12.5	50	50	50	357	364	9	36

Compositions of rMOMP adjuvanted with ADJ.SQ (*i.e.*, lacking the TLR4 agonist, E6020) elicited levels of total IgG (including IgG1 and IgG2 subclasses) similar to those elicited

with rMOMP adjuvanted with ADJ.A (an adjuvant comprising the TLR4 agonist, E6020), however, the neutralizing capacity of the anti-sera was lower in comparison to anti-sera elicited by compositions comprising rMOMP adjuvanted with ADJ.A. These ADJ.SQ compositions stimulated a Th2-biased immune response (i.e., elicited high levels of Th2 cytokines and low levels of IFN- γ). The addition of a TLR4 agonist (e.g., E6020) shifted the immune response towards a Th1 type response.

EXAMPLE 8

Immunogenic compositions comprising rMOMP adjuvanted with ADJ.A produced by one of several different processes.

Groups of female CDI mice (10 per group) (Charles River) were immunized intramuscularly on three separate occasions (at about 3 week intervals) with 50 μ l of formulations comprising either 1 or 10 μ g of rMOMP, and having 1 μ g of E6020 with the carrier comprising a squalene emulsion. In that study, the adjuvant was prepared in one of several different ways: either the product E6020 was introduced in the aqueous phase before the emulsification took place, or it was introduced in the oily phase, or even in some cases, it was simply added to the emulsion. The adjuvant effect of E6020 in the rMOMP composition was similar irrespective of which of the three preparation methods was utilized. The three adjuvants tested elicited similar levels of antigen-specific total IgG, with both IgG1 and IgG2a subclasses, similar in vitro neutralizing capacity and similar cytokine production profiles.

EXAMPLE 9

This example is related to immunogenic compositions comprising a TLR4 agonist (e.g., E6020) and aluminum hydroxide as a carrier.

Groups of female CDI mice (10 per group) (Charles River) were immunized intramuscularly on three separate occasions (at about 3 week intervals) with 50 μ l of formulations comprising 10 μ g of rMOMP and one of several adjuvants (i.e., ADJ.SQ, ADJ.A, Alum (aluminum hydroxide), and ADJ.B (an adjuvant comprising a TLR4 agonist (E6020) + Alum (aluminum hydroxide))). Immunization doses were prepared by mixing 25 μ l of the antigen solution (rMOMP in buffer (50mM Tris pH 8.0 + 0.1% NLS)) with 25 μ l of adjuvant.

The adjuvant, ADJ.B (comprising E6020 and Alum) was prepared in the following manner:

Powder E6020 (EISAI) was diluted in ethanol to reach a concentration of about 12 mg/ml. 100µl of this solution was then added to 1.9ml of water which was maintained under agitation. The aqueous solution was then filtered and mixed with buffer PBS (10X) (9 volume of E6020 solution for 1 volume of PBS (10X) to get an aqueous solution of E6020 (with some ethanol) at about 0.5mg/ml. To a Peni flask with 120µl of this aqueous E6020 solution and 930 µl of PBS (1X) was added 450 µl of an aqueous suspension of AlOOH at a concentration of 8 mg/ml. This mixture was homogenized and vortexed for 10 seconds. The prepared adjuvant comprised 2.4mg/ml of Aluminum and 40µg/ml of E6020.

The ADJ.SQ and ADJ.A adjuvants used in this example were prepared substantially as described in Example 6.

Collection of sera and splenocytes was done substantially as described in Example 6 and measurement of cytokine production and isotyping analysis was also performed substantially as described in that Example. The ability of the elicited antibodies to neutralize *C. trachomatis* serovar E was assessed by neutralization assay (conducted substantially as described in Example 6). A summary of the ELISA titres, neutralizing titres and cytokine profile by MSD analysis are set out in Tables 9 and 10.

Table 9

	ELISA Titre						% Neutralizing Mice	Average Titre	t-test
	Total IgG Avg	sd	IgG1 Avg	Sd	IgG2a Avg	sd			
rMOMP+ADJ.SQ	79840	74271	42560	36821	30755	64745	20%	80	0.028
rMOMP+Alum	3415	7857	1625	3957	205	490	0%	13	0.015
rMOMP+ADJ.A	348160	506164	57765	79520	496650	1009398	90%	769	
rMOMP+ADJ.B	558090	1079692	42735	66320	364335	1025443	80%	568	0.602

Table 10

These results demonstrated that rMOMP adjuvanted with E6020 irrespective of the carrier system used (i.e., either aluminum or emulsion) induces a Th1/Th2 balanced response whereas a Th2-orientated immune response is induced by rMOMP adjuvanted with the carrier system alone (e.g., Alum).

	MSD Analysis							
	INF γ	IL-10	IL-4	IL-5	IL-2	IL-12	IL-13	IL-17
rMOMP+ ADJ.SQ	4885.8	11953.1	90.5	5670.6	575.8	219.3	3131.2	255.6
rMOMP+Alum	9981.9	3038.8	113.2	2789.8	582.2	282.0	1879.9	130.3
rMOMP+ ADJ.A	20189.4	3626.1	72.7	1104.1	2934.0	438.8	2662.0	346.9
rMOMP+ ADJ.B	15869.9	766.3	77.7	1006.2	4832.4	395.4	4461.1	922.8

EXAMPLE 10

This example describes the biochemical and biophysical characterization of the multimeric nature of the recombinant MOMP.

Biochemical and biophysical characterization testing was performed on human serovar rMOMP samples that were prepared substantially in accordance with the method set out in Example 4. Tests included CD spectroscopy, intrinsic fluorescence spectroscopy, AUC, SEC-MALS, blue native gel electrophoresis, DSC, FTIR, and mass spectrometry. The data from these tests show that rMOMP samples are folded, and have β -sheet rich secondary structures. Their solution structures are characterized by the presence of polydisperse oligomers likely consisting of MOMP dimers, trimers, tetramers, higher order oligomers and in some samples, putative monomers.

Protein samples were diluted to the appropriate concentration for loading and then combined with sample buffer and Coomassie G-250 additive reagents (Invitrogen). Samples were loaded into wells of a pre-cast 4-16% polyacrylamide gradient gel (NativePAGE™ Novex Bis-Tris Gels, Invitrogen) and electrophoresed at 150 V for approximately 2 h. Following electrophoresis, gels were fixed and destained according to the manufacturer's instructions. For Western blot analysis, gels were transferred to PVDF membranes.

Samples of rMOMP showed a characteristic ladder pattern on BN-PAGE (see Figures 6A and 6B). Based on the mobilities of the MW standards, the lowest-MW band in the majority of rMOMP samples had a molecular weight of approximately 80,000 corresponding to a putative MOMP dimer. Similarly, the higher MW bands on the ladder presumably correspond to progressively higher-order oligomers (i.e. trimers, tetramers, pentamers, etc.). This was investigated from a theoretical standpoint by comparing the relative mobilities (Rf-values) of bands from 3 different SerD rMOMP samples with the theoretical MW's of the putative MOMP oligomers. In one of these lots (JR2886) a weak band was observed at the bottom of the ladder with mobility beyond that of the 66-kDa MW marker, and this band was assumed to be a monomer. Weak monomer bands were also observed in SerJ rMOMP lots JR3321 and JR3340 (Figure 6B). Plotting of the measured Rf-values (Figure 6C) against the logarithm of the putative MW's of the putative oligomers (ranging from monomer to heptamer) resulted in an approximately linear correlation, as would be expected for proteins migrating in a polyacrylamide gel suggesting that the ladder bands observed in rMOMP samples correspond to integer multiple oligomers of MOMP (monomer, dimer, trimer, etc.).

The specificity of the stained bands on BN-PAGE was investigated by means of Western blotting using a SerD MOMP-specific monoclonal antibody. A native MOMP sample, which was extracted and purified from the outer membranes of SerD *C. trachomatis* elementary bodies (EB-MOMP), in accordance to process described previously (2001, Infect. Immun. 69:6240-6247) was run as a positive control. All of the ladder bands in the BNG of the 3 SerD rMOMP lots were recognized by the specific mAb in the corresponding Western blot. Therefore, the bands observed in the BNG ladder are MOMP-specific.

The rMOMP samples were assessed by Far-UV CD spectroscopy. The samples all showed evidence of β -sheet rich secondary structure, characterized by a broad spectral minimum around 215 nm. By intrinsic fluorescence spectroscopy, the samples had similar emission spectra, both at 280 nM and 295 nm, suggesting a similar molecular environment for the aromatic side chains of tyrosine and tryptophan.

The samples were assessed by size-exclusion chromatography (SEC). The SEC elution profiles of the rMOMP samples were consistent with polydisperse MOMP oligomers (Figures 7, 8). Difference in profiles may reflect differences in the distribution of the various sized oligomeric species. Analysis of the eluting SE-HPLC fractions using multi-angle light scattering (MALS) was also performed and this data (not shown) was similarly consistent with the presence

of polydisperse oligomers, whereby high-MW species eluted early in the chromatogram, with lower-MW weight species eluting later.

Sedimentation velocity was conducted by AUC. The sedimentation velocity results for the rMOMP samples are consistent with the presence of oligomers. The broadness and overall shape of the AUC spectra was consistent with polydisperse samples consisting of MOMP oligomers of different sizes. This profile is consistent with results from SEC-MALS and BNG experiments. The peak apex sedimentation coefficients (*s*-values) for the lots were between 8 and 9 Svedbergs. A monomer of rMOMP (~40 kDa) would be expected to have an *s*-value of approximately 2 Svedbergs. Therefore, rMOMP samples consist of polydisperse oligomers.

EXAMPLE 11

This example describes the evaluation of human sera from subjects with Chlamydia using rMOMP.

Serum samples collected from two different cohorts of Chlamydia-infected subjects were evaluated by ELISA, to assess IgG titers, and using an *in-vitro* assay to assess neutralization capacity. The first study cohort was comprised of male and female subjects >16 years of age and attending a clinic for Chlamydia treatment. The subjects in this study cohort tested positive in a urogenital Chlamydia screening nucleic acid amplification test (the Gen-Probe Aptima Combo 2 [GP AC2, Gen-Probe, Inc., San Diego, CA]) and were enrolled in a Chlamydia study following treatment. These subjects underwent urogenital Chlamydia testing by GP AC2 and had serum collected during the baseline visit and as part of a 6-month follow-up visit. The second study cohort was comprised of female subjects (> 18 years of age) enrolled in a treatment outcome study following a positive test in a urogenital Chlamydia screening test (Ligase Chain Reaction). As part of the treatment outcome study, both a cervical Chlamydia culture and a serum sample was collected from each subject during scheduled visits (*i.e.*, at baseline, day 7, and day 21). Culture-negative genital specimens were subsequently tested by PCR (COBAS AMPLICORTM; Roche Diagnostic Systems, Inc., Branchburg, NJ).

Serum levels of total *Chlamydia trachomatis* IgG were measured using the *Chlamydia trachomatis*-IgG-ELISA-plus Medac assay (497-PLUS, Medac GmbH, Germany) as per the manufacturer's protocol. The Medac assay uses a synthetic peptide from a MOMP variable domain (an immunodominant region of the protein).

Total IgG, IgG1 and IgG3 levels were also measured using ELISA assays developed using rMOMP made in accordance to the methods of the invention (SP-IgG, SP-IgG1 and SP-IgG3, respectively). Two lots of rMOMP (lot# sp021 and sp4500), each derived from serovar E, were utilized. Lot#sp021 had been prepared substantially in accordance with the process set out in Example 4, as was Lot#sp4500 apart from a few differences (i.e., the diafiltration buffer used in the refolding procedure was 50 mM Tris, pH 8.0, 0.1% NLS and the inclusion body pellet underwent a NLS wash substantially as described in Example 5, before the purification/refolding process). Protein purity for Lot#sp021 was 91.4% and was 99.7% for Lot#sp4500.

Briefly, 96-well plates were coated with rMOMP at a concentration of 0.1µg/mL and incubated overnight at 4°C. The plate was then blocked with blocking buffer (PBS-1% BSA solution) and following incubation, residual blocking buffer was removed. Serum samples pre-diluted with assay diluent were then added and incubated at room temperature. The plate was washed and then incubated with an HRP conjugated goat anti-human IgG (H+L chain, Jackson Laboratories), mouse anti-human IgG1 (γ1 chain, Southern Biotechnology) or mouse anti-human IgG3 (γ3 chain, Southern Biotechnology). The plate was then developed as per the manufacturer's protocol using TMB substrate (Sigma) and analyzed using the Softmax™ plate reader (at absorbance 450nm-540nm). ELISA units were noted in absorbance units (AU/mL); the AU unit value was assessed by comparing the fluorescence of the test sample to an established standard control serum.

Results were analysed statistically: correlations between continuous variables were assessed using Spearman's Rank correlation and differences between means were evaluated using the Mann-Whitney U-Test for non-parametric populations. Only those study participants that were positive by the Medac IgG ELISA (26/40 (65%) study participants) were included in the statistical analysis.

Overall, total IgG titers were lower by the Medac IgG assay compared to levels measured by the SP-IgG ELISA ($\log_{10}$ IgG 1.9 vs. 2.5 respectively, $p < 0.0001$, Figure 9A), but a strong positive correlation between total IgG levels measured by the Medac ELISA and the SP-IgG ELISA (Figure 9B) was evident. In addition, total IgG levels measured using the medac and SP IgG ELISA's strongly correlated to SP-IgG3 levels in patient sera. A strong positive correlation was also noted between the total IgG and IgG1 levels as measured by the SP-ELISA ($r = 0.52$, $p = 0.005$), although no correlation was observed between SP-IgG1 levels and Medac IgG levels ($r = 0.15$, $p = 0.46$).

The neutralization capacity of sera from 26 participants sero-positive for *Chlamydia trachomatis* (by Medac IgG ELISA) was assessed using an *in vitro* neutralization assay. The assay was conducted substantially as described in Example 6 but with serum samples diluted in PBS (Invitrogen) containing 5% Guinea pig serum (VWR catalogue # CA80057-496). A similarly diluted EB stock was added to samples and following an incubation period, mixture was transferred to a previously plated monolayer of HELA cells. Plate was centrifuged and sera-EB suspension was removed. Following an incubation period, the cells were fixed with the addition of 100% methanol (Sigma, Canada), then washed with PBS and incubated in 0.1% BSA-PBS buffer with a dilution of antibodies against *C. trachomatis* MOMP. The antibodies had been derived from rabbit sera raised against 4 peptides to the variable domain of MoPn MOMP. Cells were then washed with PBS and incubated with a Donkey anti-Rabbit HRP conjugated antibody diluted in 5% FBS-PBS with goat serum (Sigma, Canada). The plate was then washed and developed with the metal enhanced DAB substrate kit (Pierce, Canada) and counted using the Zeiss Observer Z1 microscope with the AxioVision™ 4.7.1 software. Neutralization titers, which were defined as the dilution of sera able to neutralizing infection by $\geq 50\%$ of control values, ranged from 5 to 320.

The assay utilized provided a method of evaluating the effectiveness of serum IgG at preventing infection of HELA cells exposed to *Chlamydia trachomatis* elementary bodies (EB), and as such, it enabled a determination to be made as to whether the IgG titers in sera corresponded to functional activity against Chlamydia EB's. A strong positive correlation between SP-IgG levels and neutralization titers in all participants ($r=0.47$, $p=0.015$, Figure 10A) was evident and the correlation was even stronger when 4 of the outliers were removed from the analysis ($r=0.59$, $p=0.004$, Figure 10B). In all participants, levels of IgG3, but not IgG1 ($r=0.27$, $p=0.17$), as measured by the SP-ELISA correlated positively with neutralization titers ($r=0.55$, $p=0.003$, Figure 11A). When perceived outliers were excluded from the analysis, a stronger correlation was noted ($r=0.66$, $p=0.001$, Figure 11B). IgG titers as measured by the Medac ELISA kit, did not correlate with neutralization titers ($r=0.21$, $p=0.30$).

These findings show that the ELISA assay utilizing rMOMP made in accordance to the present invention was able to detect *Chlamydia trachomatis* specific IgG levels in sera from human subjects infected with *C. trachomatis*. The levels of total IgG detected by this assay correlated strongly with those measured using the commercially available Medac ELISA kit as did the levels of IgG subtypes, including IgG1 and IgG3. The levels of total IgG and IgG3 as measured using the rMOMP ELISA assay also correlated positively with neutralization titers

which suggest that the rMOMP-specific IgG and IgG3 recognized by the SP-IgG ELISA are able to neutralize serovar E infections *in vitro*. Indeed, the neutralization capacity of sera was abolished by depleting IgG from sera. Therefore, the rMOMP utilized in the ELISA (and made in accordance to the present invention) was in a substantially native-like form as it was able to detect neutralizing *C. trachomatis* specific antibodies that had been elicited by a wild-type infection with *C. trachomatis*.

While example methods, proteins, compositions and other features have been described, it is not the intention of the applicants to restrict or in any way limit the scope of the invention or application. Modifications, alterations and variations will be readily apparent to those of skill in the art. Therefore, the invention is not limited to the specific details, the representative apparatus and examples shown and described.

The contents of all references cited above are incorporated herein by reference. Use of singular forms herein, such as “a” and “the”, does not exclude indication of the corresponding plural form, unless the context indicates to the contrary. Thus, for example, if a claim indicates that use of “a” X or Y, it can also be interpreted as covering use of more than one X or Y unless otherwise indicated. To the extent that the term (or) is used in the description or claims (e.g., A or B) it is intended to mean “A or B or both”. In circumstances where the intention is to indicate “only A or B but not both” then the term “only A or B but not both” will be employed. Thus, the term “or” herein is used in the inclusive and not the exclusive sense.

Other embodiments are within the following claims.

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 Lys Pro Thr Thr Asp Thr Gly Asn Ser Ala Ala Pro Ser Thr Leu Thr
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 Phe Thr Asn Ala Ala Cys Met Ala Leu Asn Ile Trp Asp Arg Phe Asp
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 Val Glu Leu Tyr Thr Asp Thr Thr Phe Ala Trp Ser Val Gly Ala Arg
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 30 Ala Ala Glu Phe Thr Ile Asn Lys Pro Lys Gly Tyr Val Gly Lys Glu
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 Phe Pro Leu Asp Leu Thr Ala Gly Thr Asp Ala Ala Thr Gly Thr Lys
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 Asp Ala Ser Ile Asp Tyr His Glu Trp Gln Ala Ser Leu Ala Leu Ser
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	Ile Ala Val Gly Thr Thr Ile Val Asp Ala Asp Lys Tyr Ala Val Thr			
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	atcgtttctt tgcaattgaa caagatgaaa tctagaaaat cttgcggtat tgcagtagga	1020		
	acaactattg tggatgcaga caaatacgca gttacagttg agactcgctt gatcgatgag	1080		

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	acgactattg	tagatgcaga	caaatacgca	gttacagttg	agactcgctt	gatcgatgag	1080
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 50 55 60
 5 Lys Pro Thr Ser Thr Thr Gly Asn Ala Thr Ala Pro Thr Thr Leu Thr
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 Ala Arg Glu Asn Pro Ala Tyr Gly Arg His Met Gln Asp Ala Glu Met
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 Phe Thr Asn Ala Ala Cys Met Ala Leu Asn Ile Trp Asp Arg Phe Asp
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 Val Phe Cys Thr Leu Gly Ala Ser Ser Gly Tyr Leu Lys Gly Asn Ser
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 Ala Ser Phe Asn Leu Val Gly Leu Phe Gly Asp Asn Glu Asn Gln Ser
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 165 170 175
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 25 Phe Pro Leu Ala Leu Ile Ala Gly Thr Asp Ala Ala Thr Gly Thr Lys
 225 230 235 240
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 Ala Ser Phe Asp Ala Asp Thr Ile Arg Ile Ala Gln Pro Lys Ser Ala
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 35 Gly Asp Val Lys Ala Ser Ala Glu Gly Gln Leu Gly Asp Thr Met Gln
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	gccgcttgca tgacattgaa tatttgggat cgttttgatg tattctgtac attaggagcc	360
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 10 Ala Leu Ala Gly Ala Ser Gly Asn Thr Thr Ser Thr Leu Ser Lys Leu
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 Val Glu Arg Thr Asn Pro Ala Tyr Gly Lys His Met Gln Asp Ala Glu
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 Met Phe Thr Asn Ala Ala Cys Met Thr Leu Asn Ile Trp Asp Arg Phe
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 Asp Val Phe Cys Thr Leu Gly Ala Thr Ser Gly Tyr Leu Lys Gly Asn
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 Ser Ala Ser Phe Asn Leu Val Gly Leu Phe Gly Asp Gly Val Asn Ala
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 20 Thr Lys Pro Ala Ala Asp Ser Ile Pro Asn Val Gln Leu Asn Gln Ser
 145 150 155 160
 Val Val Glu Leu Tyr Thr Asp Thr Thr Phe Ala Trp Ser Val Gly Ala
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 Arg Ala Ala Leu Trp Glu Cys Gly Cys Ala Thr Leu Gly Ala Ser Phe
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 Gln Tyr Ala Gln Ser Lys Pro Lys Ile Glu Glu Leu Asn Val Leu Cys
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 Asn Ala Ala Glu Phe Thr Ile Asn Lys Pro Lys Gly Tyr Val Gly Lys
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 30 Glu Phe Pro Leu Asp Leu Thr Ala Gly Thr Asp Ala Ala Thr Gly Thr
 225 230 235 240
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 35 260 265 270
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 5 325 330 335
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 210 215 220
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 Gly Thr Lys Asp Ala Ser Ile Asp Tyr His Glu Trp Gln Ala Ser Leu
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Ala Leu Ser Tyr Arg Leu Asn Met Phe Thr Pro Tyr Ile Gly Val Lys
 260 265 270
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 5 Lys Leu Ala Glu Ala Ile Leu Asp Val Thr Thr Leu Asn Pro Thr Ile
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 Ala Gly Lys Gly Thr Val Val Ala Ser Gly Ser Glu Asn Asp Leu Ala
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 10 325 330 335
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 35 40 45
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 20 Ala Pro Thr Thr Lys Asp Ile Ala Gly Leu Glu Asn Asp Pro Thr Thr
 65 70 75 80
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 85 90 95
 Glu Met Phe Thr Asn Ala Ala Tyr Met Ala Leu Asn Ile Trp Asp Arg
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 Phe Asp Val Phe Cys Thr Leu Gly Ala Thr Thr Gly Tyr Leu Lys Gly
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 30 Ser Ser Asn Phe Asn Thr Ala Lys Leu Ile Pro Asn Ala Ala Leu Asn
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 165 170 175
 Gly Ala Arg Ala Ala Leu Trp Glu Cys Gly Cys Ala Thr Leu Gly Ala
 35 180 185 190
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 260 265 270
 Trp Ser Arg Val Ser Phe Asp Ala Asp Thr Ile Arg Ile Ala Gln Pro
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 Lys Ser Cys Gly Ile Ala Val Gly Thr Thr Ile Val Asp Ala Asp Lys
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21

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<211> 39

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<400> 21

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39

CLAIMS

What we claim is:

1. A method of obtaining a recombinant Chlamydial major outer membrane protein (MOMP) that is expressed as an insoluble aggregate in a heterologous host, in a soluble
5 and immunogenic form comprising the steps of:
 - (a) isolating the insoluble aggregated protein;
 - (b) admixing the insoluble aggregated protein from step(a) in an aqueous solution comprising a denaturing agent to denature the insoluble aggregated protein;
 - (c) purifying the denatured protein from step (b) by subjecting the mixture of step (b)
10 to at least one chromatographic purification in the presence of denaturing agent and collecting eluted solution of purified denatured protein;
 - (d) admixing the purified denatured protein solution from step (c) with a reducing agent and at least one small molecule additive to enhance protein folding and/or suppress protein aggregation;
 - 15 (e) reducing concentration of denaturing agent and reducing agent to levels sufficient to allow the protein to renature into soluble and substantially oligomeric form; and
 - (f) isolating the oligomeric form.
2. The method of claim 1, wherein the renatured, soluble protein from step (f) exhibits a
20 ladder pattern when analyzed by polyacrylamide gel electrophoresis conducted in non-reducing conditions.
3. The method of claim 1, wherein step (e) comprises subjecting the mixture of step (d) to at least two diafiltrations.
4. The method of claim 1 wherein the reducing agent is dithiothreitol (DTT).
5. The method of claim 1, wherein the isolated protein is greater than 80% pure.
- 25 6. The method of claim 1, wherein the denaturing agent is selected from the group consisting of urea and guanidine hydrochloride.
7. The method of claim 1 wherein the small molecule additive of step (d) is selected from the group consisting of: l-arginine, N-lauroyl sarcosine, sucrose, and ammonium sulfate.

8. The method of claim 1, wherein the small molecule additive of step (d) is selected from the group consisting of l-arginine and N-lauroyl sarcosine.
9. The method of claim 8, wherein l-arginine and N-lauroyl sarcosine are each added in step (d).
- 5 10. The method of claim 1, wherein the insoluble aggregated protein is isolated by lysing the heterologous host cell in which the protein was recombinantly expressed by chemical, enzymatic or physical means.
11. The method of claim 1, wherein the at least one chromatographic purification of step (d) is selected from the group consisting of: cation exchange chromatography, anion
10 exchange chromatography and hydrophobic interaction chromatography.
12. The method of claim 11, wherein step (c) comprises purifying the denatured protein from step (b) by cation exchange chromatography and further purifying the denatured protein by anion exchange chromatography.
13. The method of claim 12, wherein the cation exchange chromatography is performed to
15 collect solution of purified protein and the solution is then subjected to a pH shock treatment before being subjected to the anion exchange chromatography.
14. The method of claim 1, wherein less than 5% of the resulting renatured protein is in the monomeric form.
15. The method of claim 1, wherein the oligomeric form includes at least a trimeric form.
- 20 16. The method of claim 1, wherein the isolated protein is in a buffered solution having a pH of 7.5 to 8.5.
17. The method of claim 1, wherein the isolated protein is in a buffered solution having a residual concentration of NLS of 0.5% (w/v) or less.
18. The method of claim 1, wherein the reducing agent is DTT and the isolated protein is in a
25 buffered solution having a residual concentration of DTT of 24 µg/ml or less.
19. The method of claim 1, wherein the denaturing agent is urea and the isolated protein is in a buffered solution having a residual concentration of urea of 10 mg/ml or less.
20. A soluble and immunogenic recombinant Chlamydial MOMP produced in accordance with the method of any one of claims 1 to 19 wherein the protein elicits a neutralizing
30 antibody titre of at least 50% when administered to a subject.

21. A soluble recombinant Chlamydial MOMP produced in accordance with the method of any one of claims 1 to 19 wherein the protein elicits a neutralizing antibody titre of at least 90% when administered to a subject.
22. A soluble recombinant Chlamydial MOMP produced in accordance with the method of any one of claims 1 to 19 wherein the protein is derived from a serovar of *C. trachomatis* or *C. pneumoniae*.
23. The soluble recombinant Chlamydial MOMP of claim 22 wherein the protein is derived from a serovar of *C. trachomatis*.
24. The soluble recombinant MOMP protein of claim 22 wherein the protein has a amino acid sequence with at least 80% sequence identity to SEQ ID NO:3.
25. The soluble MOMP protein of claim 22 wherein the protein has a amino acid sequence with at least 80% sequence identity to SEQ ID NO:8.
26. The soluble MOMP protein of claim 22 wherein the protein has a amino acid sequence with at least 80% sequence identity to SEQ ID NO:11.
27. The soluble MOMP protein of claim 22 wherein the protein has a amino acid sequence with at least 80% sequence identity to SEQ ID NO:13.
28. The soluble MOMP protein of claim 22 wherein the protein has a amino acid sequence with at least 80% sequence identity to SEQ ID NO:15.
29. The soluble MOMP protein of claim 22 wherein the protein has a amino acid sequence with at least 80% sequence identity to SEQ ID NO:17.
30. A composition comprising the soluble recombinant Chlamydial MOMP of any one of claims 21 to 29 and a pharmaceutically acceptable carrier.
31. The composition of claim 30 further comprising an adjuvant.
32. The composition of claim 31 wherein the adjuvant comprises a TLR-4 agonist.
33. The composition of claim 32, wherein the TLR-4 agonist is E6020.
34. The composition of claim 31 wherein the adjuvant comprises an oil-in-water emulsion.
35. The composition of claim 32 or 33 wherein the adjuvant further comprises an oil-in-water emulsion.

36. The composition of claim 32 or 33 wherein the adjuvant further comprises an aluminum compound.
37. The composition of claim 36 wherein the aluminum compound is aluminum hydroxide.
38. A composition according to any one of claims 30 to 37, wherein the soluble recombinant Chlamydial MOMP is derived from a serovar of *C. trachomatis*.
39. A composition according to claim 38, wherein the composition comprises at least two or more soluble recombinant Chlamydial MOMP proteins each derived from a different serovar of *C. trachomatis*.
40. Use of the soluble recombinant MOMP of any one of claims 20 to 29 in the manufacture of a vaccine composition for the treatment or prevention of infection by the *C. trachomatis* serovar from which the MOMP was derived.
41. A method of inducing an immune response to *C. trachomatis* in a subject, the method comprising administering to the subject an effective amount of the composition of any one of claims 30 to 39.
42. A method of immunizing a subject against a disease caused by a *C. trachomatis* infection comprising administering to the subject an effective amount of the soluble MOMP of any one of claims 20 to 29.

FIGURE 1: Schematic diagram of an embodiment of the protein purification and folding process

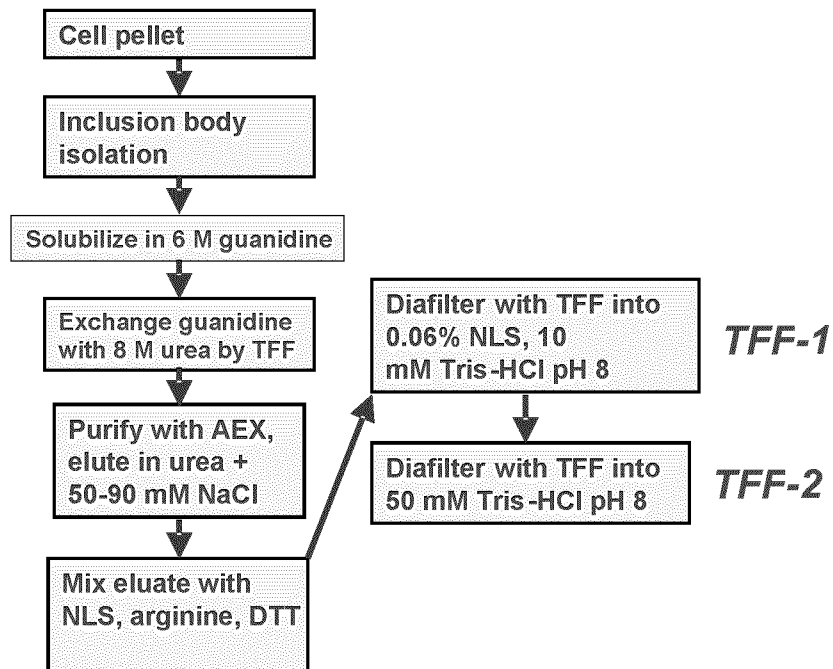
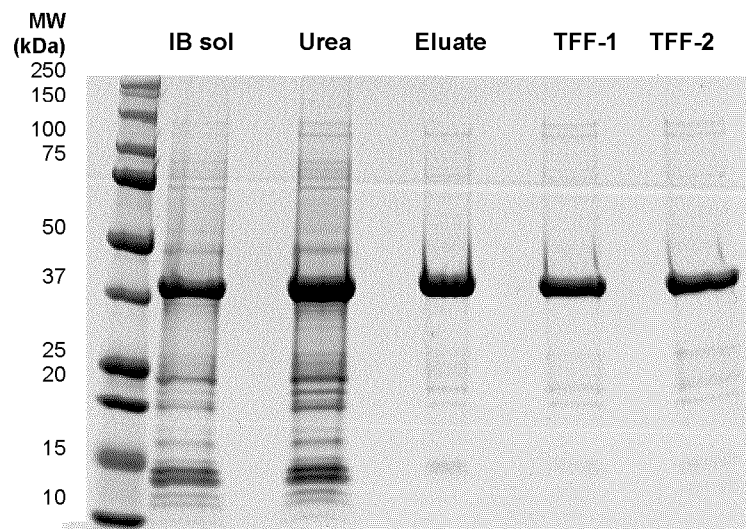


FIGURE 2:



IB sol : inclusion body pellet solubilized in 6 M guanidine

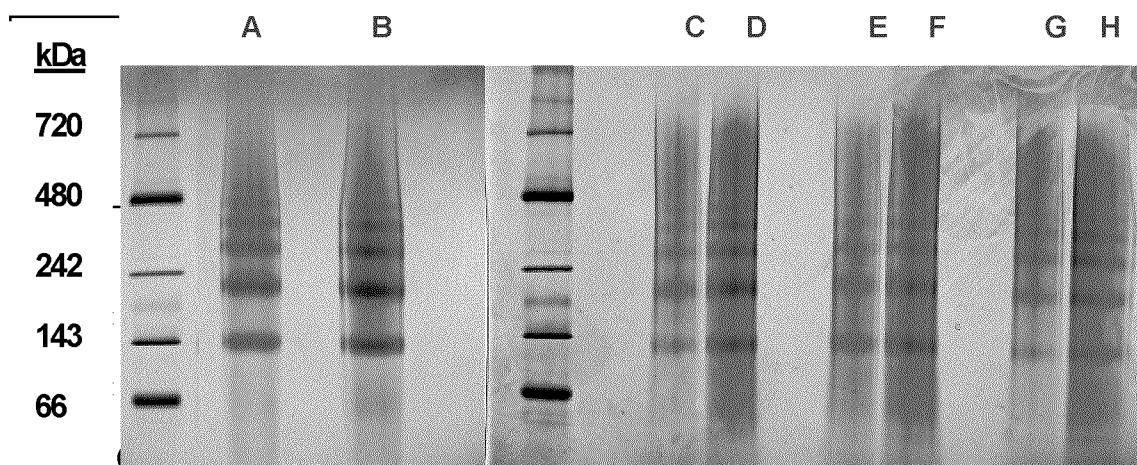
Urea : Guanidine exchanged with 8 M urea prior to column step

Eluate : Purified MOMP eluted from anion exchange column

TFF-1: First re-folding diafiltration

TFF-2: Second re-folding diafiltration – final product

FIGURE 3: Blue Native PAGE gel analysis of pilot scale (left, A,B) and three lab scale (right, C-H) Ser E MOMP protein samples.



A,B: 10&15 μ g respectively of pilot lot JR3182 Ser E MOMP
C,D: 5&10 μ g respectively of lab scale lot JR3081
E,F: 5&10 μ g respectively of lab scale lot JR3095
G,H: 5&10 μ g respectively of lab scale lot JR3097

FIGURE 4: Pilot scale Ser E MOMP purity at various steps. Shown are samples of the solubilized IBs, the purified denatured MOMP (Q pool); the folded protein (folded); and the final product (final).

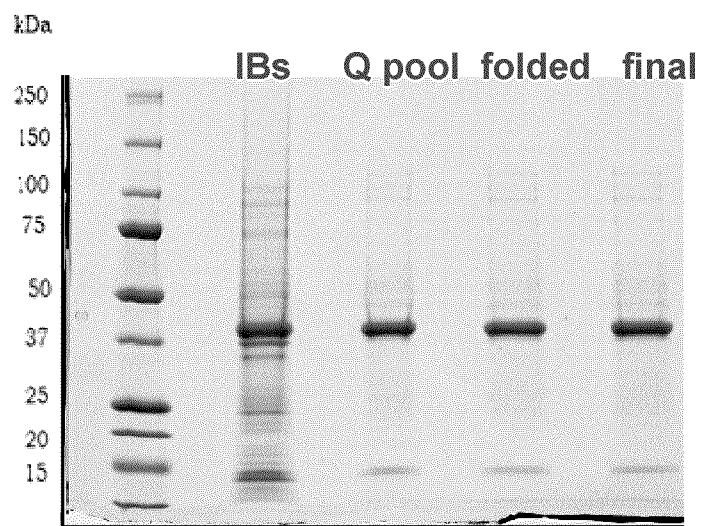


FIGURE 5: Comparison of the former (left panel) and revised (right panel) MOMP protein purification methods. In both gels, lanes are loaded in triplicate with 4 μ g of protein. The same starting material was used for both purification runs, and improved purity is evident in the right panel.

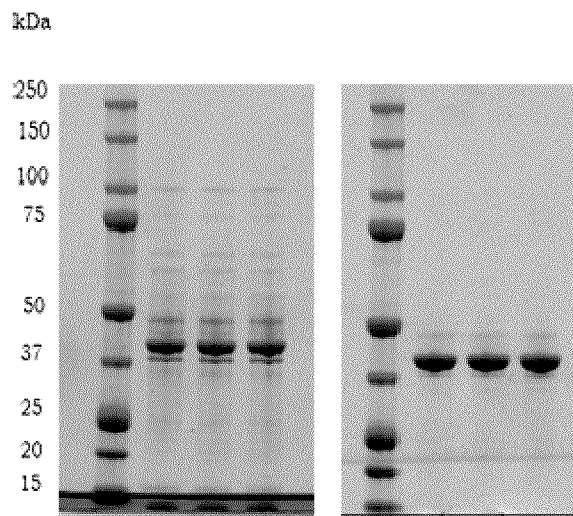


FIGURE 6A: Blue Native Gel Electrophoresis of samples of SerE rMOMP

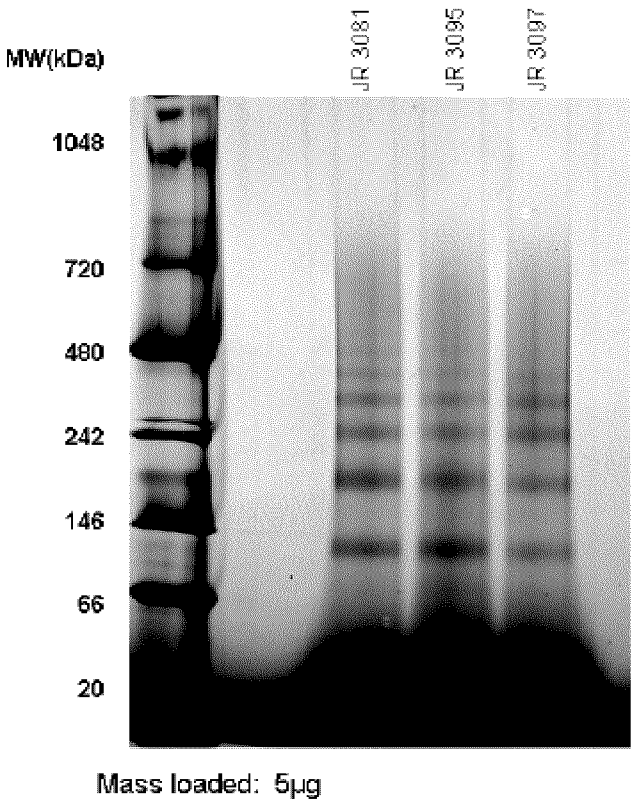


FIGURE 6B: Blue native gel electrophoresis of SerF, Ia, and J rMOMP

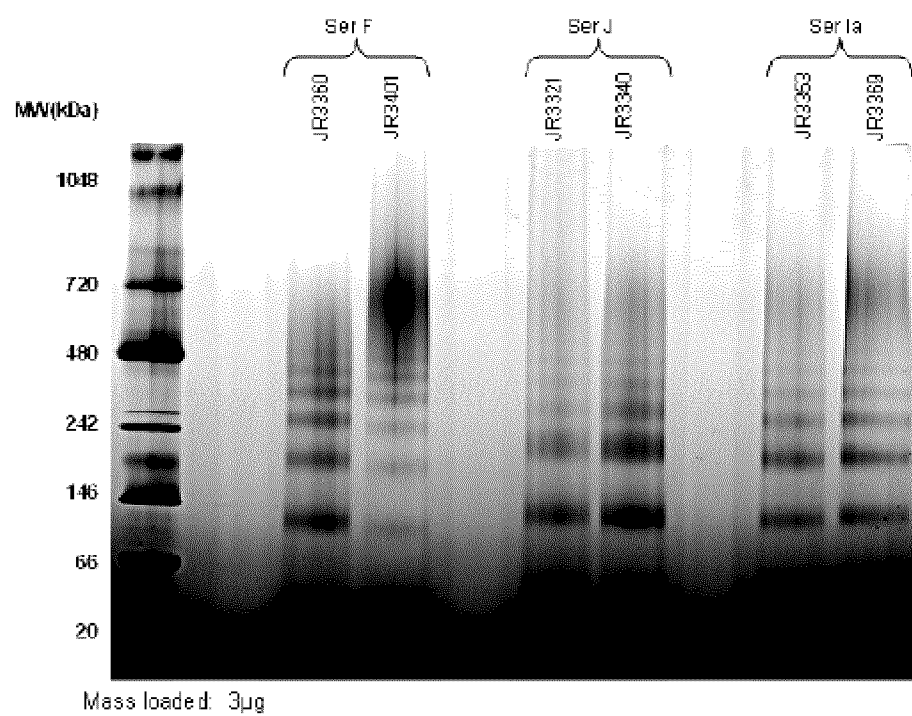


FIGURE 6C: Migration of SerD rMOMP bands on Blue native gel electrophoresis

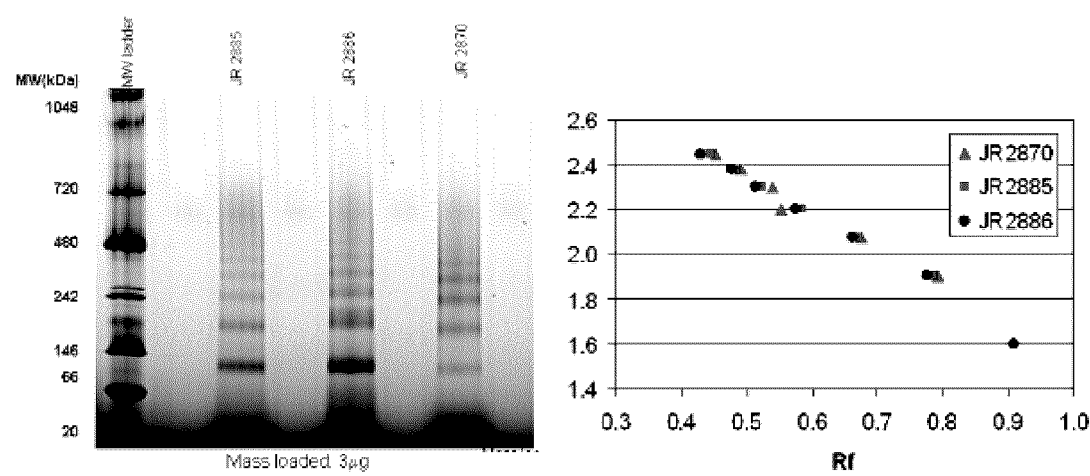


FIGURE 7

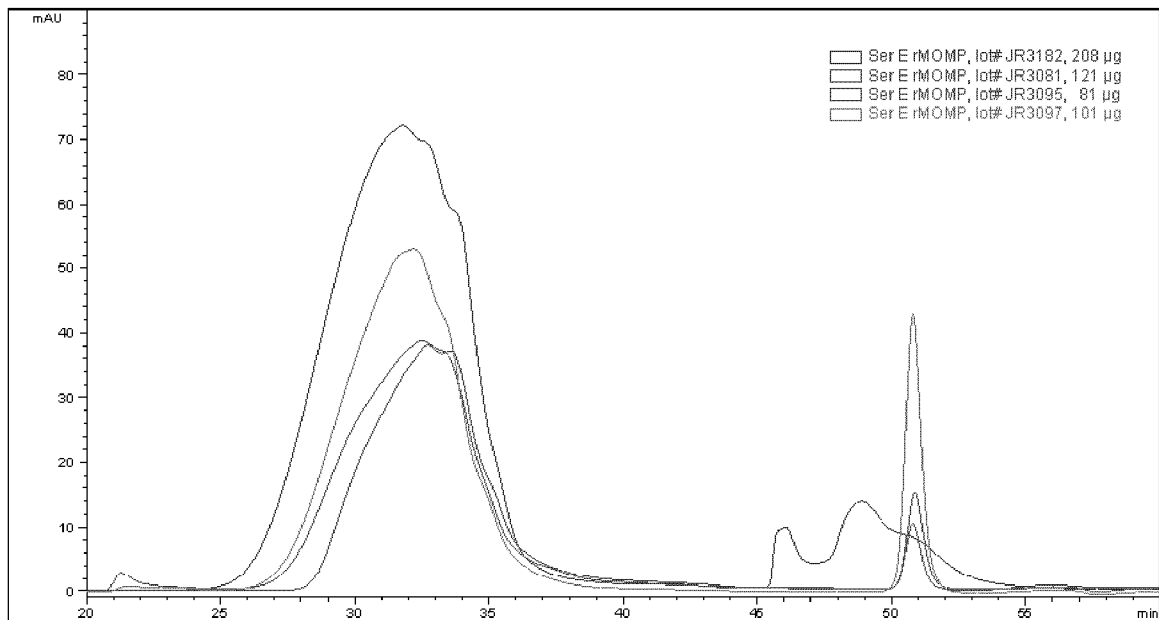
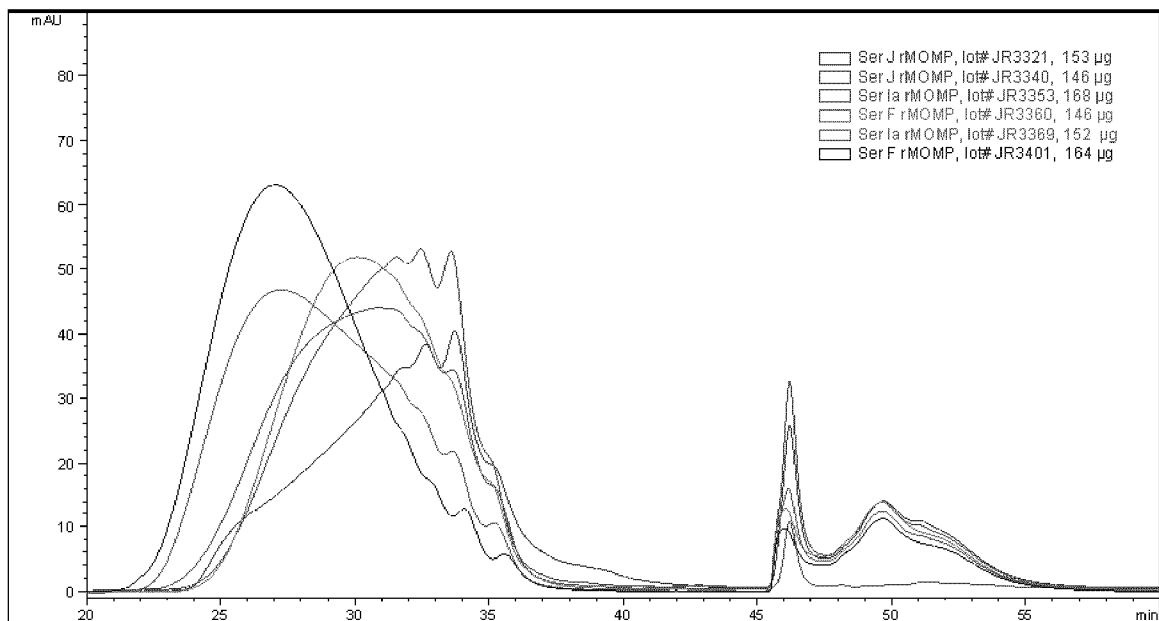


FIGURE 8



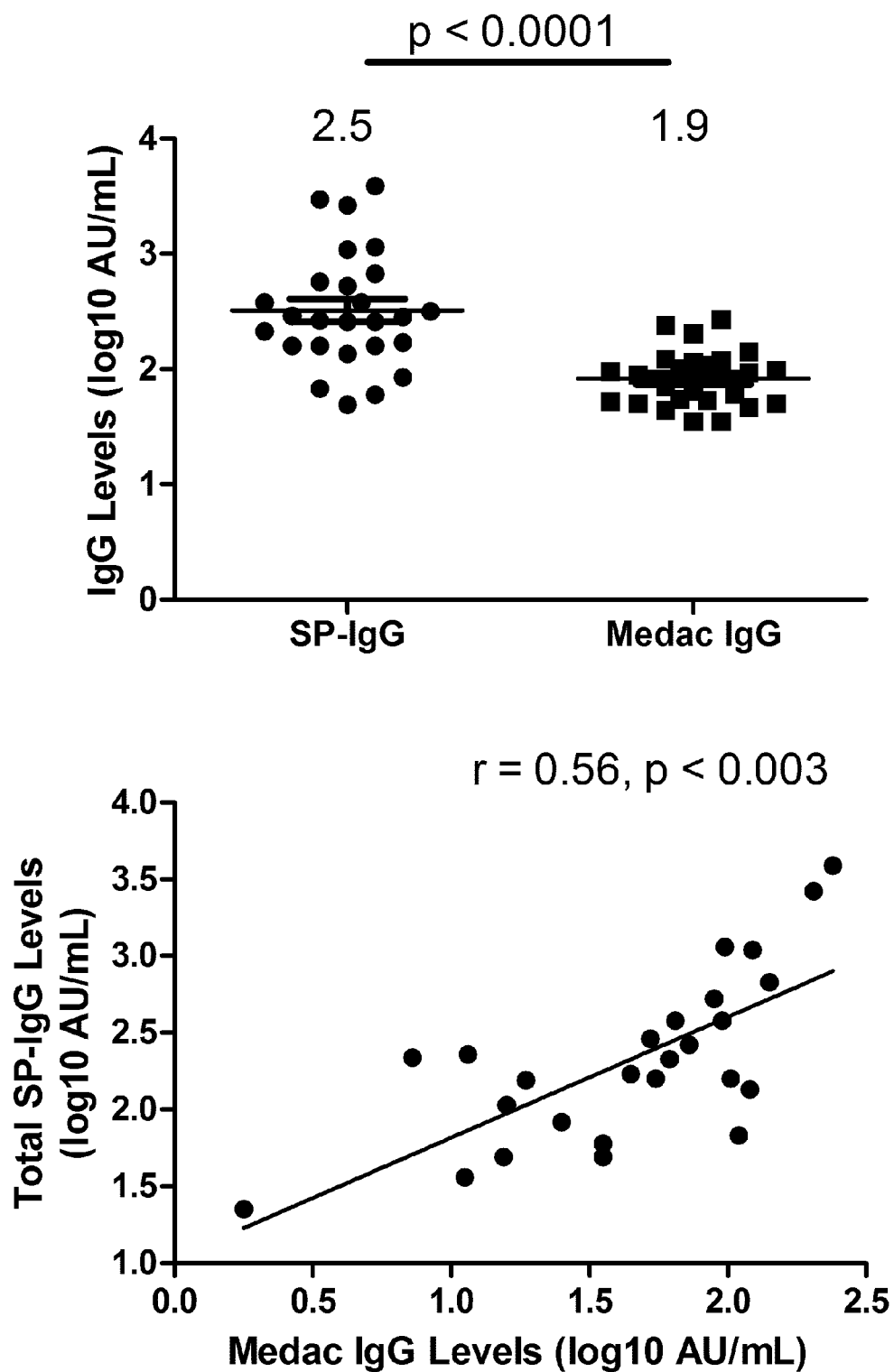


FIGURE 9 – (A) IgG titers as measured by two different ELISA methods; (B) Correlation between IgG titers as measured by the two ELISA methods.

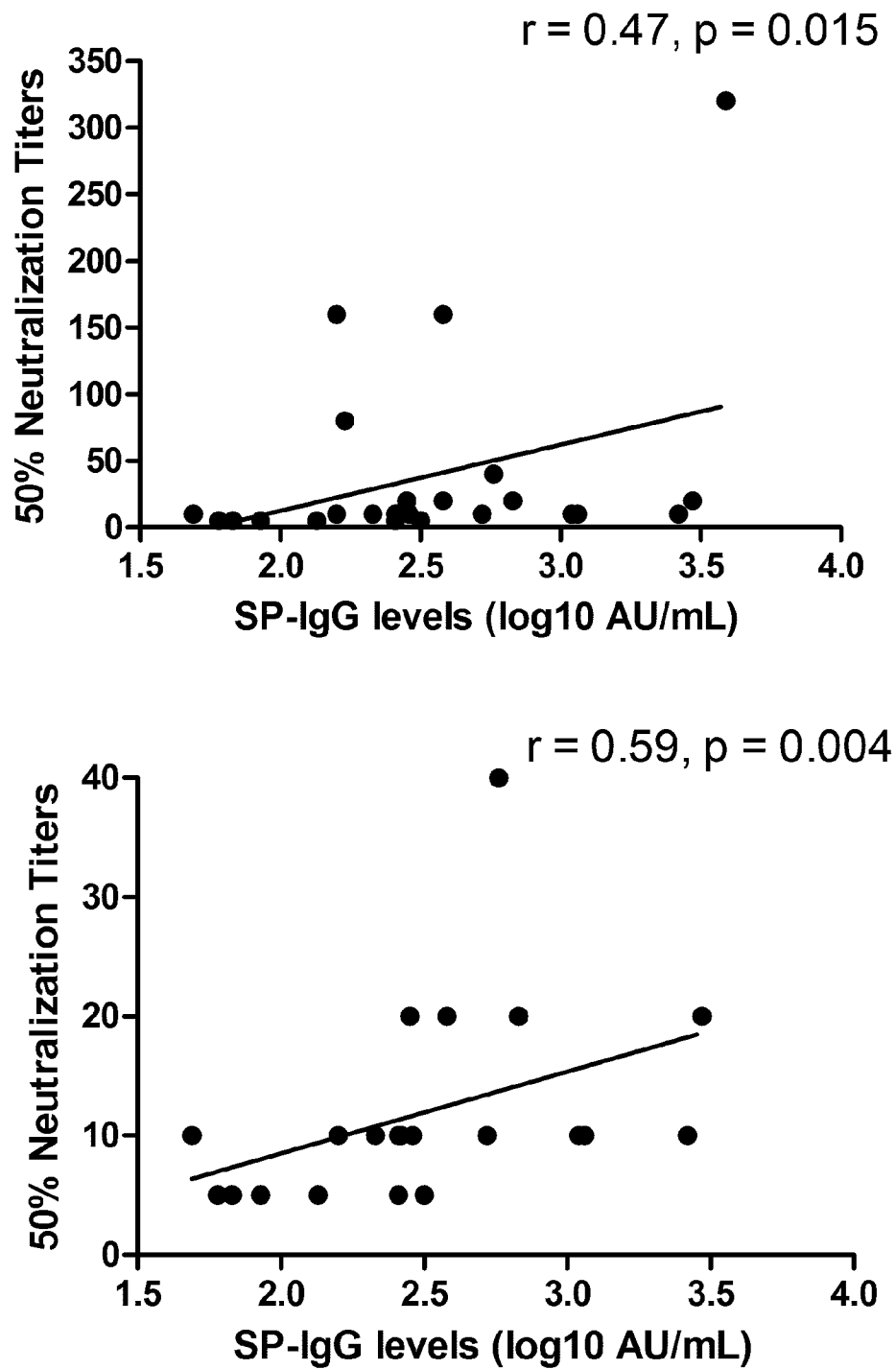


FIGURE 10 – (A) Correlation between IgG levels measured by the SP-IgG ELISA and *in-vitro* neutralization capacity of patient sera; (B) with outliers removed.

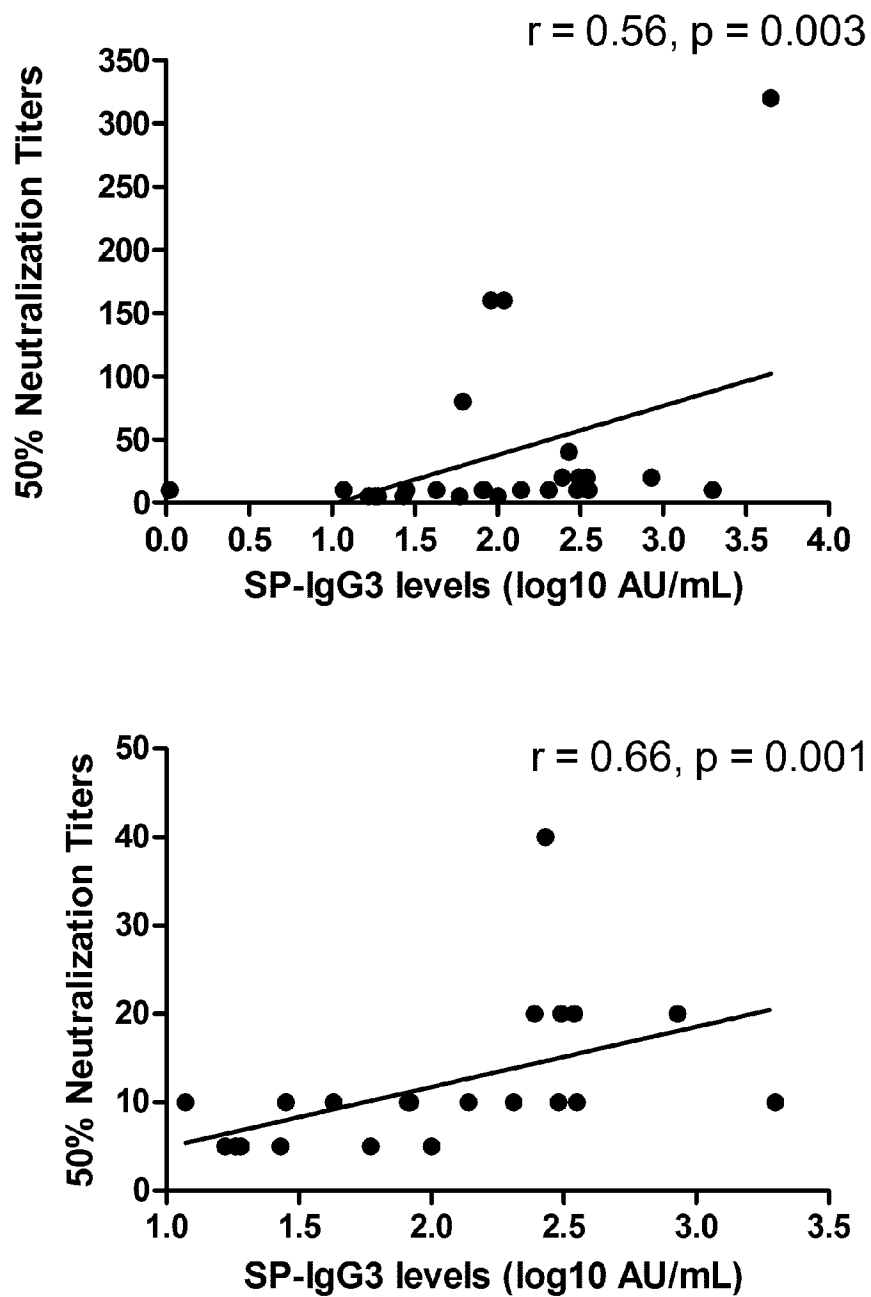


FIGURE 11 – (A) Correlation between IgG3 levels and neutralization titers in sera from all participants and (B) with the most visually obvious outliers excluded.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2011/050704

A. CLASSIFICATION OF SUBJECT MATTER

IPC: **C07K 14/295** (2006.01), **A61K 39/118** (2006.01), **A61P 31/04** (2006.01), **A61P 37/04** (2006.01), **C07K 1/16** (2006.01), **C12N 15/31** (2006.01)

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC: **C07K 14/295** (2006.01), **A61K 39/118** (2006.01), **A61P 31/04** (2006.01), **A61P 37/04** (2006.01), **C07K 1/16** (2006.01), **C12N 15/31** (2006.01)

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

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)

CAPLus, Biosis, Medline, TotalPatent, Canadian Patent Database and NCBI; Author: Riley J; Key words: insoluble, purification, urea, arginine, guanidine, refold, denature, chlamydia and major outer membrane protein

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WEN, Q. et al. Expression, purification, and refolding of recombinant fusion protein hIL-2/mGM-CSF. Biomedical and Environmental Sciences. December 2008 (12-2008), Vol. 21, No. 6, pages 509-513, ISSN 0895-3988. *see pages 509-511	1-19
Y	WAN, L. et al. Expression, purification, and refolding of a novel immunotoxin containing humanized single-chain fragment variable antibody against CTLA4 and the N-terminal fragment of human perforin. Protein Expression and Purification. 9 March 2006 (09-03-2006), Vol. 48, No. 2, pages 307-313, ISSN 1096-0279. *see pages 308-309*	1-19
Y	WARNER, L. R. et al. Expression, purification, and refolding of recombinant collagen $\alpha 1(XI)$ amino terminal domain splice variants. Protein Expression and Purification. April 2007 (04-2007), Vol. 52, No. 2, pages 403-409, ISSN 1096-0279. *see pages 404-405*	1-19

☒ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents :	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

16 January 2012 (16-01-2012)

Date of mailing of the international search report

15 February 2012 (15-02-2012)

Name and mailing address of the ISA/CA
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Authorized officer

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2011/050704

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PAL, S. et al. Vaccination with the <i>Chlamydia trachomatis</i> major outer membrane protein can elicit an immune response as protective as that resulting from inoculation with live bacteria. <i>Infection and Immunity</i> . December 2005 (12-2005), Vol. 73, No. 12, pages 8153-8160, ISSN 1098-5522. *see entire document*	20-42
Y	MANNING, D. S. and STEWART, S. J. Expression of the major outer membrane protein of <i>Chlamydia trachomatis</i> in <i>Escherichia coli</i> . <i>Infection and Immunity</i> . October 1993 (10-1993), Vol. 61, No. 10, pages 4093-4098, ISSN 1098-5522. *see entire document*	1-19
A	CALDWELL, H. D. et al. Purification and partial characterization of the major outer membrane protein of <i>Chlamydia trachomatis</i> . <i>Infection and Immunity</i> . March 1981 (03-1981), Vol. 31, No. 3, pages 1161-1176, ISSN 1098-5522. *see entire document*	1-42
A	CHEN, J. et al. Cooperative effects of urea and L-arginine on protein folding. <i>Protein Expression and Purification</i> . 20 February 2009 (20-02-2009), Vol. 66, No. 1, pages 82-90, ISSN 1096-0279. *see entire document*	1-42

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

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

1. ☒ Claim Nos. : 41 and 42

because they relate to subject matter not required to be searched by this Authority, namely :

Methods of medical treatment. Claims 41 and 42 are directed to methods of medical treatment which the Authority is not required to search under Rule 39.1 (iv) of the PCT. Nevertheless, this Authority has established a search report based on the alleged therapeutic effect derived from the use of the products defined.

2. ☐ Claim Nos. :

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

3. ☐ Claim Nos. :

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

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

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

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

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

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

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

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

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

☐ No protest accompanied the payment of additional search fees.