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2005 Research Park Circle, Suite 105, Manhattan, Kansas
66502 (US).

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(74) Agent: TRUITT, Tracey S. et al.; Armstrong Teasdale
LLP, 7700 Forsyth Blvd., Suite 1800, St. Louis, Missouri
63105-1847 (US).

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(71) Applicant: KANSAS STATE UNIVERSITY
RESEARCH FOUNDATION [US/US]; 2005 Research
Park Circle, Suite 105, Manhattan, Kansas 66502 (US).

(72) Inventors: FABURAY, Bonto; 2005 Research Park Circle,
Suite 105, Manhattan, Kansas 66502 (US). MCGILL, Jodi;

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(54) Title: EHRlichia RUMINANTium IMMUNOGENIC COMPOSITIONS AND METHODS OF USING THEREOF

Fig. 1

Antigua	MNCKKIFITSTLISLVSHLPGVSFSDVIQEDSSSPVGSVYISAKYMP TASHFGKMSIKEDS	60
Gardel	MNCKKIFITSTLISLVSHLPGVSFSDVIQEDSSPAGSVYISAKYMP TASHFGKMSIKEDS	60
Antigua	RDTKVVPGLKKDWDGVKTSSS-----NTIFTEKDYSFKYENNPFLGFAGAI GYSMNGPRI	115
Gardel	KNTQTVPGLKKDWDGVKTSSSDSGNNSI IFTEKDYSFKYENNPFLGFAGAI GYSMNGPRI	120
Antigua	EFEISYETFDVKNP GGNYNKNDAHMYCALDTATS--SGGAAASTVMVKENLTDISIMLN	173
Gardel	EFEVSYETFDVKNP GGNYNKNDAHMYCALDTGTPGSTQGATLNGSUMVKENLTDIALMLN	180
Antigua	ACYDIMLDGMPVSPYVCAGIGTDLVSVINSTNPKLSYQKLGISYSINPEASIFIGGHFH	233
Gardel	ACYDITLEGMPVSPYVCAGIGTDLVSVINATNPKLSYQKLGISYSINPEASIFIGGHFH	240
Antigua	RVIGNEFKDITTSKIFNTSN--TGCATPGFASAILDVCHFGIEIGGRFVF	281
Gardel	RVIGNEFKDITTSKIFSTGKLATAASPGFASATLDVCHFGIEIGGRFVF	290

(57) Abstract: The present disclosure provides compositions and methods for reducing the incidence of and/or severity of diseases associated with tick-borne pathogens. In preferred forms, the compositions comprise a recombinant antigenic protein subunit that has been glycosylated. Some preferred subunits include the MAPI protein of Ehrlichia ruminantium, the p30-1 sequence from Ehrlichia canis, the p28-Omp19 protein from Ehrlichia chaffeensis, and the MSP4 protein from Anaplasma marginale. Administration of such compositions to an animal in need thereof provides protection against clinical signs of infection in susceptible animals.



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EHRlichia RUMINANTIUM IMMUNOGENIC
COMPOSITIONS AND METHODS OF USING
THEREOF

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] The current patent application is related to U.S. Patent Application Serial No. 62/533,550, which is hereby incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION

[0002] The Heartwater or cowdriosis is a tick-borne disease caused by an intracellular rickettsial pathogen *Ehrlichia ruminantium* and transmitted by ticks of the genus *Amblyomma*. The disease can cause high mortality rates of up to 90% in susceptible ruminants. Heartwater is endemic in sub-Saharan Africa where it presents a serious constraint to livestock improvement programs. Through its occurrence in the Caribbean, there is significant threat of introducing heartwater into North America, where the presence of competent vectors, *A. cajennense*, *A. maculatum*, and *A. dissimile* have been confirmed. Additionally, other tick-borne diseases including *Ehrlichia chaffeensis*, *Ehrlichia canis*, *Ehrlichia ewingii*, *Ehrlichia muris*, *Anaplasma phagocytophilum*, *Anaplasma platys*, and *Anaplasma marginale* present serious problems for livestock

[0003] There are no safe or reliable vaccines available for these pathogens. Due to safety concerns associated with the production and manufacture of live attenuated and inactivated vaccines, subunit vaccines are considered appropriate to control the disease in endemic and non-endemic areas. Antigenic diversity amongst different isolates of *E. ruminantium*, resulting in lack of protection between heterologous strains, is the single major obstacle to heartwater vaccine development. However, the observation that inactivated vaccines stimulate protective immunity indicates that the development of a successful recombinant vaccine, using selected *E. ruminantium* genes, is possible. The Major Antigenic Protein 1 (MAP1), encoded by

map1 gene, is an immunodominant surface protein of *E. ruminantium* and a target for subunit vaccine development. However, sequence polymorphism of the gene among different isolates suggests that any vaccine based on this gene would have to include variants of all important strains. For the American continent, variants of these genes should ideally be obtained from strains in the Caribbean, which due to geographic proximity, pose the greatest threat of imminent introduction. Previous studies using MAP1 as a subunit vaccine involved DNA vaccine constructs encoding MAP1 of the Crystal Springs isolate from Zimbabwe. This vaccine formulation induced partially protective T_{H1} type immune responses in mice; and a subsequent study to improve protection involved boosting with recombinant MAP1 (rMAP1) resulting in survival rate of mice up to 67%. Protection was associated with induction of T_{H1} type immune responses characterized by production of IgG2a and IgG3 anti-MAP1 specific antibodies. However, when delivered as recombinant protein, MAP1 induced a less protective immune response, which was characterized by anti-MAP1 antibodies of predominantly IgG1 isotype. Although, the mechanism by which the *map1* DNA vaccine elicits protection is not fully elucidated, it clearly involves utilization of the host (eukaryotic) expression machinery to generate MAP1 antigen that induces the host anti-pathogen immune response.

[0004] MAP1 has been demonstrated to be a glycoprotein, and the protein is expressed in glycosylated form in eukaryotic (endothelial cells) expression systems. Glycosylation of immunodominant surface proteins has also been reported in other *Ehrlichia* species, whereby weak immunoreactivity has been reported for unglycosylated immunodominant proteins of *E. canis* (gp36) and *E. chaffeensis* (gp47). This suggests that glycans are important epitope determinants. The rMAP1 protein used in prime-boost studies were expressed in a prokaryotic (*E. coli*) expression system, which is known to produce nonglycosylated proteins. The present disclosure postulated that lack of protein glycosylation could account for the suboptimal protective immune response observed in mice vaccinated with the rMAP1 protein. The overall hypothesis was that vaccination with glycosylated forms of rMAP1 proteins produced in a eukaryotic (baculovirus) expression system will elicit a

more robust immune response that will confer protection against virulent *E. ruminantium* challenge. We have recently reported the successful use of a baculovirus expression system to produce Rift Valley fever virus (RVFV) glycoproteins as constituents of an efficacious vaccine against RVFV infection in a target host. Here, we used a similar baculovirus expression system to produce glycosylated MAP1 proteins from *E. ruminantium* and report for the first time the immunogenicity of a glycosylated rMAP1 subunit vaccine in sheep.

BRIEF DESCRIPTION OF THE INVENTION

[0005] To develop a protective DIVA (differentiate infected from vaccinated animals) subunit vaccine for heartwater, we targeted the *E. ruminantium* immunodominant major antigenic protein1 (MAP1) with the hypothesis that MAP1 is a glycosylated protein and glycans contained in the antigenic protein are important epitope determinants. Using a eukaryotic recombinant baculovirus expression system, we expressed and characterized, for the first time, a glycoform profile of MAP1 of two Caribbean *E. ruminantium* isolates, Antigua and Gardel. We have shown that the 37-38 kDa protein corresponded to a glycosylated form of the MAP1 protein, whereas the 31-32 kDa molecular weight band represented the non-glycosylated form of the protein frequently reported in scientific literature. Additionally, the signal peptide common to all *E. ruminantium*, was included and cleavage site identified, which differs from previous attempts to solve this problem. This ensured the glycosylation of the expressed recombinant MAP1 protein. Without being bound to any theory of action, it is postulated that the glycan residues are antigenic determinants and are key to the induction of protective T cell and humoral immune response. It is understood that other methods of glycosylation, such as expression through any mammalian or eukaryotic system, would also be effective. Three groups of sheep ($n = 3-6$) were vaccinated with increasing doses of a bivalent (Antigua and Gardel MAP1) rMAP1 vaccine cocktail formulation with montanide ISA25 as an adjuvant. The glycosylated recombinant subunit vaccine induced *E. ruminantium*-specific humoral and Th1 type T cell responses, which are critical for controlling intracellular pathogens, including

E. ruminantium, in infected hosts. These results provide an important basis for development of a subunit vaccine as a novel strategy to protect susceptible livestock against heartwater in non-endemic and endemic areas.

[0006] The present disclosure provides for an immunogenic composition or vaccine providing protection against *E. ruminantium*. Further, a method for eliciting an immune response in an animal is provided, where the steps include administration of the immunogenic composition or vaccine disclosed herein to an animal or human in need thereof. A method for reducing the incidence and/or severity of clinical signs associated with *E. ruminantium* infection is also provided as an aspect of the present disclosure. Such a method comprises the steps of administration of the immunogenic composition or vaccine providing protection against *E. ruminantium* to an animal or human in need thereof.

[0007] Additionally, it is understood that other immunogenic compositions and vaccines against other pathogens, and especially tick-borne pathogens, could be made using the same antigenic protein glycosylation approach as that demonstrated herein. For example, the same approach will be used and effective against infection by a pathogen selected from the group consisting of *Ehrlichia chaffeensis*, *Ehrlichia canis*, *Ehrlichia ewingii*, *Ehrlichia muris*, *Anaplasma phagocytophilum*, *Anaplasma platys*, and *Anaplasma marginale* (together with *Ehrlichia ruminantium*, referred to as “tick-borne pathogens of the disclosure”). In preferred forms, the glycosylated protein will be a homolog of the MAP1 protein of *Ehrlichia ruminantium*. Particularly preferred homolog proteins include glycosylated forms of the p30-1 sequence from *Ehrlichia canis*, the p28-Omp19 protein from *Ehrlichia chaffeensis*, and the MSP4 protein from *Anaplasma marginale*. Preferably, the glycosylation would result from the inclusion of the signal peptide of the same pathogen or from *Ehrlichia ruminantium*.

[0008] The immunogenic composition or vaccine of the present disclosure is preferably killed/inactivated bacteria, modified live bacteria, a protein of which can be modified, is also provided herewith. In a preferred aspect, the

immunogenic composition or vaccine includes a protein subunit selected from the MAP1 protein subunit of *Ehrlichia ruminantium*, the p30-1 protein from *Ehrlichia canis*, the p28-OMP19 protein from *Ehrlichia chaffeensis*, the MSP4 protein from *Anaplasma marginale*, and any combination thereof. In some preferred forms the MAP1 protein will be derived from the Antigua or Gardel strains of *Ehrlichia ruminantium*. In other preferred forms, the protein subunit will be encoded by a nucleotide sequence having at least 80%, at least 90% or least 95%, 96%, 97%, 98%, 99%, or even 100% sequence homology to a nucleic acid sequence encoding any of the protein subunits described herein including the MAP1 protein subunit of *Ehrlichia ruminantium*, the p30-1 protein from *Ehrlichia canis*, the p28-OMP19 protein from *Ehrlichia chaffeensis*, the MSP4 protein from *Anaplasma marginale*, and any combination thereof.. In other preferred forms, the nucleic acid is selected from sequences having at least 80%, 90%, 95%, 96%, 97%, 98%, 99%, or even 100% sequence homology with a sequence selected from the group consisting of SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 10, SEQ ID No. 11, or any combination thereof. In some forms, the immunogenic composition or vaccine includes a protein having at least 80%, 90%, 95%, 96%, 97%, 98%, 99%, or even 100% sequence homology with a sequence selected from the group consisting of the MAP1 protein subunit of *Ehrlichia ruminantium*, the p30-1 protein from *Ehrlichia canis*, the p28-OMP19 protein from *Ehrlichia chaffeensis*, the MSP4 protein from *Anaplasma marginale*, and any combination thereof. In some preferred forms, these sequences have at least 80%, 90%, 95%, 96%, 97%, 98%, 99%, or even 100% sequence homology with a sequence selected from the group consisting of SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 7, SEQ ID No. 12, SEQ ID No. 12, and any combination thereof.

[0009] In one aspect, the immunogenic composition or vaccine of the present disclosure further comprises at least one additional element. The at least one additional element is preferably selected from, but not limited to, pharmaceutical carriers, adjuvants, pathogens other than the tick-borne pathogens of the disclosure (such as *E. ruminantium*), additional antigens, preservatives, stabilizers, colors,

flavors, and combinations thereof. In a further embodiment, the at least one additional element is an antigenic protein or peptide. In such an embodiment, the antigenic protein or peptide can be used as a positive marker for the immunogenic composition or vaccine.

[0010] A method for reducing the incidence or severity of clinical symptoms of heartwater or cowdriosis (*E. ruminantium* infection) or infection and clinical symptoms or signs caused by a tick-borne pathogen of the disclosure is also provided. The method preferably includes the steps of administration of the immunogenic composition or vaccine of the present disclosure to an animal or human in need thereof. The dosage is preferably provided in an effective amount. Preferably, clinical symptoms are selected from, but not limited to, death, fever, respiratory distress, which may be exaggerated, paroxysmal convulsions, anorexia, depression, respiratory congestion, friable mucous membranes, respiratory distress, nervous signs, hyperaesthesia, high-stepping stiff gate, exaggerated blinking, exaggerated chewing movements, and diarrhea. The clinical symptoms are preferably reduced in incidence or severity by about 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or even by 100% when compared to those animals or humans not provided the immunogenic composition or vaccine of the present disclosure.

[0011] A method for eliciting an immune response against heartwater or a different disease caused by a tick-borne pathogen of the disclosure is also provided. The method preferably includes the steps of administration of the immunogenic composition or vaccine of the present disclosure to an animal or human in need thereof. The immunogenic composition or vaccine is preferably a subunit; however, the method is not so limited. The composition or vaccine can be administered once or several times. When administered more than once, the second or subsequent doses will be administered at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 days, or more after the initial or previous administration. In preferred forms, the immune response will lessen the severity, frequency, and/or duration of at least one clinical sign of the disease by at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 96%,

97%, 98%, 99%, or even 100% in comparison to a group of animals or humans that did not receive an administration of the vaccine or immunogenic composition.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] Figure 1 is an illustration of the sequences of the present disclosure, showing the Antigua and Gardel isolates wherein the rectangular box indicates the signal peptide sequence (SEQ ID No. 14) and the underlined portions are the predicted N- and O-linked glycosylation sites. The top (Antigua) sequence is SEQ ID No 2 and the bottom sequence (Gardel) is SEQ ID No. 11;

[0013] Fig. 2A is a photograph of an electrophoresis gel confirming expression of *E. ruminantium* rMAP1 (Antigua and Gardel isolates) protein in glycosylated form utilizing a eukaryotic expression system and demonstrating a distinct glycosylation profile of the protein;

[0014] Fig. 2B is a photograph of an electrophoresis gel similar to Fig. 2A;

[0015] Fig. 3A is a graph showing three groups each having a different dose of the immunogenic composition (low =A (200 ug), intermediate =B (400 ug), and high=C (600ug), where the graph shows that there was no significant difference between the dose groups in inducing antibody responses, as assessed by the level of IgG antibody responses;

[0016] Fig. 3B is a graph showing three groups each having a different dose of the immunogenic composition (low =A (200 ug), intermediate =B (400 ug), and high=C (600ug), where the graph shows that there was no significant difference between the dose groups in inducing antibody responses, as assessed by the level of IgG antibody responses;

[0017] Fig. 3C is a graph showing three groups each having a different dose of the immunogenic composition (low =A (200 ug), intermediate =B (400 ug), and high=C (600ug), where the graph shows that there was no significant difference between the dose groups in inducing antibody responses, as assessed by the level of IgG antibody responses;

[0018] Fig. 3D is a graph showing three groups each having a different dose of the immunogenic composition (low =A (200 ug), intermediate =B (400 ug), and high=C (600ug), where the graph shows that there was no significant difference between the dose groups in inducing antibody responses, as assessed by the level of IgG antibody responses;

[0019] Fig. 4 is a series of graphs illustrating the results of endpoint titration of MAP1-specific antibody activity in all three vaccine dose groups, exhibited in a time dependent increase in antibody titers, with 28 dpv sera showing highest endpoint titers and overall antibody reactivity showed persistence of MAP1-specific antibody titers in all vaccinated animals through the endpoint;

[0020] Fig. 4A illustrates the endpoint titration of MAP1-specific antibody activity in all three vaccine dose groups of the Antigua isolate at 7 dpv, exhibited a time dependent increase in antibody titers;

[0021] Fig. 4B illustrates the endpoint titration of MAP1-specific antibody activity in all three vaccine dose groups of the Antigua isolate at 14 dpv, exhibited a time dependent increase in antibody titers;

[0022] Fig. 4C illustrates the endpoint titration of MAP1-specific antibody activity in all three vaccine dose groups of the Antigua isolate at 21 dpv, exhibited a time dependent increase in antibody titers;

[0023] Fig. 4D illustrates the endpoint titration of MAP1-specific antibody activity in all three vaccine dose groups of the Antigua isolate at 28 dpv, exhibited a time dependent increase in antibody titers;

[0024] Fig 4E illustrates the endpoint titration of MAP1-specific antibody activity in all three vaccine dose groups of the Antigua isolate at 35 dpv, exhibited a time dependent increase in antibody titers;

[0025] Fig. 4F illustrates the endpoint titration of MAP1-specific antibody activity in all three vaccine dose groups of the Antigua isolate at 42 dpv, exhibited a time dependent increase in antibody titers;

[0026] Fig. 4G illustrates the endpoint titration of MAP1-specific antibody activity in all three vaccine dose groups of the Gardel isolate at 7dpv, exhibited a time dependent increase in antibody titers;

[0027] Fig. 4H illustrates the endpoint titration of MAP1-specific antibody activity in all three vaccine dose groups of the Gardel isolate at 14 dpv, exhibited a time dependent increase in antibody titers;

[0028] Fig. 4I illustrates the endpoint titration of MAP1-specific antibody activity in all three vaccine dose groups of the Gardel isolate at 21 dpv, exhibited a time dependent increase in antibody titers;

[0029] Fig. 4J illustrates the endpoint titration of MAP1-specific antibody activity in all three vaccine dose groups of the Gardel isolate at 28 dpv, exhibited a time dependent increase in antibody titers;

[0030] Fig. 4K illustrates the endpoint titration of MAP1-specific antibody activity in all three vaccine dose groups of the Gardel isolate at 35 dpv, exhibited a time dependent increase in antibody titers;

[0031] Fig. 4L illustrates the endpoint titration of MAP1-specific antibody activity in all three vaccine dose groups of the Gardel isolate at 42 dpv, exhibited a time dependent increase in antibody titers;

[0032] Fig. 5 is a graph illustrating a characterization of the immunoglobulin G (IgG) isotype responses in vaccinated sheep showed induction of

both IgG1 and IgG2 antibody responses, with the IgG1 isotype showing comparatively higher titers as determined by endpoint titration;

[0033] Fig. 6A is a series of graphs illustrating the percentage of antigen-specific proliferation of CD4⁺ and CD8⁺ T-cells in each of the vaccinated groups for the Gardel isolate, where Group D is the control;

[0034] Fig. 6B is a series of graphs illustrating the percentage of antigen-specific proliferation of CD4⁺ and CD8⁺ T-cells in each of the vaccinated groups for the Antigua isolate, where Group D is the control;

[0035] Fig. 7 is a series of graphs illustrating the percentage of IFN γ -positive CD4⁺ cells for each of the vaccination groups, where Group A responded with a higher percentage of IFN γ -positive CD4⁺ cells than any of the other vaccination groups or the control group (Group D);

[0036] Fig. 8A illustrates a Western blot analysis of recombinant expression and reactivity of ER type IV (T4) and type VI (T6) secretion systems and showing detection of protein expression using mouse anti-His (C-terminal)-HRP monoclonal antibody;

[0037] Fig. 8B illustrates a Western blot analysis of recombinant expression and reactivity of specific immunoreactivity of T4 (93 kDa), T6 (38 kDa) and MAP1 (32 – 39 kDa) using sheep *E. ruminantium* polyclonal antiserum. Arrows indicate specific reactive bands;

[0038] Fig. 9A illustrates an analysis of immunoreactivity of *E. ruminantium* type IV outer membrane protein by indirect enzyme-linked immunosorbent assay (ELISA) using polyclonal ER antiserum obtained from a sheep experimentally infected with virulent heartwater agent. The polyclonal antiserum shows significantly IgG OD readings (higher antibody reactivity ($P < 0.05$)) compared to serum obtained from a naïve uninfected sheep. A MAP1 positive control antiserum from sheep vaccinated with a recombinant Gardel-Antigua MAP1 subunit vaccine (Faburay et al., 2017) shows strong reactivity with MAP1 protein;

[0039] Fig. 9B illustrates an analysis of immunoreactivity of *E. ruminantium* type VI outer membrane protein by indirect enzyme-linked immunosorbent assay (ELISA) using polyclonal ER antiserum obtained from a sheep experimentally infected with virulent heartwater agent. The polyclonal antiserum shows significantly IgG OD readings (higher antibody reactivity ($P < 0.05$)) compared to serum obtained from a naïve uninfected sheep. A MAP1 positive control antiserum from sheep vaccinated with a recombinant Gardel-Antigua MAP1 subunit vaccine (Faburay et al., 2017) shows strong reactivity with MAP1 protein;

[0040] Fig. 9C illustrates an analysis of immunoreactivity of *E. ruminantium* MAP1 outer membrane protein by indirect enzyme-linked immunosorbent assay (ELISA) using polyclonal ER antiserum obtained from a sheep experimentally infected with virulent heartwater agent. The polyclonal antiserum shows significantly IgG OD readings (higher antibody reactivity ($P < 0.05$)) compared to serum obtained from a naïve uninfected sheep. A MAP1 positive control antiserum from sheep vaccinated with a recombinant Gardel-Antigua MAP1 subunit vaccine (Faburay et al., 2017) shows strong reactivity with MAP1 protein;

[0041] Fig. 10A illustrates an analysis of immunological cross-reactivity of recombinant *E. ruminantium* type IV secretion proteins with *E. canis* anti-sera from infected dogs. Serum from an uninfected dog was used as negative control. Cross-reactivity with recombinant *E. ruminantium* MAP1 is included as a positive control. IgG OD readings the *E. canis*-positive antisera and the naïve uninfected serum are significantly different ($P < 0.005$);

[0042] Fig. 10B illustrates an analysis of immunological cross-reactivity of recombinant *E. ruminantium* type VI secretion proteins with *E. canis* anti-sera from infected dogs. Serum from an uninfected dog was used as negative control. Cross-reactivity with recombinant *E. ruminantium* MAP1 is included as a positive control. IgG OD readings the *E. canis*-positive antisera and the naïve uninfected serum are significantly different ($P < 0.005$);

[0043] Fig. 10C illustrates an analysis of immunological cross-reactivity of recombinant *E. ruminantium* MAP1 secretion proteins with *E. canis* antisera from infected dogs. Serum from an uninfected dog was used as negative control. Cross-reactivity with recombinant *E. ruminantium* MAP1 is included as a positive control. IgG OD readings the *E. canis*-positive antisera and the naïve uninfected serum are significantly different ($P < 0.005$);

DETAILED DESCRIPTION OF THE INVENTION

[0044] The present disclosure provides for an immunogenic composition or vaccine providing protection against *E. ruminantium* infection or heartwater, *Ehrlichia chaffeensis*, *Ehrlichia canis*, *Ehrlichia ewingii*, *Ehrlichia muris*, *Anaplasma phagocytophilum*, *Anaplasma platys*, and *Anaplasma marginale*. Further, a method for eliciting an immune response in an animal is provided, where the steps include administration of the immunogenic composition or vaccine disclosed herein to an animal or human in need thereof. A method for reducing the incidence or severity of clinical signs associated with a tick-borne pathogen of the disclosure including *E. ruminantium* infection or heartwater, *Ehrlichia chaffeensis*, *Ehrlichia canis*, *Ehrlichia ewingii*, *Ehrlichia muris*, *Anaplasma phagocytophilum*, *Anaplasma platys*, and *Anaplasma marginale* is also provided as an aspect of the present disclosure. Such a method comprises the steps of administration of the immunogenic composition or vaccine providing protection against *E. ruminantium* infection or heartwater or a tick-borne pathogen of the disclosure to an animal or human in need thereof.

[0045] The immunogenic composition or vaccine of the present disclosure can either be a killed or live bacterium, a modified live or killed bacterium, a nucleic acid based immunogenic composition; a protein based immunogenic composition; a chimeric composition; or any combination thereof. In a preferred embodiment, the immunogenic composition or vaccine is a protein based subunit vaccine.

[0046] In one embodiment, a subunit based immunogenic composition or vaccine is provided. The subunit is preferably encoded by a sequence

selected from the group consisting of a modified live or killed sequence of SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 10, SEQ ID No. 11, and any sequence having at least 80%, at least 90%, or at least 95%, 96%, 97%, 98%, 99%, or even 100%% sequence homology with any one of the recited sequences. Alternatively, the subunit is selected from the group consisting of SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 7, SEQ ID No. 12, SEQ ID No. 13, any sequence having at least 80%, at least 90%, or at least 95%, 96%, 97%, 98%, 99%, or even 100%% sequence homology with any one of the recited sequences, or any combination thereof. In preferred forms, the protein subunit is recombinantly-produced.

[0047] In a further embodiment, a protein based composition is provided. Preferably, the protein component is selected from, but not limited to, a recombinant protein, a harvested protein, a purified protein, and combinations thereof. In one embodiment, the protein component is a modified MAP1 protein of *E. ruminantium*. In another embodiment, the protein component is a modified homolog protein of MAP1 from a tick-borne pathogen of the disclosure. In another embodiment, the protein component is selected from the group consisting of a modified MAP1 protein of *Ehrlichia ruminantium*, the p30-1 protein from *Ehrlichia canis*, the p28-OMP19 protein from *Ehrlichia chaffeensis*, the MSP4 protein from *Anaplasma marginale*, or any combination thereof. In preferred forms, the modification includes glycosylation such that the modified protein includes more glycosylation than an unmodified protein.

[0048] A method for reducing the clinical symptoms associated with heartwater or *E. ruminantium*, *Ehrlichia chaffeensis*, *Ehrlichia canis*, *Ehrlichia ewingii*, *Ehrlichia muris*, *Anaplasma phagocytophilum*, *Anaplasma platys*, or *Anaplasma marginale* infection also provided. The step of the method preferably includes administration of an immunogenic composition or vaccine described herein to an animal or human. The clinical symptoms preferably include, but are not limited to, death, fever, respiratory distress, which may be exaggerated, paroxysmal convulsions, anorexia, depression, respiratory congestion, friable mucous membranes, respiratory distress, nervous signs, hyperaesthesia, high-stepping stiff gate,

exaggerated blinking, exaggerated chewing movements, diarrhea, and combinations thereof. The method preferably includes the step of administration of the immunogenic composition or vaccine to an animal or human thereof. Preferably the clinical symptoms associated with heartwater or *E. ruminantium*, *Ehrlichia chaffeensis*, *Ehrlichia canis*, *Ehrlichia ewingii*, *Ehrlichia muris*, *Anaplasma phagocytophilum*, *Anaplasma platys*, and *Anaplasma marginale* infection are reduced in frequency and/or severity by at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or reduced by 100%. This reduction is in comparison to an animal or human not receiving the immunogenic composition or vaccine of the present disclosure. With reference to *E. chaffeensis* in humans, typical symptoms include: fever, headache, fatigue, and muscle aches. Usually, these symptoms occur within 1-2 weeks following a tick bite. With reference to *E. chaffeensis* in dogs, the incubation period for monocytic ehrlichiosis is 8 to 20 days; for granulocytic ehrlichiosis, it is 1 to 14 days after a tick bite. Dogs usually present with fever, lethargy, anorexia, swollen lymph nodes, enlarged spleen, and weight loss. With reference to *E. canis* in dogs, a variety of symptoms during the acute phase of canine monocytic ehrlichiosis can occur, including: fever, lethargy, poor appetite, lymph node enlargement, abnormal bruising and bleeding, chronic eye inflammation, neurologic abnormalities, and occasionally lameness. *E. canis* is so far not considered a human pathogen. With respect to *E. ewingii* in dogs (similar to *E. canis*), clinical illness most often manifests as an acute febrile condition associated with musculoskeletal signs. Reluctance to stand or walk, lameness, a stiff or stilted gait, and joint effusion are common findings in *E. ewingii*-infected dogs and may be quite severe. Lethargy, anorexia, and central nervous system signs (*e.g.*, head tilt, tremors, and anisocoria) may also be present. In humans, typical symptoms of *E. ewingii* infection include: fever, headache, fatigue, and muscle aches. With respect to Anaplasmosis (*A. marginale*), bovine anaplasmosis causes important economic loss in most countries, mainly due to the high morbidity and mortality in susceptible cattle herds. The losses due to anaplasmosis are measured through several parameters: low weight gain, reduction in milk production, abortion, the cost of anaplasmosis treatments, and mortality.

[0049] In one aspect of the disclosure, a method for reducing the incidence or severity of clinical symptoms of heartwater or *E. ruminantium*, *Ehrlichia chaffeensis*, *Ehrlichia canis*, *Ehrlichia ewingii*, *Ehrlichia muris*, *Anaplasma phagocytophilum*, *Anaplasma platys*, and/or *Anaplasma marginale* infection is provided. The method includes the step of administering the immunogenic composition or vaccine of the present disclosure to an animal or human. Preferably, the symptoms of heartwater or *E. ruminantium*, *Ehrlichia chaffeensis*, *Ehrlichia canis*, *Ehrlichia ewingii*, *Ehrlichia muris*, *Anaplasma phagocytophilum*, *Anaplasma platys*, and/or *Anaplasma marginale* infection are selected from, but not limited to, the group consisting of death, fever, respiratory distress, which may be exaggerated, paroxysmal convulsions, anorexia, depression, respiratory congestion, friable mucous membranes, respiratory distress, nervous signs, hyperaesthesia, high-stepping stiff gate, exaggerated blinking, exaggerated chewing movements, diarrhea, and combinations thereof or any of the symptoms described above for any of the other tick-borne pathogens of the disclosure.

[0050] The recipient of the composition and method of the present disclosure may be a human or an animal. The animal is preferably selected from, but not limited to porcine, pigs, cows, ruminants, goats, horses, dogs, cats, poultry, and other related wild and domestic animals.

[0051] In one aspect, the immunogenic composition may also comprise additional elements, antigens, pharmaceutical carriers, adjuvants, preservatives, stabilizers, or combinations thereof.

[0052] Modified or modified live nucleotide sequence will be understood as meaning any nucleotide sequence obtained by mutagenesis according to techniques well known to the person skilled in the art, and containing modifications with respect to the normal sequences according to the disclosure, for example mutations in the regulatory and/or promoter sequences of polypeptide expression, especially leading to a modification of the rate of expression of said polypeptide or to a modulation of the replicative cycle. In some forms, the nucleotide sequence will be

used to express a recombinant protein or subunit selected from the group consisting of SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 7, SEQ ID No. 12, SEQ ID No. 13, any sequence having at least 80%, at least 90%, or at least 95%, 96%, 97%, 98%, 99%, or even 100%% sequence homology with any one of the recited sequences, or any combination thereof.

[0053] Nucleotide, polynucleotide or nucleic acid sequence will be understood according to the present disclosure as meaning both a double-stranded or single-stranded RNA or DNA in the monomeric and dimeric (so-called in tandem) forms and the transcription products of said DNAs.

[0054] It must be understood that the present disclosure does not relate to the genomic nucleotide sequences taken in their natural environment, that is to say, in the natural state. It concerns sequences for which it has been possible to isolate, purify or partially purify, starting from separation methods such as, for example, ion-exchange chromatography, by exclusion based on molecular size, or by affinity, or alternatively fractionation techniques based on solubility in different solvents, or starting from methods of genetic engineering such as amplification, cloning and subcloning, it being possible for the sequences of the disclosure to be carried by vectors. Further, the sequences have been altered from what is found in nature to include mutations induced through site-directed mutagenesis or other attenuation techniques, such as serial passaging, further demonstrating that the sequences are made by the hand of man and not found in nature. In some forms, the expression of protein subunits from the nucleotide sequences results in glycosylated forms of the protein, as described above. Such glycosylated forms of the expressed proteins are not found in nature.

[0055] The vaccine or immunogenic composition of the present invention does not contain a nucleotide or amino acid sequence found in nature, as it has been constructed by the hand of man. Therefore, the immunogenic composition or vaccine of the present invention is markedly different from what is found in nature. Similar to Example 5 for the Nature-Based Product Examples of eligible subject

matter under 35 U.S.C. 101 issued by the US Patent Office in 2014, the immunogenic composition or vaccine of the present invention is like claim 2 of that example because the immunogenic composition or vaccine gene has additional elements, such as the mutations within the sequence or inactivation of the bacteria that provides it with a functionally different characteristic than naturally occurring *E. ruminantium* strains or any of the tick-borne pathogens of the disclosure.

[0056] Pharmaceutically acceptable vehicle is understood as designating a compound or a combination of compounds entering into a pharmaceutical composition or vaccine which does not provoke secondary reactions and which allows, for example, the facilitation of the administration of the active compound, an increase in its duration of life and/or its efficacy in the body, an increase in its solubility in solution or alternatively an improvement in its conservation. These pharmaceutically acceptable vehicles are well known and will be adapted by the person skilled in the art as a function of the nature and of the mode of administration of the chosen active compound. At least one pharmaceutically acceptable vehicle can be used with the immunogenic compositions and methods of the present disclosure.

[0057] As far as the vaccine and immunogenic composition formulations of the present disclosure are concerned, these can comprise adjuvants of the appropriate immunity which are known to the person skilled in the art, such as, for example, aluminum hydroxide, a representative of the family of muramyl peptides such as one of the peptide derivatives of N-acetyl muramyl, a bacterial lysate, carbomers, montanide ISA25, or alternatively Freund's incomplete adjuvant.

[0058] The immunogenic composition and vaccine described herein can be administered by the systemic route, in particular by the intravenous route, by the intramuscular, intradermal or subcutaneous route, or by the oral or nasal, or intranasal route. In a more preferred manner, the immunogenic composition or vaccine composition according to the disclosure will be administered by the

intramuscular route, through the food, or by nebulization several times, staggered over time.

[0059] Their administration modes, dosages and optimum pharmaceutical forms can be determined according to the criteria generally taken into account in the establishment of a treatment adapted to an animal such as, for example, the age or the weight, the seriousness of its general condition, the tolerance to the treatment and the secondary effects noted. Preferably, the vaccine of the present disclosure is administered in an amount that is protective against heartwater or *E. ruminantium*, *Ehrlichia chaffeensis*, *Ehrlichia. canis*, *Ehrlichia ewingii*, *Ehrlichia muris*, *Anaplasma phagocytophilum*, *Anaplasma platys*, and/or *Anaplasma marginale* infection. Whether the vaccine or immunogenic composition is protective can be determined by one of skill in the art based on whether there is a reduction in the incidence of or severity of clinical symptoms caused by infection by a tick-borne pathogen of the disclosure.

[0060] The administration of the immunogenic composition or vaccine according to the present disclosure may be administered one times, two times, three times, four times, five times, six times, seven times, eight times, nine times, or at least 10 times. In one aspect, the immunogenic composition or vaccine of the present disclosure is effective at reducing the severity of and/or incidence of clinical signs of infection after a single dose administration.

[0061] For example, the immunogenic composition or vaccine according to the present disclosure may be administered one time or several times, spread out over time in an amount of about 0.1 to 1000 µg per kilogram weight of the animal or human, where values and ranges such as, but not limited to, 0.5 to 800 µg per kilogram weight of the animal or human, 1 to 1000 µg per kilogram weight of the animal or human, 1 to 500 µg per kilogram weight of the animal or human, 1 to 300 µg per kilogram weight of the animal or human, 1 to 200 µg per kilogram weight of the animal or human, 1 to 150 µg per kilogram weight of the animal or human, 1 to 125 µg per kilogram weight of the animal or human, 1 to 100 µg per kilogram weight

of the animal or human, 5 µg per kilogram weight of the animal or human, 10 µg per kilogram weight of the animal or human, 15 µg per kilogram weight of the animal or human, 20 µg per kilogram weight of the animal or human, 25 µg per kilogram weight of the animal or human, 30 µg per kilogram weight of the animal or human, 35 µg per kilogram weight of the animal or human, 40 µg per kilogram weight of the animal or human, 45 µg per kilogram weight of the animal or human, 50 µg per kilogram weight of the animal or human, 55 µg per kilogram weight of the animal or human, 60 µg per kilogram weight of the animal or human, 65 µg per kilogram weight of the animal or human, 70 µg per kilogram weight of the animal or human, 75 µg per kilogram weight of the animal or human, 80 µg per kilogram weight of the animal or human, 85 µg per kilogram weight of the animal or human, 90 µg per kilogram weight of the animal or human, 95 µg per kilogram weight of the animal or human, 100 µg per kilogram weight of the animal or human, 125 µg per kilogram weight of the animal or human, 150 µg per kilogram weight of the animal or human, 200 µg per kilogram weight of the animal or human, 250 µg per kilogram weight of the animal or human, 300 µg per kilogram weight of the animal or human, 400 µg per kilogram weight of the animal or human, 500 µg per kilogram weight of the animal or human, 600 µg per kilogram weight of the animal or human, 700 µg per kilogram weight of the animal or human, 800 µg per kilogram weight of the animal or human, 900 µg per kilogram weight of the animal or human, and 1000 µg or more per kilogram weight of the animal or human are envisioned. In other preferred forms, the above amounts are also provided without reference to the weight of the animal or human. Alternatively, the dosage can be between 5 – 2000 ug, more preferably 10 – 1800 ug, still more preferably between 15 – 1600 ug, even more preferably between 20 – 1400 ug, still more preferably between 25 – 1200 ug, even more preferably 30 to 1000 ug, still more preferably 35 – 800 ug, even more preferably 35 – 600 ug, still more preferably 40 – 400 ug, even more preferably 45 – 300 ug, and most preferably 50 - 200 ug per animal.

[0062] According to the present disclosure, the immunogenic composition or vaccine may include an antigen from at least one further pathogen

other than *E. ruminantium*, making it a combination vaccine or immunogenic composition. In such an embodiment, an effective amount of a vaccine or immunogenic composition administered provides effective protection including a reduction in the severity or incidence of clinical signs of infection up to and including immunity against infections caused by *E. ruminantium* and at least one further disease-causing organism. In some preferred forms, the combination vaccine or immunogenic composition includes at least one antigen from a group of pathogens selected from the group consisting of *Ehrlichia chaffeensis*, *Ehrlichia canis*, *Ehrlichia ewingii*, *Ehrlichia muris*, *Anaplasma phagocytophilum*, *Anaplasma platys*, and *Anaplasma marginale*. In some of these forms, the antigen is similar to the *E. ruminantium* antigen described herein. In some forms, that antigen is the equivalent of the MAP1 protein of *E. ruminantium*. In other preferred forms, the further pathogen is preferably selected from the group consisting of *Rickettsia rickettsia*, *Rickettsia helevatica*, *Ehrlichia equi*, *A. americanum*, and combinations thereof. In still other preferred forms, the other antigen can be from a pathogen that is not from a tick-borne disease.

[0063] In another aspect, the present disclosure provides an immunogenic composition or vaccine that is effective against more than one of the tick-borne pathogens of the disclosure. In other words, there is cross protection across the different species. In preferred forms, the cross protection occurs when using a glycosylated form of a protein selected from the group consisting of the MAP1 protein of *Ehrlichia ruminantium*, the p30-1 sequence from *Ehrlichia canis*, the p28-Omp19 protein from *Ehrlichia chaffeensis*, and the MSP4 protein from *Anaplasma marginale*. Use or administration of any of these antigens as described herein will provide some protection including a reduction in the incidence of or severity of clinical signs or symptoms of infection caused by any of these tick-borne pathogens (*Ehrlichia ruminantium*, *Ehrlichia canis*, *Ehrlichia chaffeensis*, and *Anaplasma marginale*).

[0064] “Homologous nucleotide sequence” or “having sequence homology” in the sense of the present disclosure is understood as meaning a

nucleotide sequence having at least a percentage identity with the bases of a nucleotide sequence according to the disclosure of at least 80%, where ranges and values, including but not limited to, from 80% to 85%, 85% to 96%, 80% or 95%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 95.5%, 96%, 96.5%, 97%, 97.5%, 98%, 98.5%, 99%, 99.5%, 99.9%, and higher are envisioned for the present disclosure, where this percentage being purely statistical and it being possible to distribute the differences between the two nucleotide sequences at random and over the whole of their length.

[0065] An “immunogenic composition” as used herein, means an *E. ruminantium*, *Ehrlichia chaffeensis*, *Ehrlichia. canis*, *Ehrlichia ewingii*, *Ehrlichia muris*, *Anaplasma phagocytophilum*, *Anaplasma platys*, and/or *Anaplasma marginale* composition which elicits an “immunological response” in the host of a cellular and/or antibody-mediated immune response to the *E. ruminantium*, *Ehrlichia chaffeensis*, *Ehrlichia. canis*, *Ehrlichia ewingii*, *Ehrlichia muris*, *Anaplasma phagocytophilum*, *Anaplasma platys*, and/or *Anaplasma marginale* sequence, whether the sequence is killed/inactivated, modified live, a subunit of the nucleotide sequence or peptide expressed by that sequence, or in a vector. Preferably, this immunogenic composition is capable of conferring protective immunity against heartwater or *E. ruminantium*, *Ehrlichia chaffeensis*, *Ehrlichia canis*, *Ehrlichia ewingii*, *Ehrlichia muris*, *Anaplasma phagocytophilum*, *Anaplasma platys*, and/or *Anaplasma marginale* infection and the clinical symptoms associated therewith. Preferred subunits are those described herein.

[0066] “Sequence Identity” as it is known in the art refers to a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, namely a reference sequence and a given sequence to be compared with the reference sequence. Sequence identity is determined by comparing the given sequence to the reference sequence after the sequences have been optimally aligned to produce the highest degree of sequence similarity, as determined by the match between strings of such sequences. Upon such alignment, sequence identity is ascertained on a position-by-position basis, e.g., the sequences

are “identical” at a particular position if at that position, the nucleotides or amino acid residues are identical. The total number of such position identities is then divided by the total number of nucleotides or residues in the reference sequence to give % sequence identity. Sequence identity can be readily calculated by known methods, including but not limited to, those described in Computational Molecular Biology, Lesk, A. N., ed., Oxford University Press, New York (1988), Biocomputing: Informatics and Genome Projects, Smith, D.W., ed., Academic Press, New York (1993); Computer Analysis of Sequence Data, Part I, Griffin, A.M., and Griffin, H. G., eds., Humana Press, New Jersey (1994); Sequence Analysis in Molecular Biology, von Heinge, G., Academic Press (1987); Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M. Stockton Press, New York (1991); and Carillo, H., and Lipman, D., SIAM J. Applied Math., 48: 1073 (1988), the teachings of which are incorporated herein by reference. Preferred methods to determine the sequence identity are designed to give the largest match between the sequences tested. Methods to determine sequence identity are codified in publicly available computer programs which determine sequence identity between given sequences. Examples of such programs include, but are not limited to, the GCG program package (Devereux, J., et al., Nucleic Acids Research, 12(1):387 (1984)), BLASTP, BLASTN and FASTA (Altschul, S. F. et al., J. Molec. Biol., 215:403-410 (1990)). The BLASTX program is publicly available from NCBI and other sources (BLAST Manual, Altschul, S. et al., NCVI NLM NIH Bethesda, MD 20894, Altschul, S. F. et al., J. Molec. Biol., 215:403-410 (1990), the teachings of which are incorporated herein by reference). These programs optimally align sequences using default gap weights in order to produce the highest level of sequence identity between the given and reference sequences. As an illustration, by a polynucleotide having a nucleotide sequence having at least, for example, 85%, preferably 90%, even more preferably 95% “sequence identity” to a reference nucleotide sequence, it is intended that the nucleotide sequence of the given polynucleotide is identical to the reference sequence except that the given polynucleotide sequence may include up to 15, preferably up to 10, even more preferably up to 5 point mutations per each 100 nucleotides of the reference nucleotide sequence. In other words, in a polynucleotide having a

nucleotide sequence having at least 85%, preferably 90%, even more preferably 95% identity relative to the reference nucleotide sequence, up to 15%, preferably 10%, even more preferably 5% of the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or a number of nucleotides up to 15%, preferably 10%, even more preferably 5% of the total nucleotides in the reference sequence may be inserted into the reference sequence. These mutations of the reference sequence may occur at the 5' or 3' terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence. Analogously, by a polypeptide having a given amino acid sequence having at least, for example, 85%, preferably 90%, even more preferably 95% sequence identity to a reference amino acid sequence, it is intended that the given amino acid sequence of the polypeptide is identical to the reference sequence except that the given polypeptide sequence may include up to 15, preferably up to 10, even more preferably up to 5 amino acid alterations per each 100 amino acids of the reference amino acid sequence. In other words, to obtain a given polypeptide sequence having at least 85%, preferably 90%, even more preferably 95% sequence identity with a reference amino acid sequence, up to 15%, preferably up to 10%, even more preferably up to 5% of the amino acid residues in the reference sequence may be deleted or substituted with another amino acid, or a number of amino acids up to 15%, preferably up to 10%, even more preferably up to 5% of the total number of amino acid residues in the reference sequence may be inserted into the reference sequence. These alterations of the reference sequence may occur at the amino or the carboxy terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in the one or more contiguous groups within the reference sequence. Preferably, residue positions which are not identical differ by conservative amino acid substitutions. However, conservative substitutions are not included as a match when determining sequence identity.

[0067] The definition of sequence identity given above is the definition that would be used by one of skill in the art. The definition by itself does not need the help of any algorithm, said algorithms being helpful only to achieve the optimal alignments of sequences, rather than the calculation of sequence identity.

[0068] From the definition given above, it follows that there is a well-defined and only one value for the sequence identity between two compared sequences which value corresponds to the value obtained for the best or optimal alignment.

[0069] In the BLAST N or BLAST P “BLAST 2 sequence”, software which is available in html at the web site ncbi.nlm.nih.gov/gorf/b12, and habitually used by the inventors and in general by the skilled man for comparing and determining the identity between two sequences, gap cost which depends on the sequence length to be compared is directly selected by the software (i.e. 11.2 for substitution matrix BLOSUM-62 for length >85).

[0070] A “conservative substitution” refers to the substitution of an amino acid residue or nucleotide with another amino acid residue or nucleotide having similar characteristics or properties including size, hydrophobicity, etc., such that the overall functionality does not change significantly.

[0071] “Isolated” means altered “by the hand of man” from its natural state, i.e., if it occurs in nature, it has been changed or removed from its original environment, or both. For example, a polynucleotide or polypeptide naturally present in a living organism is not “isolated,” but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is “isolated”, as the term is employed herein.

[0072] This written description uses examples to disclose the invention, including the best mode, and also to enable any person skilled in the art to practice the invention, including making and using any devices or systems and performing any incorporated methods. The patentable scope of the invention is

defined by the claims, and may include other examples that occur to those skilled in the art. Such other examples are intended to be within the scope of the claims if they have structural elements that do not differ from the literal language of the claims, or if they include equivalent structural elements with insubstantial differences from the literal languages of the claims.

EXAMPLES

EXAMPLE 1

Materials and methods

Production of recombinant MAP1 protein

Cloning and recombinant protein expression

[0073] A recombinant baculovirus expression system was used to express glycosylated MAP1 proteins of the *E. ruminantium* isolates, Antigua and Gardel. Briefly, the complete map1 coding sequences of both isolates were codon-optimized including insertion of a Kozak sequence to achieve high level of target protein expression in *Spodoptera frugiperda*, Sf9, insect cells. The sequences were synthesized and cloned into pUC57 vector by a manufacturer (Genewiz). The resulting recombinant plasmid, pUC57-map1 was used as template in high fidelity PCR using a proof-reading DNA polymerase, Accuprime DNA polymerase (Life Technologies) and gene-specific primers BYF101F 5'-CACCATGAACTGCAAGAAGATCTTCATCACCTCC-3' (SEQ ID No. 8) and BYF102R 5'-GAACACGAAACGACCACCGATCTCGATACC-3' (SEQ ID No. 9). The PCR was performed per manufacturer's instruction. The PCR amplicon was cloned into pFastBac/CT/TOPO to create donor plasmids, pFastBac-Antigua and pFastBac-Gardel, containing map1 coding sequences of Antigua and Gardel, respectively. The recombinant donor plasmids were used to create recombinant bacmid via site-specific transpositioning following transfection into an *E. coli* strain, DH10Bac. Recombinant bacmids were purified using HiPure Plasmid kit (Life

Technologies) and used to transfect Sf9 cells to rescue respective recombinant baculoviruses. Recombinant baculoviruses, P2 and above, were used to express recombinant *E. ruminantium* MAP1 in Sf9 cells.

Recombinant protein purification

[0074] Recombinant MAP1 proteins were expressed with a C-terminal 6xHis-tag fusion protein that allowed purification by affinity chromatography using Ni-NTA superflow resin (Novagen, Rockland, MA). Purification was performed per manufacturer's instruction. Briefly, recombinant baculovirus-infected Sf9 cells expressing recombinant *E. ruminantium* MAP1 proteins were pelleted by centrifugation at 500 x g for 5 min. The pellet was resuspended in Ni-NTA binding buffer (300 mM NaCl, 50 mM Na₃PO₄, pH 8.0, and 10 mM imidazole) containing 1x complete protease inhibitor. Insect Popculture Reagent (Novagen) was then added at 0.05 volumes of original culture volume. The lysate was incubated at room temperature for 15 min and the supernatant further clarified by centrifugation at 1000 rpm for 10 min. The clarified lysate was mixed with previously equilibrated Ni-NTA superflow resin (Novagen-EMD Millipore, Billerica, MA). Binding was performed for 1 hour at 4°C. The suspension was loaded into a column and washed with 10 volumes of wash buffer (300 mM NaCl, 50 mM Na₃PO₄, pH 8.0, and 20 mM imidazole). Bound protein was eluted with elution buffer (300 mM NaCl, 50 mM Na₃PO₄, pH 8.0, and 250 mM imidazole). The eluate was dialyzed overnight against storage buffer, phosphate-buffered saline (PBS; pH 7.4), and 5% glycerol. Concentration of the purified proteins was determined using the bicinchoninic acid (BCA) assay (ThermoScientific) at an absorbance of 562 nm, using bovine serum albumin (BSA; Sigma-Aldrich) as the protein standard. Aliquots were stored at -80°C until used.

Protein analysis

Western blot

[0075] To assess expression of the target protein, approximately 5 µg of each purified recombinant MAP1 protein (Antigua and Gardel) was subjected to electrophoresis in 12% Bis-Tris polyacrylamide gel in 1x MOPS running buffer (Life Technologies). The proteins were transferred onto PVDF membranes per standard protocols. The membrane was blocked in blocking solution containing 0.1% Tween-20 in PBS (pH 7.4) and 3% bovine serum albumin (BSA) for 1 hour at room temperature or overnight at 4°C. After 3 subsequent washes of 5 min each in 0.1% Tween-20 in PBS, the membrane was incubated with anti-His-(C-Terminal)-HRP monoclonal antibody (Life Technologies) diluted 1: 5,000 in blocking solution. Expression of the rMAP1 was further confirmed using mouse anti-E. ruminantium monoclonal antibody, 4F10B4 (Abcam) at a dilution of 1: 2,000. Following three washing steps, the membrane was probed with a secondary antibody conjugate, goat anti-mouse IgG-HRP (1: 5,000) (Santa Cruz, Biotechnologies). Detection of specific reactivity was performed using ECL enhanced chemiluminescent detection system.

Analysis of protein glycosylation

[0076] To analyze glycosylation of rMAP1 proteins, approximately 50 µg of purified rMAP1 was pelleted at 100,000 x g for 30 min at 4°C. The pellet was resuspended in 2 µl of 10x denaturing buffer (New England Biolabs, Ipswich, MA) and 18 µl distilled water. An untreated rMAP1 protein was included as a negative control. The mixtures were heat-denatured at 100°C for 10 min. Thereafter, the following reagents were added to the reaction: 4 µl of 10x reaction buffer, 4 µl 10% NP40, 4 µl distilled water and 6 µl PNGase F (500,000 U/ml) (New England Biolabs). The mixture was incubated at 37°C for 1 hour and the reaction stopped by addition of SDS loading buffer. The samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in NuPAGE 12% Bis-Tris gels (LifeTech) and transferred onto polyvinylidene difluoride (PVDF) membranes per standard protocols. The membranes were probed with mouse anti-E. ruminantium monoclonal antibodies as described above. Anti-mouse-HRP conjugated secondary antibody (Santa Cruz Biotechnology) was used for detection. Detection of differentially migrating protein bands (treated and untreated rMAP1) was performed

to determine glycosylation or non-glycosylation of the MAP1 proteins. Additional glycosylation analysis of rMAP1 was performed by glycan staining using Pierce in-gel glycan staining protocol (Pierce Thermo Scientific) per manufacturer's instructions.

Vaccine preparation

[0077] Recombinant *E. ruminantium* subunit vaccine was composed of purified glycosylated rMAP1 of Caribbean *E. ruminantium* isolates, Gardel and Antigua (a bivalent vaccine). To prepare the vaccine formulations, purified rMAP1 proteins from either isolate were mixed in equal ratios in three different doses: (i) 200 µg (low), (ii) 400 µg (intermediate) and (iii) 600 µg (high). The vaccine was adjuvanted with an montanide ISA25 VG (Seppic, France) per manufacturer's instruction.

Animals and Vaccination

[0078] Twenty sheep (Dorper x Katahdin cross) aged 3-4 months were purchased from a private farm in Kansas and assigned to 4 groups. Group A (n = 5), group B (n = 6), group C (n = 6) and Group D (n = 3). Pre-vaccination blood was collected in EDTA from all animals and tested by nested pCS20 PCR to confirm their *E. ruminantium*-free status. To assess the appropriate dose response, the vaccine was administered subcutaneously in three different doses. Sheep in group A were vaccinated with 200 µg of rMAP1 subunit vaccine, group B vaccinated with 400 µg, and group C with 600 µg. Group D served as mock-vaccinated control and received adjuvant only. At 21 days post-vaccination, each sheep was boosted with the same vaccine dose with adjuvant administered previously. Animals were bled weekly for serum on day 0 (pre-vaccination), days 7, 14, 21, 28, 35 and 42 post-vaccination. Additionally, at 35 post vaccination, blood was collected in acid citrate dextrose (ACD) tubes for isolation of peripheral blood mononuclear cells (PBMCs) to measure T cell responses as described below. Injection sites and rectal temperatures were examined daily until day 3 post-vaccination for vaccine induced reactions.

Detection of vaccine-induced antibody responses

[0079] Induction of MAP1-specific antibody responses was measured in the animals to assess vaccine-induced seroconversion. An indirect ELISA format using recombinant MAP1 of Antigua and Gardel isolates as diagnostic antigens was used in separate assays. Briefly, flat-bottom 96-well microtiter plates (Nunc, MaxiSorp) were coated with 150 ng per well of recombinant MAP1 antigen in 100 µl of antigen coating buffer (Dulbecco's phosphate buffered saline, DPBS, pH 7.3) and incubated overnight at 4°C. The plates were blocked with PBS pH 7.3, supplemented with 0.1% Tween-20 and 1% non-fat dry milk before adding 100 µl per well of diluted (1:200) serum. Each sample was tested in duplicate and each plate contained duplicate negative and positive control sera obtained from an experimentally infected sheep at Utrecht University in The Netherlands. After washing, plates were incubated with Protein G-HRP (Abcam), diluted 1: 50,000 in blocking solution, at 37°C for 1 hour. Thereafter, 100 µl of substrate buffer containing 0.1 mg/ml 3,3',5,5'-tetramethyl-benzidine (TMB) (ThermoScientific) and H₂O₂ was added and plates were incubated in the dark for 25 min. The reaction was stopped with 2M H₂SO₄ and optical densities (OD) were measured at 450 nm using microplate reader Fluostar Omega (BMG Labtech). For each experimental group, the cut-off OD value was determined by addition of 2 standard deviations to the mean OD value of serum obtained from the animals prior to vaccination.

IgG isotype response

[0080] IgG isotype profiling was performed by determining the end titers of IgG1 and IgG2. Mean endpoint titer was determined for animals in group C (vaccinated with the highest vaccine dose 600 µg of rMAP1 subunit vaccine) using day 28 post vaccination sera. Briefly, 5-fold serial dilutions of test serum were initially made and then incubated with rMAP1 antigen (Gardel) coated in 96-well ELISA plates as described above. After three washings, the plate was incubated for 1 hour with mouse anti-bovine IgG1-HRP (Cat. No. MCA2440P; 1:100 dilution) or mouse anti-bovine IgG2 (Cat. No. MCA626; 1:100) (BioRad); both antibodies

showed cross-reactivity with sheep. The mouse anti-bovine IgG2 was further probed with Protein G-HRP (1:50,000) for 1 hr. After washing, reactivity was detected by addition of TMB and H₂O₂. The reaction was stopped with 2M H₂SO₄ and OD values measured at 450 nm as described above.

Measurement of T-cell responses

[0081] Antigen-specific CD4⁺ and CD8⁺ T cell proliferation and cytokine production was measured in peripheral blood on day 35 post vaccination using protocols we have previously published, modified for use in sheep. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation, labeled with Cell Trace Violet proliferation dye (Life Technologies) and placed in culture for 6 days with 1 µg/ml of purified rMAP1. Pokeweed mitogen was included as a positive control and used at 1 µg/ml. On day 6, cells were labeled with the Live/Dead Fixable Aqua Dead Cell Stain Kit (Invitrogen) per manufacturer's instructions. Samples were then stained with 10 µg/ml mouse anti-ovine CD4 (clone GC1A, isotype IgG2a), and mouse anti-ovine CD8 (clone CACT80C, clone IgG1), both from Washington State University. After washing, samples were labeled with 5 µg/ml of the following secondary antibodies: anti-mouse IgG2a-Alexa Fluor 488 and anti-mouse IgG1-Alexa Fluor 647 (both from Southern Biotech). Cells were then collected on a BD LSR Fortessa X20 Flow Cytometer and analyzed using FlowJo Software (Treestar Inc.). Live cells were gated based upon expression of CD4⁺ or CD8⁺ and proliferation was assessed by dilution of the CellTrace Violet dye. T cell responses were measured by comparing responses to stimulation with rMAP1 from the respective E. ruminantium strains to mock-stimulated cultures and results are presented as change-over mock. For intracellular cytokine production, PBMC were stimulated overnight with 1 µg/mL E. ruminantium rMAP1-specific antigen in the presence of 10 µg/mL brefeldin A. After 18-24 hours, cells were surface stained as above. After washing, cells were then fixed with the BD Cytofix/Cytoperm kit per manufacturer's instructions and stained with 10 µg/mL mouse anti-ovine IFN γ -PE (BioRad Antibodies). Cells were analyzed via flow cytometry as above, gating on live, IFN γ ⁺ CD4 and CD8 T cells. The frequency of antigen-specific cells was

determined by comparing responses to stimulation with rMAP1 from the respective *E. ruminantium* strains to mock-stimulated cultures, and results are presented as change-over mock.

Statistics

[0082] Statistical analysis was performed using Prism V6.0f software (Graphpad Software, Inc.). Differences in antibody responses at various post-vaccination time-points between the vaccine-dose groups in response to either vaccine antigens, Antigua or Gardel, were analyzed using a 2-way repeated measures ANOVA. T cell responses were analyzed using a 1-way ANOVA followed by Tukey's Multiple Comparisons post-test analysis.

Results and Conclusions

Expression of glycosylated recombinant MAP1 proteins

[0083] In silico analysis of MAP1 amino acid sequences of Antigua and Gardel *E. ruminantium* isolates revealed the presence of putative sites for N- and O-linked glycosylation (Figure 1). To ensure that rMAP1 proteins were expressed in glycosylated form, cloning of map1 gene sequences included signal peptide sequences at the N-terminus of the coding sequence (Fig. 1), which allowed translocation of the protein into the lumen of endoplasmic reticulum where signal peptidases and glycosylation enzymes are located. To rescue respective recombinant baculoviruses for expression of rMAP1 proteins, purified recombinant bacmids encoding map1 genes of either isolate, Antigua and Gardel, were used to transfect Sf9 cells. The recombinant baculoviruses expressed the MAP1 proteins as demonstrated by detection of 32-33 kDa MAP1 proteins of *E. ruminantium*, and a previously undescribed 37-38 kDa protein using anti-His-HRP conjugated and anti-*E. ruminantium* monoclonal antibodies (Fig. 2A and B). These figures show a glycoform profile. The detection of two (shown here for the first time) shows that MAP1 is expressed in two forms. One of the forms of the protein is glycosylated, which is demonstrated by treating with deglycosylation enzymes that cleave the sugar/glycan

residues resulting in a shift in electrophoretic migration. To confirm that the recombinant MAP1 proteins of both isolates were expressed in glycosylated form, a deglycosylation assay was performed. Enzymatic de-glycosylation treatment of rMAP1 proteins of both *E. ruminantium* isolates resulted in reduction in the molecular weight of the upper band demonstrated by a shift in electrophoretic migration (Fig. 2B). The upper band corresponded to a 37-38 kDa glycosylated form of the immunodominant MAP1 (Fig. 2B), whereas the 31-32 kDa molecular weight band represented a non-glycosylated form of the protein (Fig. 2B). An in-gel glycan staining confirmed glycosylation of the rMAP1 protein of both *E. ruminantium* isolates as indicated by the appearance of specific magenta bands in the stained gel.

Analysis of serological response

[0084] To assess vaccine induced seroconversion and the kinetics of the antibody response, sera were collected from the vaccinated sheep at various time points post-vaccination (day 0, 7, 14, 21, 28, 35, 42) and were tested using MAP1-specific indirect ELISAs. In response to vaccination with the three vaccine dose regimens (Group A = 200 µg, Group B = 400 µg and Group C = 600 µg in equal ratios of rMAP1 proteins of Antigua and Gardel isolates) of the recombinant subunit vaccine, 100% of the animals in all three dose categories seroconverted to both vaccine antigens, Antigua and Gardel rMAP1. Specifically, at day 14 post vaccination all vaccinated animals in the three vaccine dose groups exhibited detectable seroconversion to both vaccine antigens (Fig. 3A and B). A strong anamnestic response occurred at 28 dpv, which corresponded to peak antibody activity, following administration of a booster at 21 dpv (Fig. 3A and B). Detectable MAP1-specific antibody titers were maintained in animals in all dose groups until day 42, the study endpoint (Fig. 3A and B). In response to both vaccine antigens, optical density (OD) values detected at various post-vaccination time-points for the different dose groups were not significantly different ($P = 0.0606$ response to Antigua antigens; $P = 0.4227$, response to Gardel antigen). The magnitude of seroconversion to either vaccine antigen was manifested by marked differences in the absorbance (OD) values in 28 dpv sera ($P < 0.05$) (Figure 3C and D).

[0085] Endpoint titration of MAP1-specific antibody activity in all three vaccine dose groups exhibited a time-dependent increase in antibody titers, with 28 dpv sera showing highest endpoint titers (Fig. 4). Overall, antibody reactivity data showed persistence of MAP1-specific antibody titers in all vaccinated animals through the study endpoint. Characterization of immunoglobulin G (IgG) isotype responses in vaccinated sheep showed induction of both IgG1 and IgG2 antibody responses, with the IgG1 isotype showing comparatively higher titers as determined by endpoint titration (Fig. 5).

Analysis of CD4+ and CD8+ T cell responses

[0086] PBMC isolated from the vaccinated animals demonstrated significant antigen-specific CD4+ and CD8+ T cell proliferation in recall response to either recombinant antigen (Antigua or Gardel rMAP1) (Fig. 6A and B). Animals vaccinated with 200 µg (group A) of rMAP1 demonstrated the highest frequency of proliferating CD4+ T cells compared to mock vaccinated animals (group D) when stimulated with rMAP1 of either *E. ruminantium* isolates, Antigua or Gardel ($P < 0.05$) (Fig. 6A). The frequency of proliferating CD4+ T cells detected in animals in group B (vaccinated with 400 µg of subunit vaccine) and group C (vaccinated with 600 µg subunit vaccine) was not statistically different from the mock-vaccinated control group D ($P > 0.05$) (Fig. 6A). Furthermore, vaccinated animals in groups A, B and C exhibited a higher frequency of proliferating CD8+ T cells following antigenic stimulation with Antigua rMAP1 than mock-vaccinated control group D ($P < 0.05$) (Fig. 6B). Vaccinated animals in groups A and B also exhibited higher percentage proliferating CD8+ T cells than the mock control group D in response to stimulation with Gardel rMAP1 antigen ($P < 0.05$) (Fig. 6B); however, the frequency of proliferating CD8+ T cells from animals in group C were not statistically different from the mock-vaccinated control group D ($P > 0.05$) (Fig. 6B). Comparison of the frequency of proliferating CD4+ or CD8+ T cells between the various vaccine-dose groups (A, B, C) showed no statistically significant differences ($P > 0.05$).

[0087] To assess antigen-specific IFN γ production, CD4 $^{+}$ T cells were subjected to intracellular cytokine staining following antigenic stimulation with Antigua or Gardel rMAP1. Group A animals responded with higher percent of IFN γ -positive CD4 $^{+}$ T cells than the mock-vaccinated control group D ($P < 0.05$) (Fig. 7). The frequency of IFN γ -positive CD4 $^{+}$ T cells detected in groups B and C animals was higher but statistically not different from the control group D animals ($P > 0.05$) (Fig. 7). Comparison of the percent IFN γ -positive CD4 $^{+}$ T cells between the various vaccine-dose groups (A, B, C) revealed no statistically significant differences ($P > 0.05$). Similarly, a comparison of the frequency of IFN γ -positive CD8 $^{+}$ T cells revealed no statistically significant differences between the various vaccine-dose groups (data not shown).

Discussion

[0088] Current vaccines developed and evaluated for protecting ruminant livestock against heartwater are based on inactivated and live-attenuated vaccines. These vaccines suffer from low immunogenicity (for killed vaccines), lack of cross-protection and/or safety concerns, especially for use in non-endemic areas. Beside a few sheep studies, most subunit vaccine studies were performed in mouse models. This represents the first study wherein the immunogenicity of glycosylated forms of the major antigenic protein 1 (MAP1) of *E. ruminantium* is assessed in a ruminant model. The *E. ruminantium* MAP1 is an immunodominant membrane protein and considered a good target for vaccine development. Like most membrane proteins with potential sites for glycosylation, in silico analysis of *E. ruminantium* map1 gene revealed putative N-linked and O-linked glycosylation sites (Figure 1). O-linked sites have been reported to serve as sites for O-linked mannosylation, which has been shown, in eukaryotes, to enhance immunogenicity of membrane proteins. In this study, we confirmed expression of *E. ruminantium* rMAP1 (Antigua and Gardel isolates) protein in glycosylated form utilizing a eukaryotic expression system and demonstrated a distinct glycosylation profile of the protein (Figure 2). Vaccination of sheep with a subunit vaccine formulation induced both antibody and Th1 T cell responses, which are critical to controlling intracellular pathogens, including *E.*

ruminantium, in infected hosts; suggesting that a glycosylated rMAP1 subunit vaccine could be potentially efficacious against virulent heartwater challenge.

[0089] Specifically, the recombinant MAP1 subunit vaccine induced a strong Th1 T cell response characterized by increased proliferation of CD4⁺ and CD8⁺ T cells, including production of IFN- γ , and induction of IgG2 isotype antibodies. The significance of this cellular response in terms of protective immunity in the ruminant host is unknown, as we were unable to perform efficacy testing in this study. However, in a mouse model, a polarized Th1 response characterized by induction of IgG2a and IgG3 antibodies, following a prime-boost vaccination regimen (DNA prime and *E. coli*-expressed rMAP1 boost), was associated with increased protection against virulent heartwater challenge. Increased protection is attributed to in vivo (eukaryotic) expression of glycosylated MAP1 from the DNA vector construct, since immunization with unglycosylated *E. coli*-expressed MAP1 proteins resulted in lower protective efficacy characterized by a Th2 response of predominantly IgG1 isotype. Indeed, the potential role of glycans in antigenicity has been reported for glycoproteins of *E. ruminantium* (MAP1), and antigenically related pathogens, *E. canis* (gp36) and *E. chaffeensis* (gp47), for which glycosylated forms of the proteins exhibited stronger immunoreactivity in comparison to unglycosylated forms. The enhanced T cell response observed in our study could be attributed to the presence of O-linked glycans, by facilitating recognition by mannose receptors on dendritic cells, resulting in efficient uptake, processing, and presentation of antigen to T cells. Taken together, these results suggest that glycan residues in *E. ruminantium* MAP1 proteins are important antigenic determinants. Further studies are required to support this hypothesis including the potential ability to confer broad-spectrum protection amongst antigenically diverse strains of the pathogen.

[0090] Although immunity to *E. ruminantium* infection (heartwater), like other intracellular parasites, is mainly cell-mediated, the role of the humoral immune response has not been fully elucidated. In this study, the recombinant MAP1 subunit vaccine also induced strong anamnestic MAP1-specific antibody response in sheep; and even though antibodies produced in response to *E. ruminantium* infection

have been reported to not correlate with protective immunity against heartwater, we speculate that their induction is an important vaccine attribute and could play a role as opsonizing agents and contribute to enhancing cell-mediated immunity.

[0091] With the aim of assessing the dosage of the subunit vaccine that induces enhanced immune response in sheep, we tested three doses (low, intermediate and high) of the vaccine. There was no significant difference between the different dose groups in inducing antibody responses, as assessed by the level of IgG antibody responses ($P > 0.05$) (Fig. 3). In contrast, the dose of the antigen/vaccine appeared to influence the level of cellular responses; showing vaccination with a low dose inducing higher frequency of proliferating CD4⁺ and CD8⁺ T cells, including higher levels of secreted cytokine, IFN- γ , compared to vaccination with intermediate and high doses. The lack of positive correlation is unknown; however, studies in mice have shown that the dose of antigen may be a critical factor in determining what type of immune response will be elicited; with susceptible mice immunized with very low doses of *Leishmania* major demonstrating higher levels of protection than did mice immunized with higher doses of the organism. It has been postulated that very small doses of antigen could imprint the immune response into a Th1-like cell-mediated mode or select T cells with high functional avidity, whereas higher immunizing doses inhibit this response. It is unknown if the comparatively low T cell response observed in the intermediate and high-dose vaccinated groups could be attributed to a similar phenomenon.

EXAMPLE 2

[0092] Recombinant expression of novel *E. ruminantium* type IV and type VI secretion proteins and assessment of their potential use as vaccine and diagnostic antigens

Materials and Methods

Summary

[0093] In this study, we cloned and expressed the genes encoding the *E. ruminantium* type IV (locus tag: ERUM_RS0013 in Welgevonden genome) and type VI (locus tag: ERUM_RS00140 in Welgevonden genome) secretion system proteins using a recombinant baculovirus expression system. We also cloned and expressed the gene encoding the immunodominant major antigenic protein 1 (MAP1) of Kerr Seringe *E. ruminantium* strain (GenBank Accession No. DQ333230) for use as a control in immunoreactivity experiments. To assess the potential use of the recombinant proteins as targets for vaccine and diagnostics development, we examined their reactivity with an antiserum obtained from a sheep experimentally infected with virulent *E. ruminantium* (heartwater agent) by Western blot/immunoblot analysis and indirect enzyme-linked immunosorbent assay (ELISA). Considering that immunoreactive epitopes of *E. ruminantium* MAP1 are conserved in *Ehrlichia canis* (causative agent of canine ehrlichiosis), an antigenically closely related pathogen, we assessed cross-reactivity of the type IV and type VI secretion proteins via indirect ELISA using the recombinant proteins as coating antigens, with antiserum from dogs that tested serologically positive for *E. canis* infection. Serum from a naïve uninfected dog was used as control.

Cloning, recombinant protein expression and purification

[0094] The coding sequences of *E. ruminantium* type IV and type VI secretion proteins, and Kerr Seringe MAP1 were codon optimized and synthesized as insert in pUC57 recombinant plasmid. The sequences were amplified by Accuprime Supermix high fidelity PCR using the respective recombinant plasmids as templates and gene specific primers including the addition of a Kozak sequence. The target PCR amplicons were subsequently cloned into a pFastBac plasmid to create recombinant donor plasmids, pFastBacT4, pFastBacT6 pFastBacMAP1. The donor plasmids were used to create recombinant bacmids via site specific transpositioning using DH10 Bac *E. coli* strain (Life Technologies) according to manufacturer's instruction. The recombinant bacmids were purified and used to transfect *Spodoptera frugiperda* (Sf9) insect cells to rescue the respective recombinant baculoviruses expressing the various target proteins. The proteins were purified using Ni-NTA affinity chromatography

and dialyzed against PBS PH 7.4 according to standard protocol (Faburay et al. 2013). Concentration of the purified proteins was measured by BCA method and proteins stored at -80C until used.

Western blot analysis

[0095] To assess expression of the recombinant proteins, approximately 5 µg of each purified protein was run through a 12% Bis-Tris polyacrylamide gel in 1x 3-(N-morpholino) propanesulfonic acid (MOPS) running buffer, and transfer was performed by electroblotting onto PVDF membranes per standard protocol. The membranes were blocked with 0.1% Tween-20 in PBS (pH 7.4) containing 3% BSA for 1 hr at room temperature. The blot was probed with either anti-His (C-terminal)-HRP monoclonal antibody at 1:5,000 dilution to detect specific recombinant protein expression, or with a primary *E. ruminantium* anti-serum (at 1:50 dilution) obtained from a sheep experimentally infected with virulent isolate of heartwater agent, to detect specific immunoreactivity. Next, the membranes were probed with a Protein G-HRP (1:5, 000) or with a secondary antibody, rabbit anti-sheep IgG-HRP (1:2,000). Detection of immunoreactive bands was performed by ECL chemiluminescence detection reagent and visualized using a Biorad.

Enzyme-linked immunosorbent assay

[0096] The ELISA plates were coated overnight at 4°C with approximately 150 ng per well of either purified recombinant protein type IV, type VI or MAP1. The assay was performed as described previously (Faburay et al.). Briefly, all washing steps were performed three times with 1% Tween 20 in PBS and all samples were tested in duplicate. Following blocking with PBS (pH 7.4) containing 1% skim milk and 0.1% Tween 20 for 15 min at 37°C, the plates were probed at 37°C for 1 hr with 1:200 dilution of *E. ruminantium*-antiserum obtained from a sheep experimentally infected with virulent heartwater agent, diluted 1:200 (for probing type IV and type VI antigens) or 1:100 (for probing MAP1 antigen), or with 1:200 dilution of *Ehrlichia canis* antiserum obtained from a naturally infected dog, which was confirmed positive by an immunofluorescence antibody test. Negative sheep

and dog sera were used as controls. The MAP1 antigen was also probed with 1:200 dilution of antiserum obtained from sheep vaccinated day 42 post vaccination with Gardel-Antigua MAP1 subunit vaccine (Faburay et al., 20017) as positive control. Next, plates were incubated with either 1: 5,000 dilution of rabbit anti-sheep-HRP (Invitrogen, Cat. No. 81-8620) for probing sheep antiserum or 1:10,000 dilution of goat anti-dog-HRP (Invitrogen, Cat. No. A18763) for probing *E. canis* dog antiserum. After washing, plates were incubated with TMB substrate for 25 min in the dark at room temperature and reaction was terminated with 2M H₂SO₄. Optical density (OD) values were measured at 450 nm wavelengths.

Results

Analysis of reactivity with *E. ruminantium* anti-serum

[0097] The results indicated successful expression of the target proteins, ER type IV (T4), type VI (T6) and MAP1 as determined by detection of the estimated molecular size proteins using mouse anti-His (C-terminal)-HRP monoclonal antibody (Fig. 8A). Further probing of the recombinant proteins with ER antiserum from sheep demonstrated reactivity as indicated by detection of estimated molecular size proteins, 93 kDa (type IV), 38 kDa (type VI) and 32-39 kDa (MAP1) corresponding to the expected molecular sizes of the target proteins (Figure 8B).

[0098] Further assessment of immunoreactivity of the recombinant protein in indirect ELISA exhibited significantly stronger reactivity of both type IV and type VI secretion proteins ($P < 0.05$) with the ER antiserum compared with serum from a naïve uninfected sheep. rMAP1 as a control, demonstrated similar strong reactivity (Fig. 9A, 9B, 9C).

Analysis of reactivity with *E. canis* anti-serum

[0099] To analyze potential cross-reactivity between *E. ruminantium* type IV and type VI secretion proteins with *E. canis* antiserum, ELISA plates were

coated with either recombinant protein and subsequently probed with antisera obtained from dogs that tested serologically positive for canine ehrlichiosis. The results indicated that both type IV and type VI secretion proteins exhibited significantly higher IgG OD readings with the positive *E. canis* anti-sera than with serum from naïve uninfected dog (Figure 10A, 10B, 10C). As expected, IgG OD readings with the control rMAP1 was similarly higher with the positive *E. canis* antisera compared to the naïve control serum (Fig. 10A, 10B, 10C).

Conclusions

[0100] Demonstration of immunoreactivity of recombinant ER type IV and type VI secretion proteins with antiserum obtained from sheep experimentally infected with virulent heartwater agent presents a strong indication that these proteins are immunogenic and could elicit specific immune responses in sheep. This suggests their potential use as a vaccine and diagnostic target for protection and/or detection of heartwater in susceptible hosts. Detection of cross-reactivity of recombinant proteins with anti-sera from *E. canis* infected dogs suggests conservation of the immunoreactive epitopes in *E. canis*, an antigenically closely related pathogen, and the potential use of these recombinant proteins as subunit vaccine and/or diagnostic antigens for protection and detection of canine ehrlichiosis, respectively, in dogs.

WHAT IS CLAIMED IS:

1. A composition comprising a recombinant antigenic protein subunit from a tick-borne pathogen selected from the group consisting of *Ehrlichia ruminantium*, *Ehrlichia chaffeensis*, *Ehrlichia canis*, *Ehrlichia ewingii*, *Ehrlichia muris*, *Anaplasma phagocytophilum*, *Anaplasma platys*, *Anaplasma marginale*, and any combination thereof, wherein the subunit has been glycosylated.

2. The composition of claim 1, further comprising an additional element selected from the group consisting of a pharmaceutical carrier, an adjuvant, at least one additional antigen from a pathogen other than one of the tick-borne pathogens from claim 1, a preservative, a stabilizer, a color, a flavor, and any combination thereof.

3. The composition of claim 1, wherein the protein has been glycosylated from expression through a mammalian or eukaryotic system.

4. The composition of claim 1, wherein the recombinant antigenic protein subunit includes a signal peptide sequence.

5. The composition of claim 4, wherein the signal peptide sequence is from the same tick-borne pathogen as the recombinant antigenic protein subunit.

6. The composition of claim 4, wherein the signal peptide sequence is from *Ehrlichia ruminantium*.

7. The composition of claim 4, wherein said signal peptide sequence has at least 80% sequence homology with SEQ ID No. 14.

8. The composition of claim 1, wherein said recombinant antigenic protein subunit is selected from the group consisting of the MAP1 protein of *Ehrlichia ruminantium*, the p30-1 sequence from *Ehrlichia canis*, the p28-Omp19 protein from *Ehrlichia chaffeensis*, the MSP4 protein from *Anaplasma marginale*, and any combination thereof.

9. The composition of claim 1, wherein said recombinant antigenic protein subunit has at least 80% sequence homology with a sequence selected from the group consisting of SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 7, SEQ ID No. 12, SEQ ID No. 12, and any combination thereof.

10. The composition of claim 2, wherein the adjuvant is montanide ISA25.

11. A method for reducing the incidence of and/or severity of at least one clinical sign caused by infection from a tick-borne pathogen comprising the step of administering the composition of claim 1 to an animal in need thereof.

12. The method of claim 11, wherein at least one clinical sign of infection is reduced by at least 20% in severity or incidence in comparison to an animal not receiving an administration of the composition of claim 1.

13. The method of claim 11, wherein clinical sign is selected from the group consisting of death, fever, respiratory distress, which may be exaggerated, paroxysmal convulsions, anorexia, depression, respiratory congestion, friable mucous membranes, respiratory distress, nervous signs, hyperaesthesia, high-stepping stiff gate, exaggerated blinking, exaggerated chewing movements, diarrhea, headache, fatigue, muscle aches, lethargy, swollen lymph nodes, enlarged spleen, weight loss, poor appetite, abnormal bruising and bleeding, chronic eye inflammation, neurologic abnormalities, lameness, reluctance to stand or walk, a stiff or stilted gait, joint effusion, central nervous system signs (*e.g.*, head tilt, tremors, and anisocoria), low weight gain, reduction in milk production, abortion, and any combination thereof.

14. The method of claim 11, wherein the composition is administered more than one time.

15. The method of claim 11, wherein the administration comprises 5 – 2000 ug of the recombinant antigenic protein subunit.

16. The method of claim 11, wherein the administration is via intramuscular injection.

17. The method of claim 11, wherein the composition is administered together with at least one additional antigen from a pathogen other than one from a tick-borne pathogen of the disclosure.

18. The method of claim 17, wherein the composition comprising the recombinant antigenic protein subunit is combined with the at least one additional antigen from a pathogen other than one from a tick-borne pathogen of the disclosure prior to administration.

19. The method of claim 11, wherein the recombinant antigenic protein subunit is glycosylated.

20. A method of making a glycosylated recombinant antigenic protein subunit of a tick-borne pathogen of the disclosure comprising the steps of:

inserting a nucleotide sequence including the coding sequence for the recombinant antigenic protein subunit and a nucleotide coding sequence for a signal peptide into an appropriate expression system;

causing the expression system to express the nucleotide sequence and thereby generate a recombinant protein together with the signal peptide; and

recovering the expressed recombinant protein.

21. The method of claim 20, wherein the signal peptide is from the tick-borne pathogen of the disclosure.

22. The method of claim 20, wherein the signal peptide is from the MAP1 of *Ehrlichia ruminantium*.

23. The method of claim 20, wherein the signal peptide has at least 80% sequence homology with SEQ ID No. 14.

24. The method of claim 20, wherein the signal peptide causes the glycosylation of the recombinant protein.

25. The method of claim 20, wherein the recombinant antigenic protein subunit is selected from the group consisting of the MAP1 protein of *Ehrlichia ruminantium*, the p30-1 sequence from *Ehrlichia canis*, the p28-Omp19 protein from *Ehrlichia chaffeensis*, the MSP4 protein from *Anaplasma marginale*, and any combination thereof.

26. The method of claim 20, wherein the coding sequence for the recombinant antigenic protein subunit has at least 80% sequence homology with a sequence selected from the group consisting of SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 10, SEQ ID No. 12, or a sequence coding for a sequence selected from the group consisting of SEQ ID No. 3, SEQ ID No. 12, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 7, and any combination thereof.

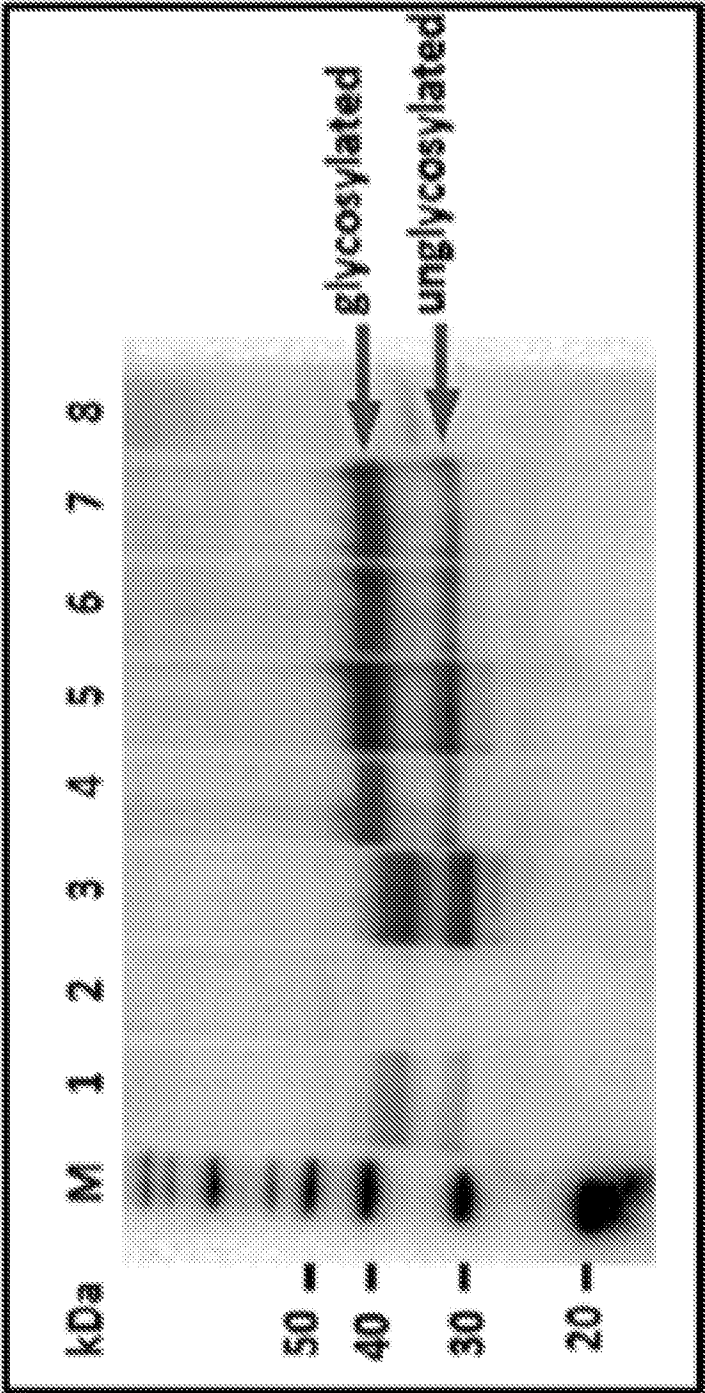
27. The method of claim 20, wherein the expression system is a baculovirus expression system.

Fig. 1

Antigua	MNCKKIFITSTLISLVSH	60
Gardel	MNCKKIFITSTLISLVSH	60

Antigua	RDTKVVFGLKKDWDGVKTSS--NTIFTEKDYSFKYENNPFLGFAGAGYSMNGPRI	115
Gardel	KNTQTVFGLKKDWDGVKT PSSDSGNNСИ FTEKDYSFKYENNPFLGFAGAGYSMNGPRI	120
	;;.*****	
Antigua	EF EISYETFDVKNP GGNKND AHMYCALDTATS--SGGAAASTSV MVKNENLTDISLMIN	173
Gardel	EF EVSYETFDVKNP GGNKND AHMYCALDTGTGTPGSTQGATLNSSVMVKNENLTDIALMIN	180
	:**	
Antigua	ACYDIMLDGMPVSPYVCAGIGTDLVSVINSTNPKLSYQGLGISYNPEASIFIGGFH	233
Gardel	ACYDITLEGMPVSPYVCAGIGTDLVSVINATNPKLSYQGLGISYNPEASIFIGGFH	240
	*** *;*****	
Antigua	RVIGNEFKDIITTSKIFNTSN--TGGATPGFASAILDVCHFIEIGGRFVF	281
Gardel	RVIGNEFKDIITTSKIFTSTGKLATAASPGFASATLDVCHFIEIGGRFVF	290
	*****:;. ; *;*****	

Fig. 2A



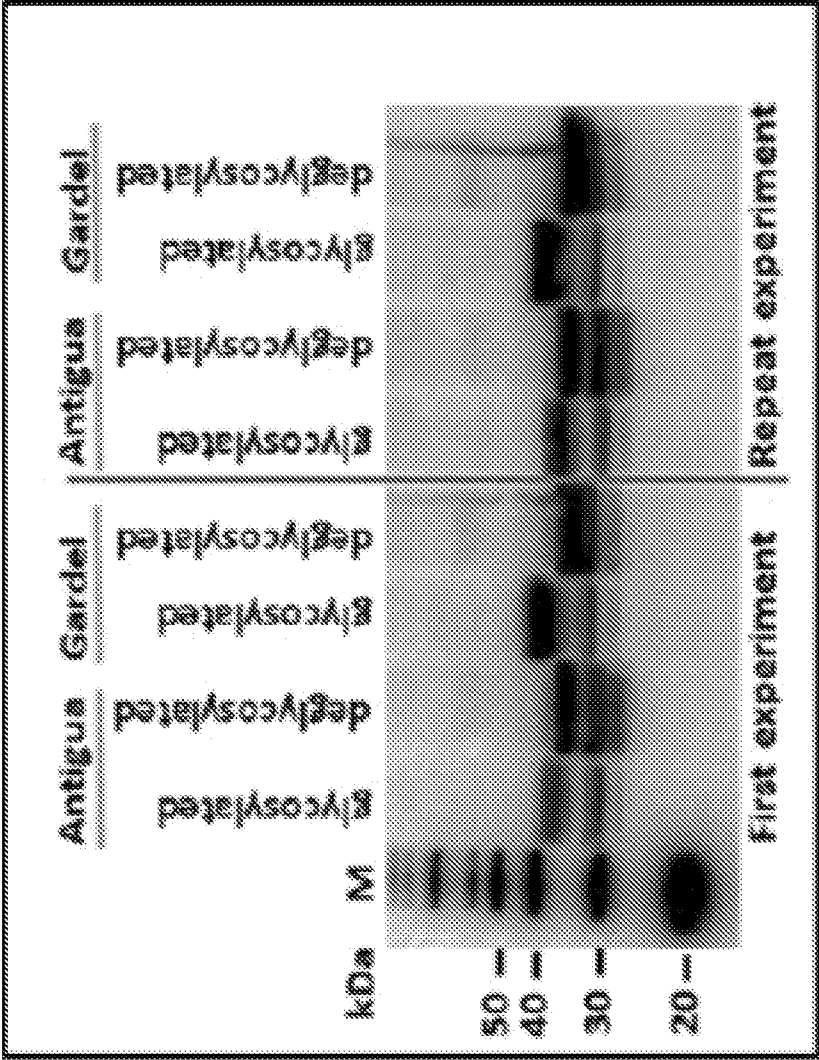


Fig. 2B

Fig. 3B

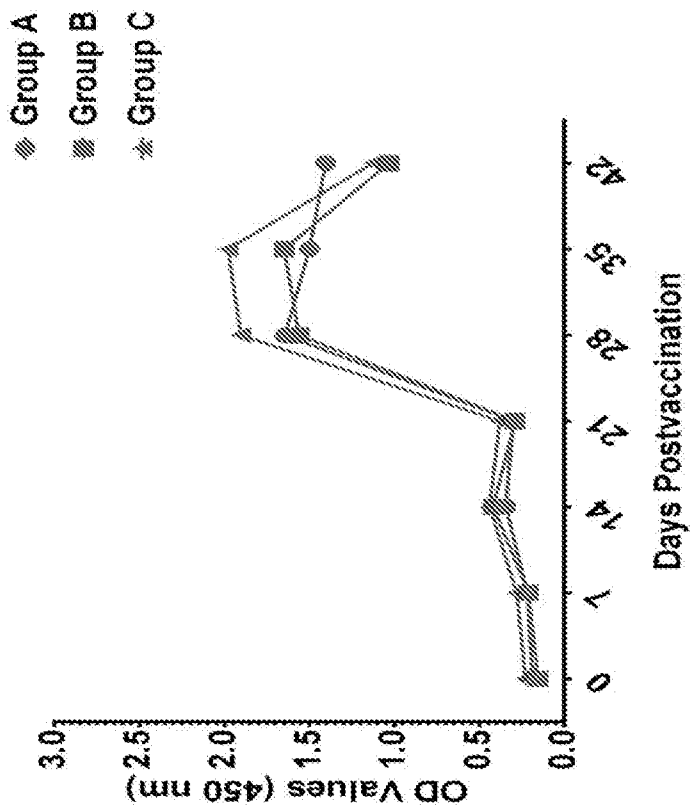


Fig. 3A

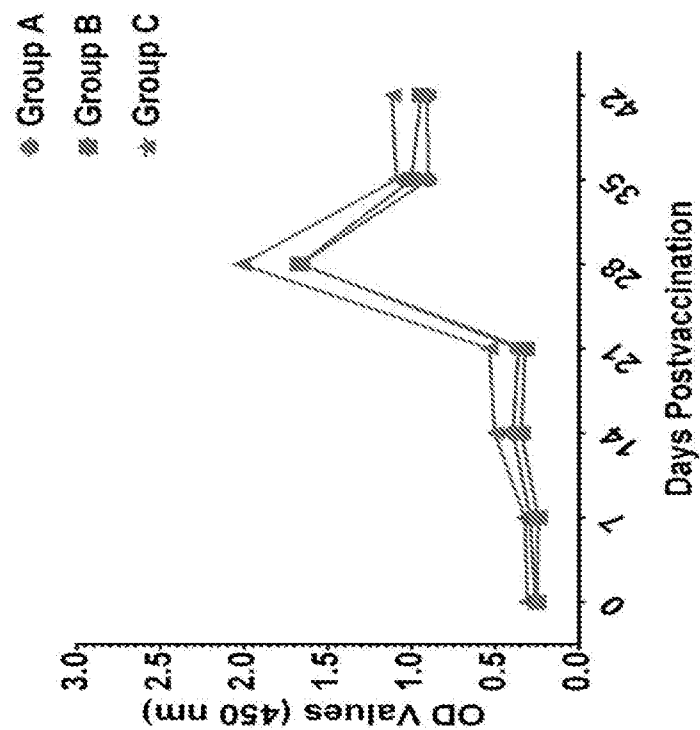


Fig. 3D

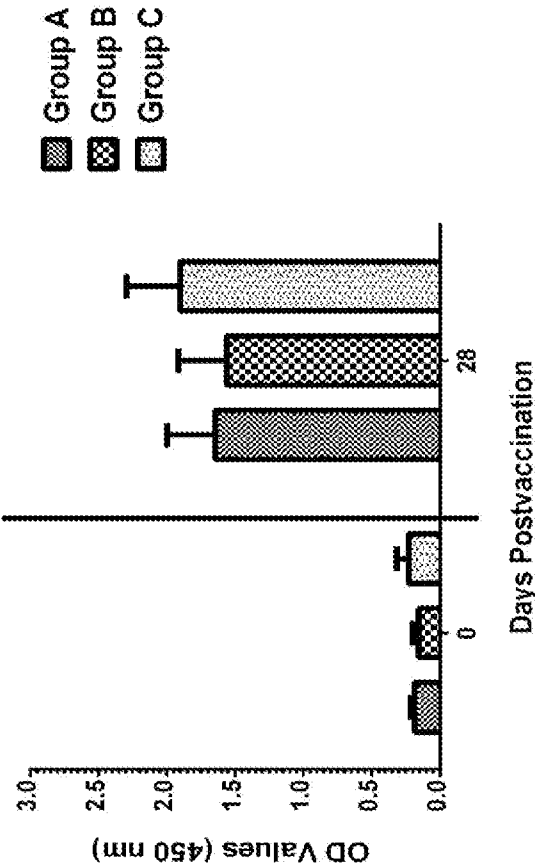


Fig. 3C

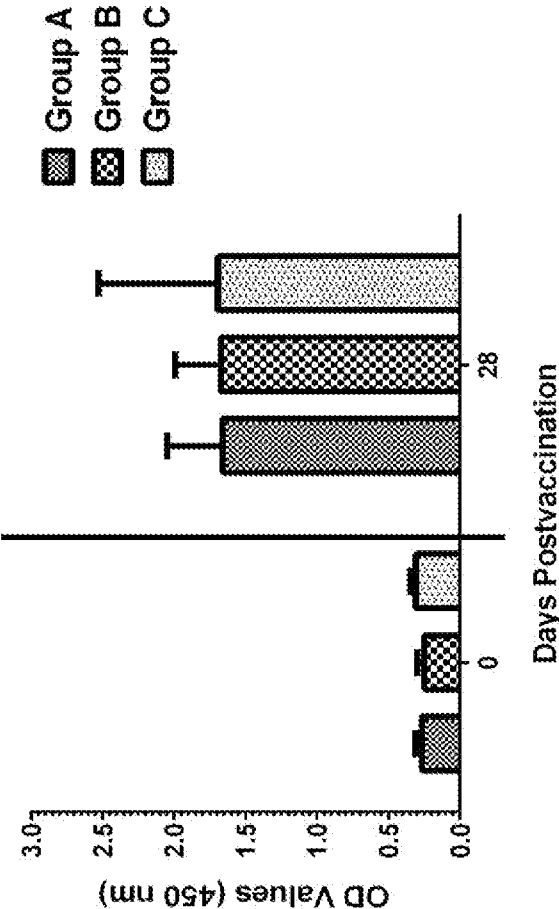


Fig. 4B

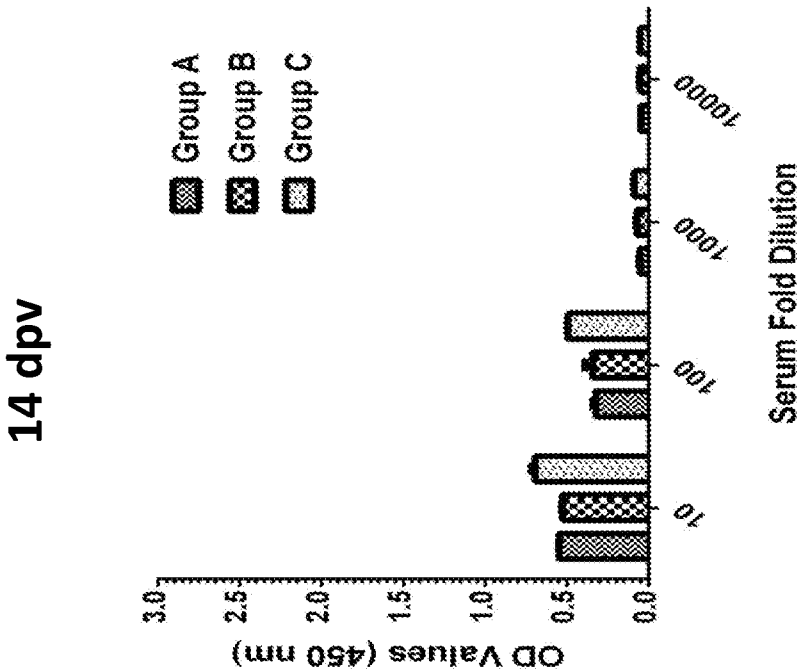


Fig. 4A

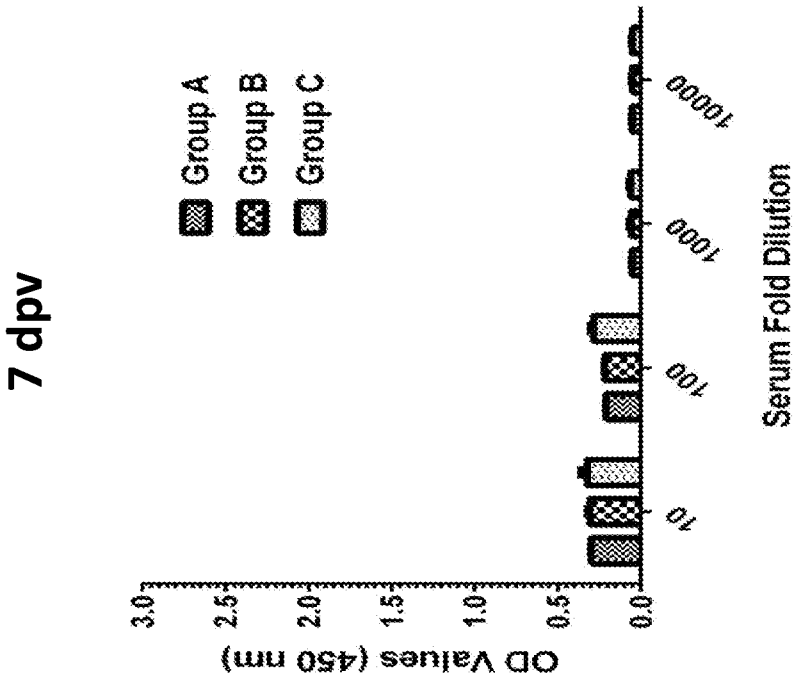


Fig. 4D

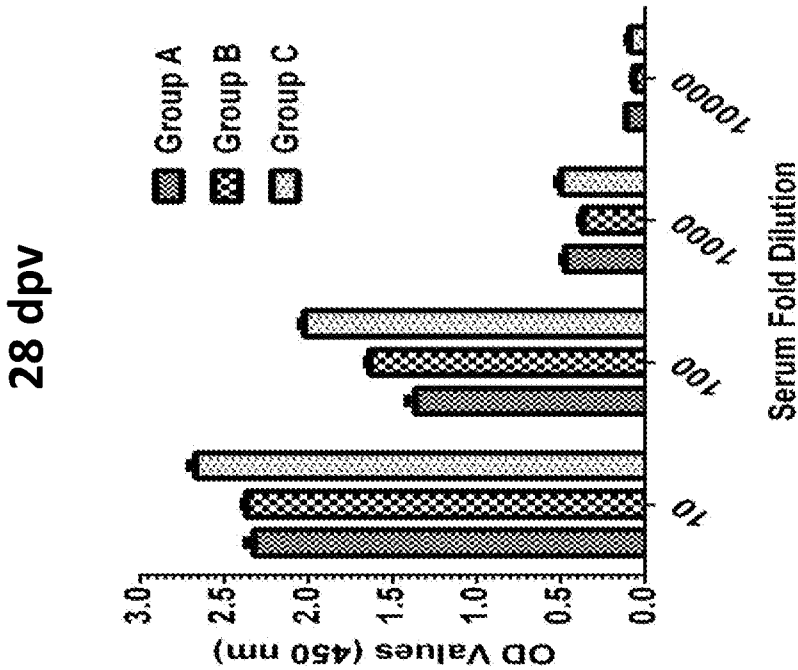


Fig. 4C

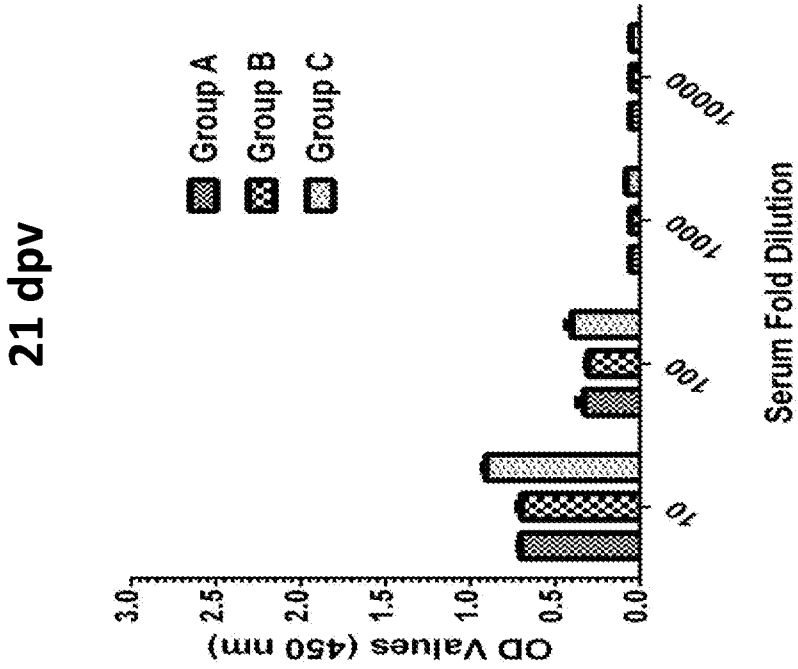


Fig. 4F

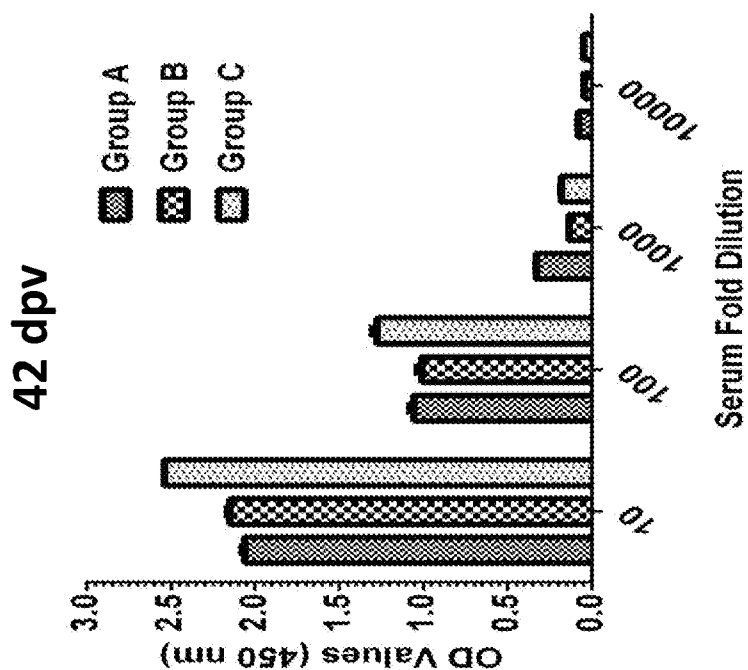


Fig. 4E

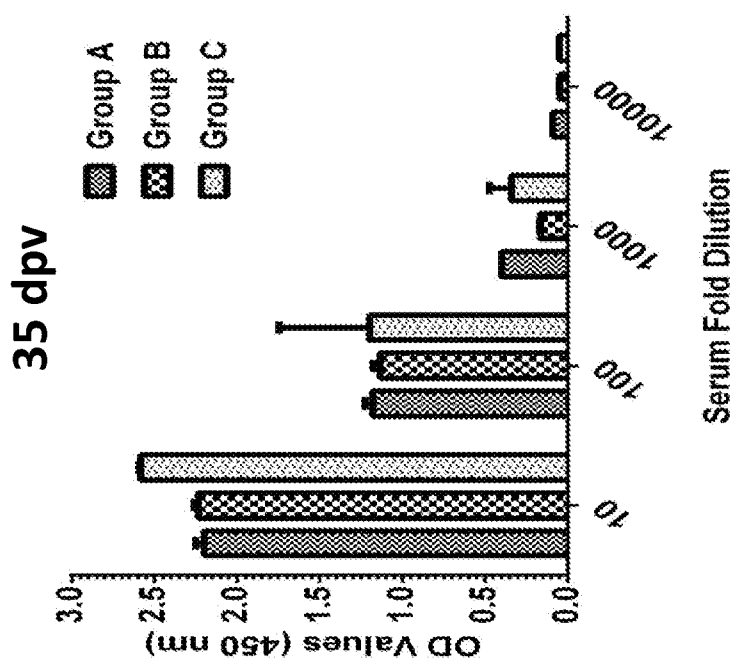


Fig. 4H

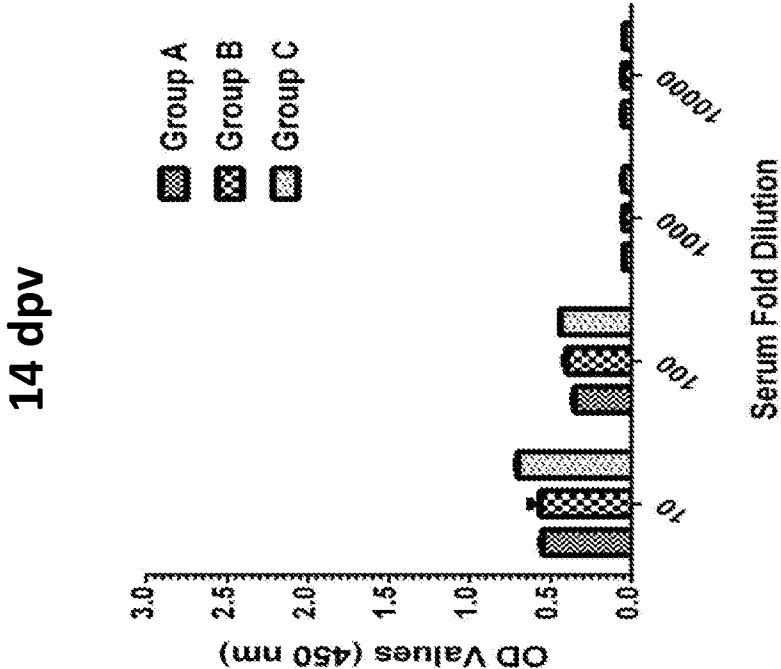


Fig. 4G

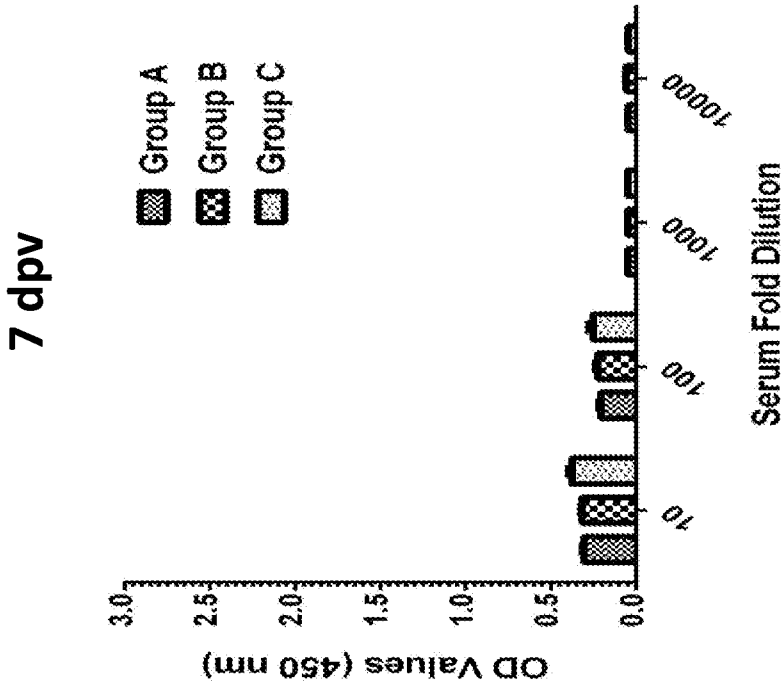


Fig. 4J

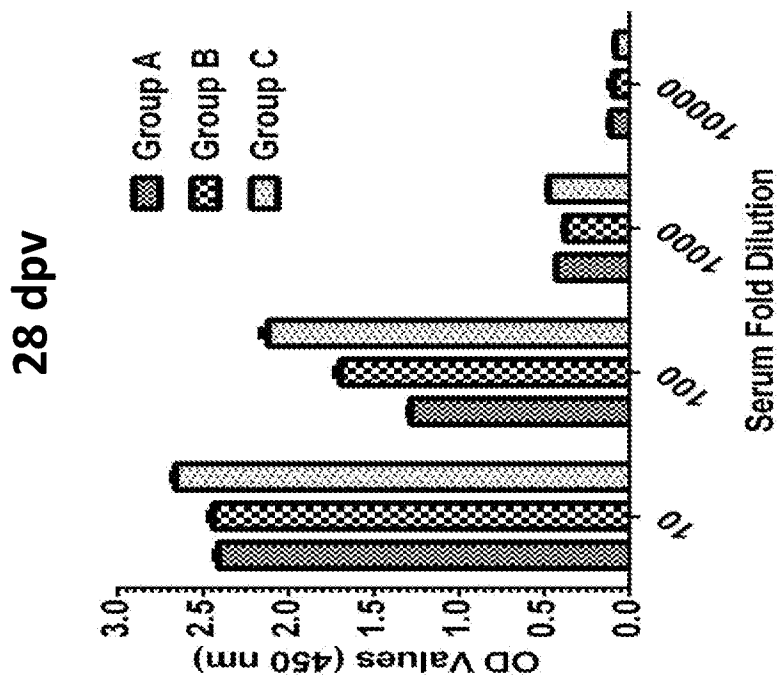


Fig. 4I

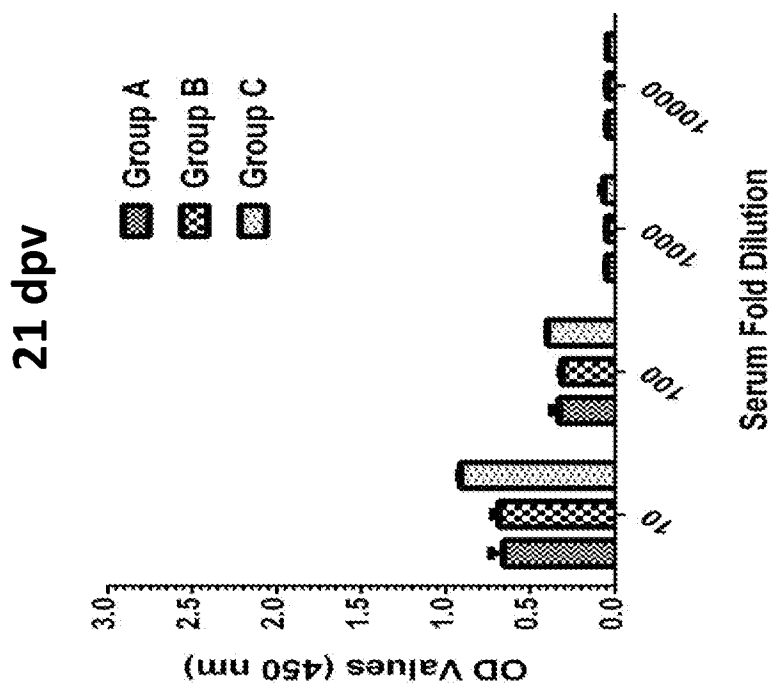


Fig. 4L

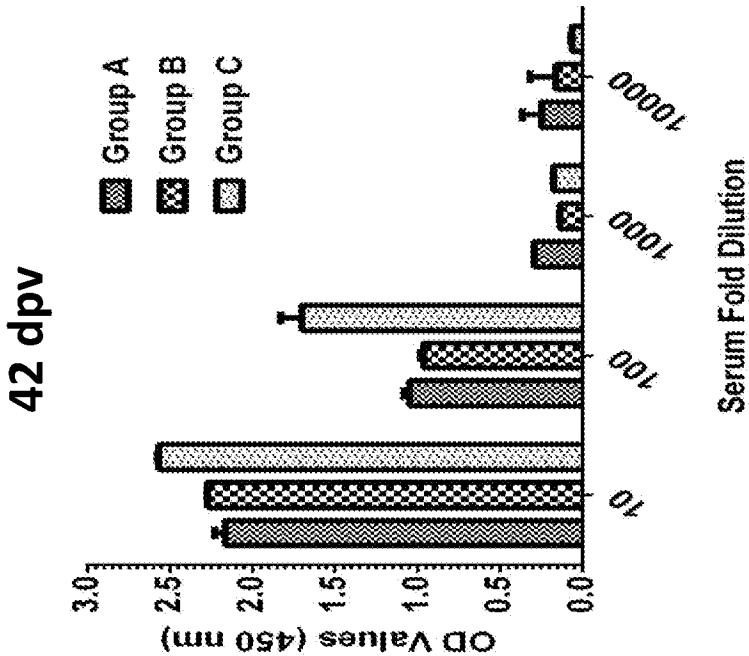
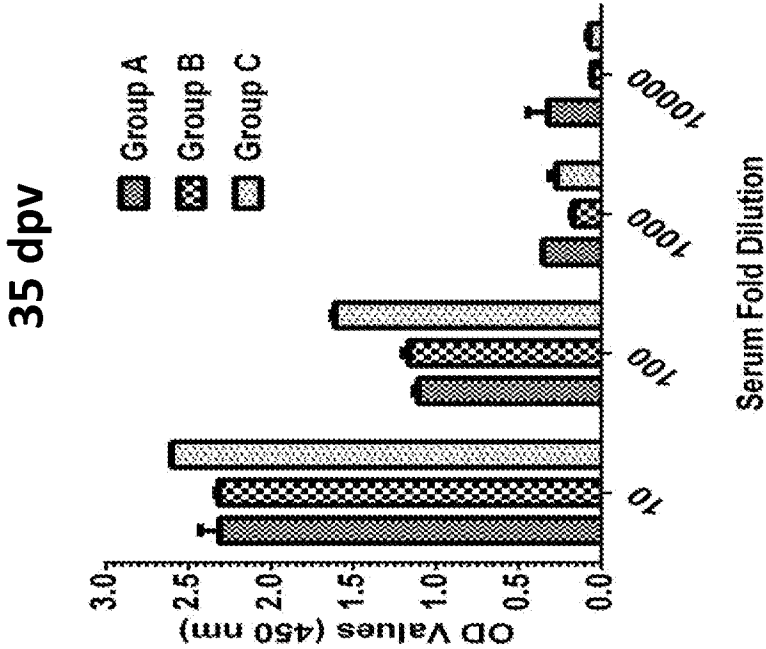


Fig. 4K



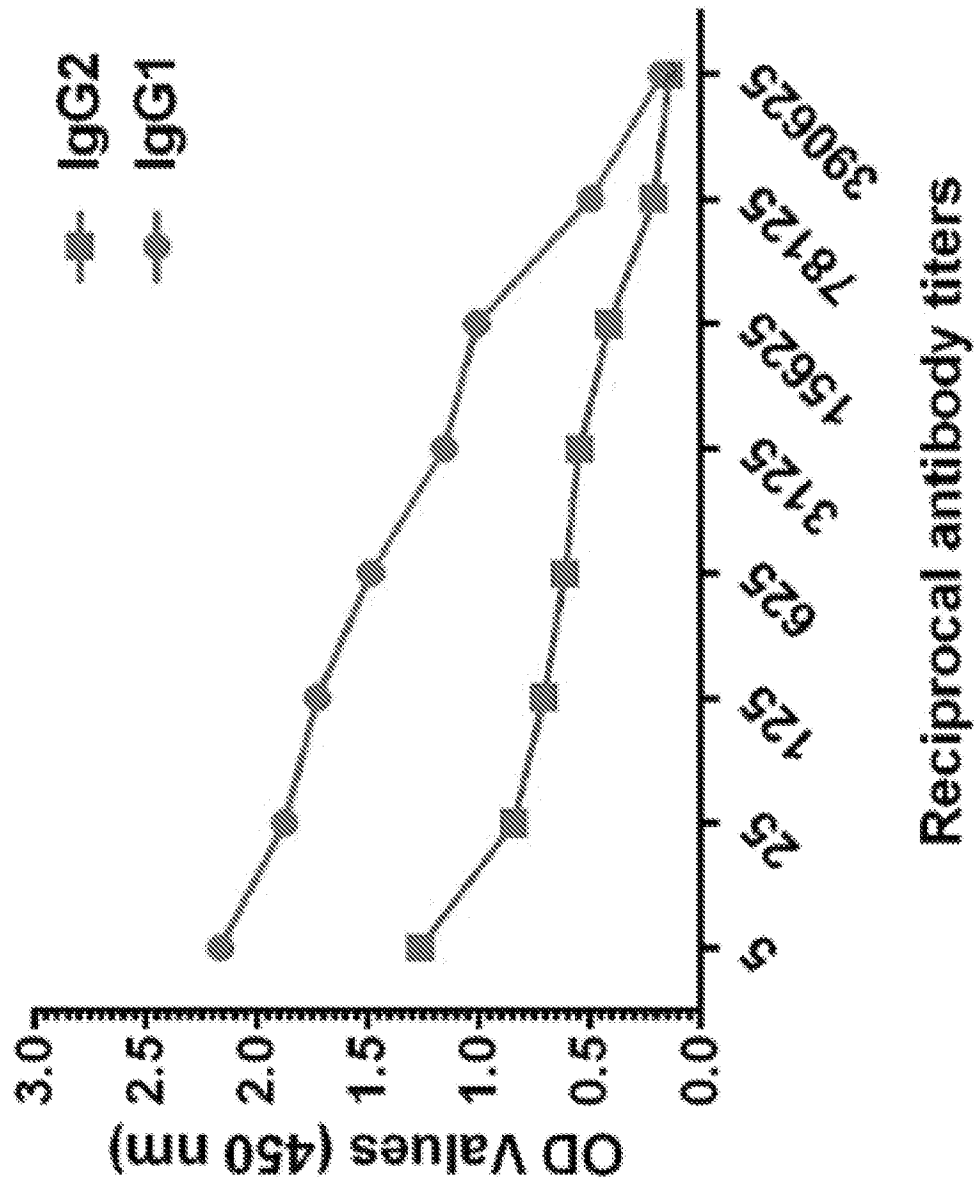


Fig. 5

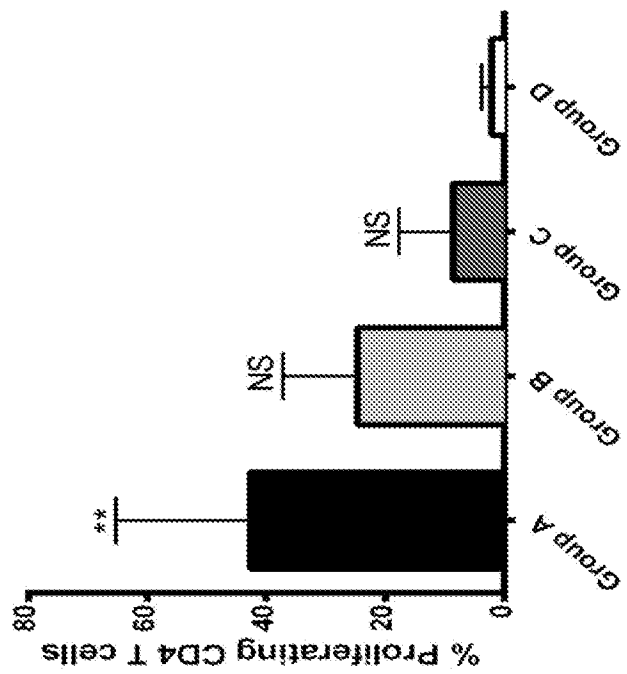
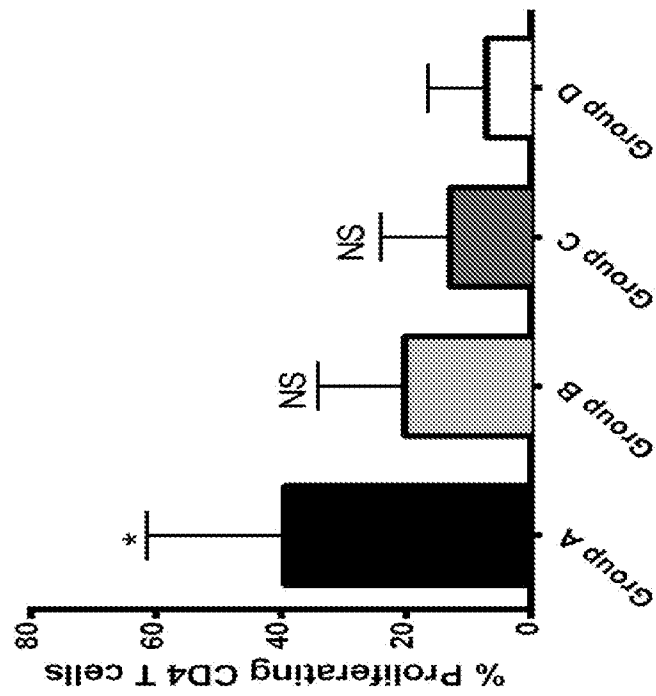


Fig. 6A

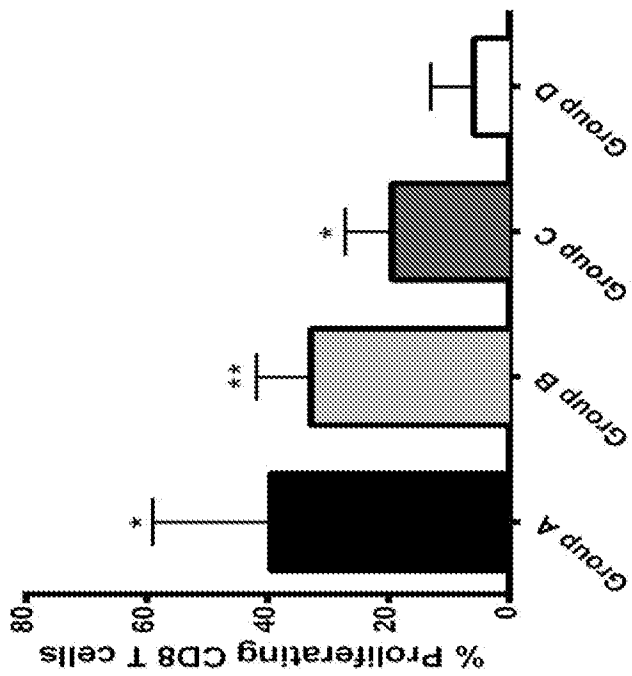
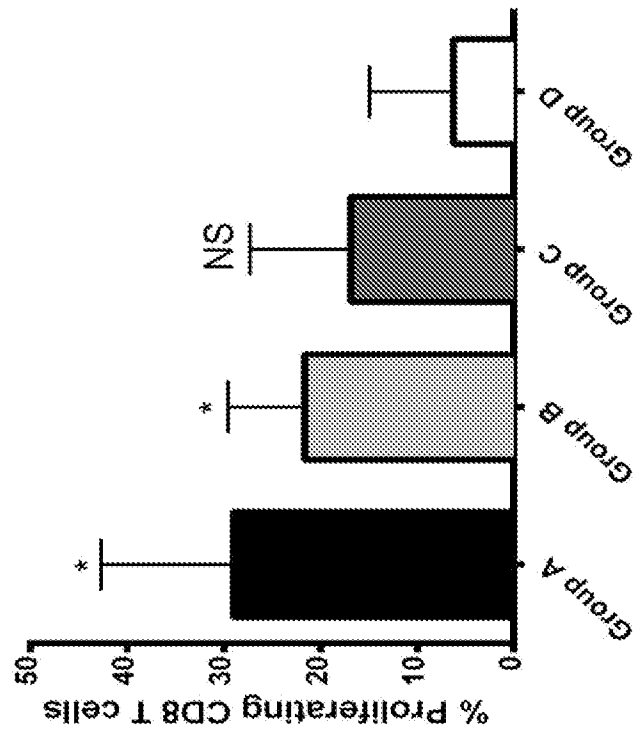


Fig. 6B

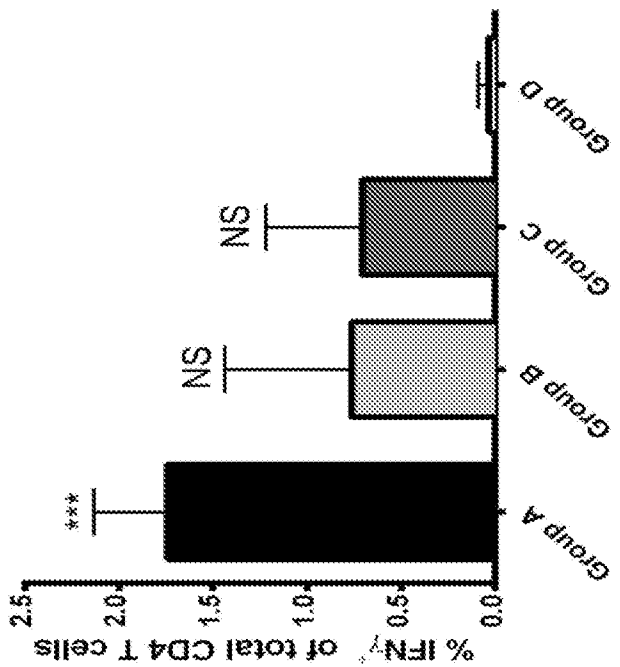
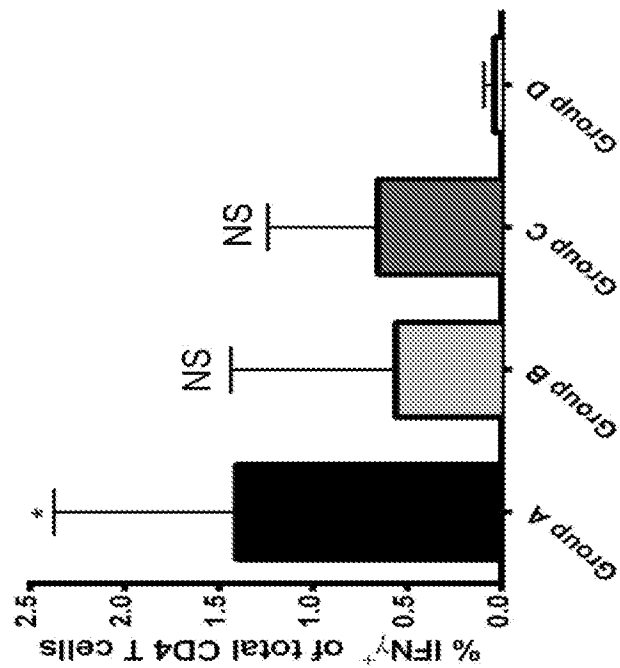


Fig. 7

Fig. 8B

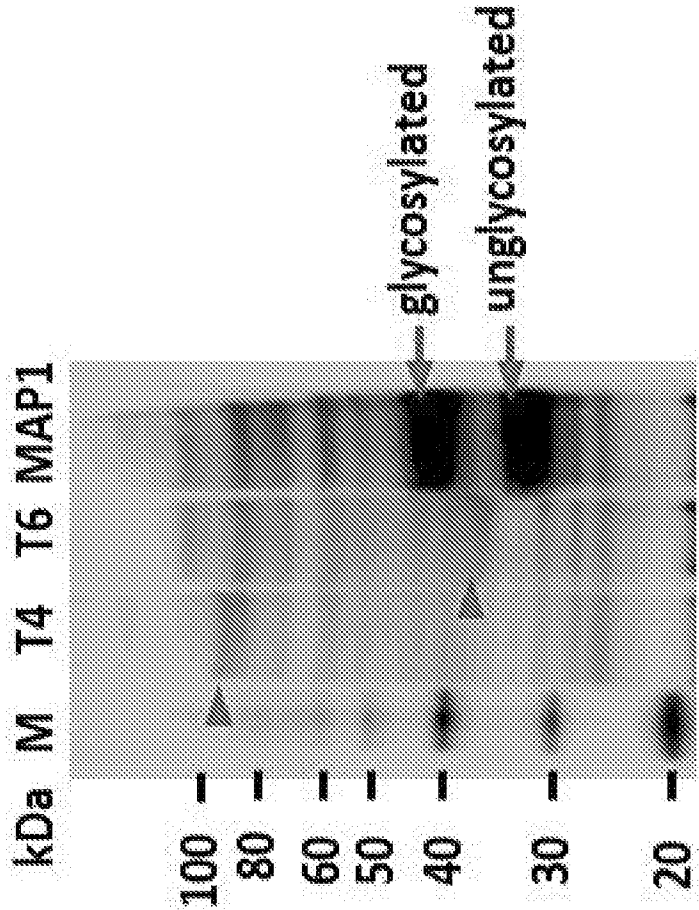


Fig. 8A

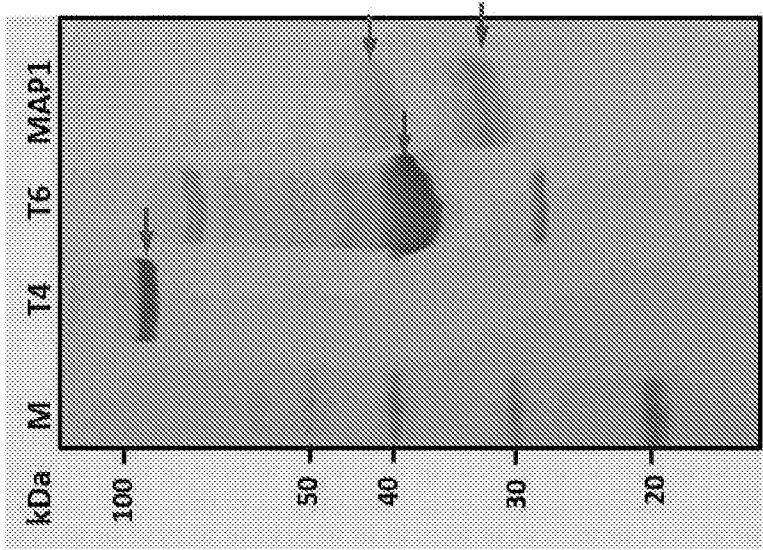


Fig. 9A

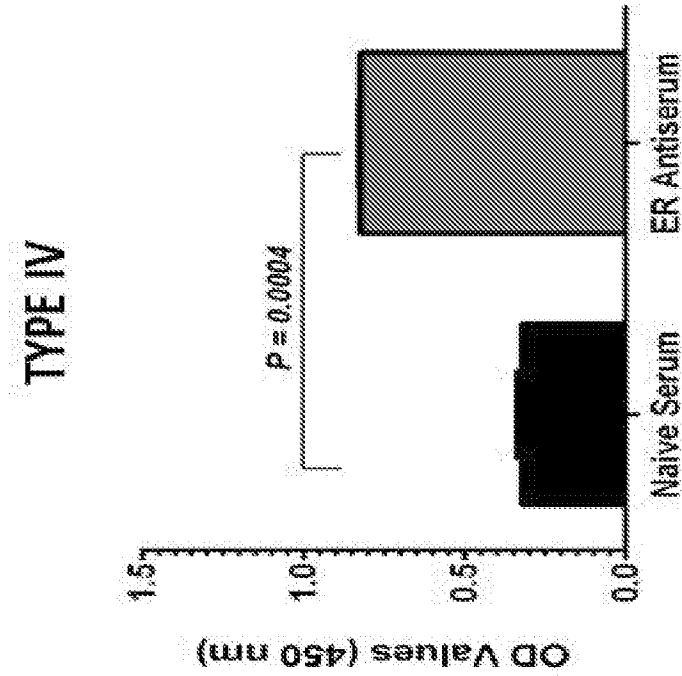
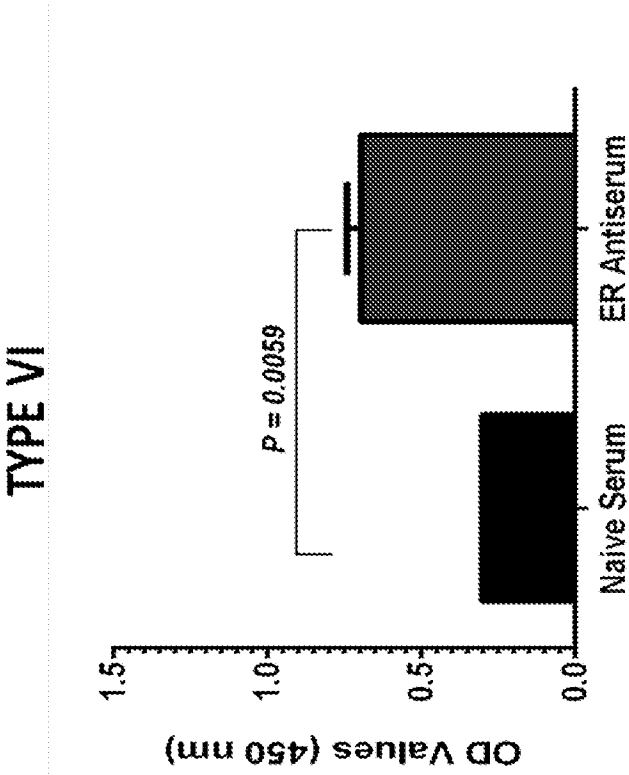


Fig. 9B



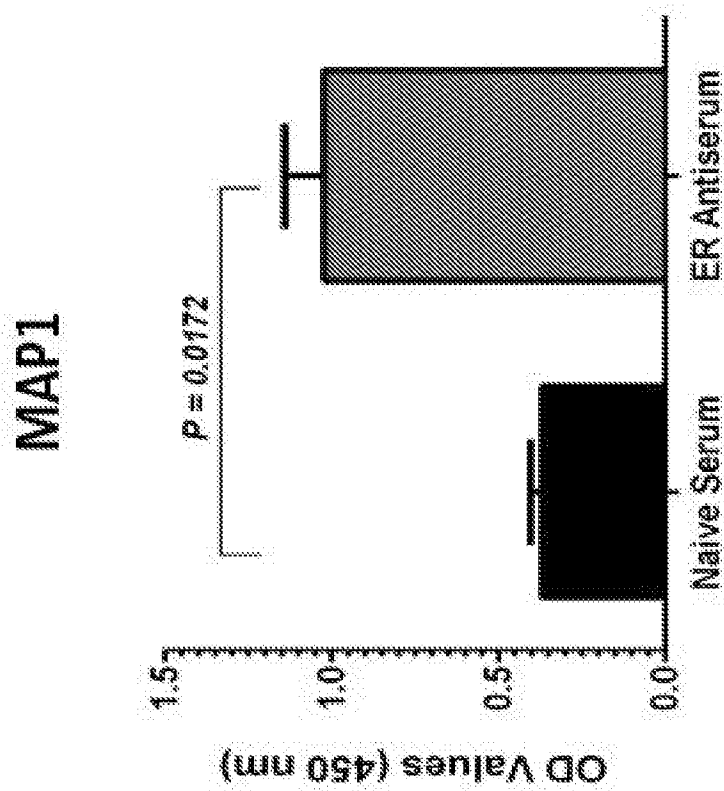


Fig. 9C

Fig. 10A

Type IV

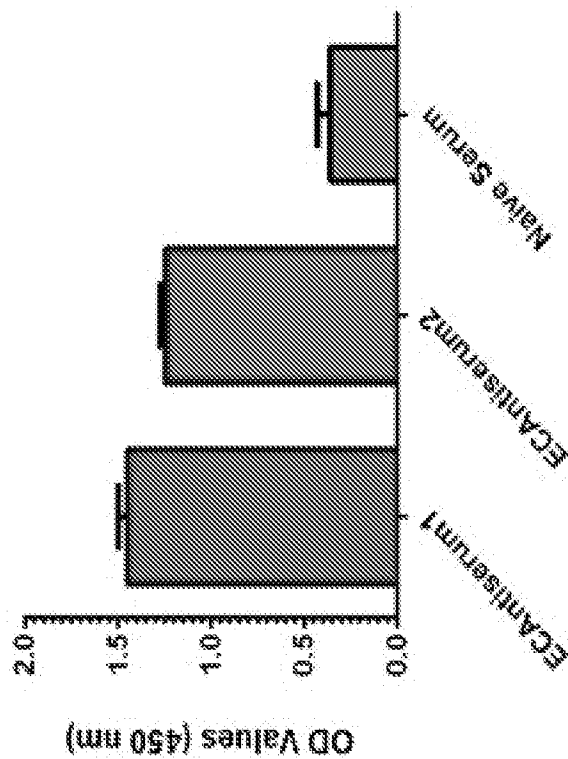


Fig. 10B

Type VI

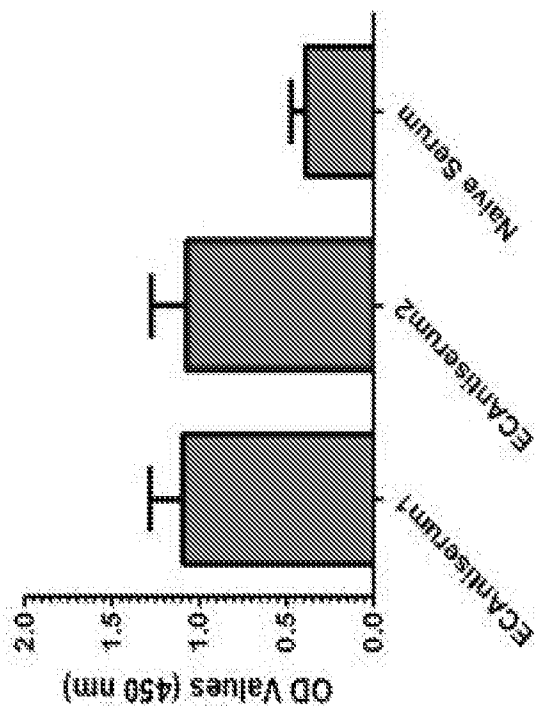
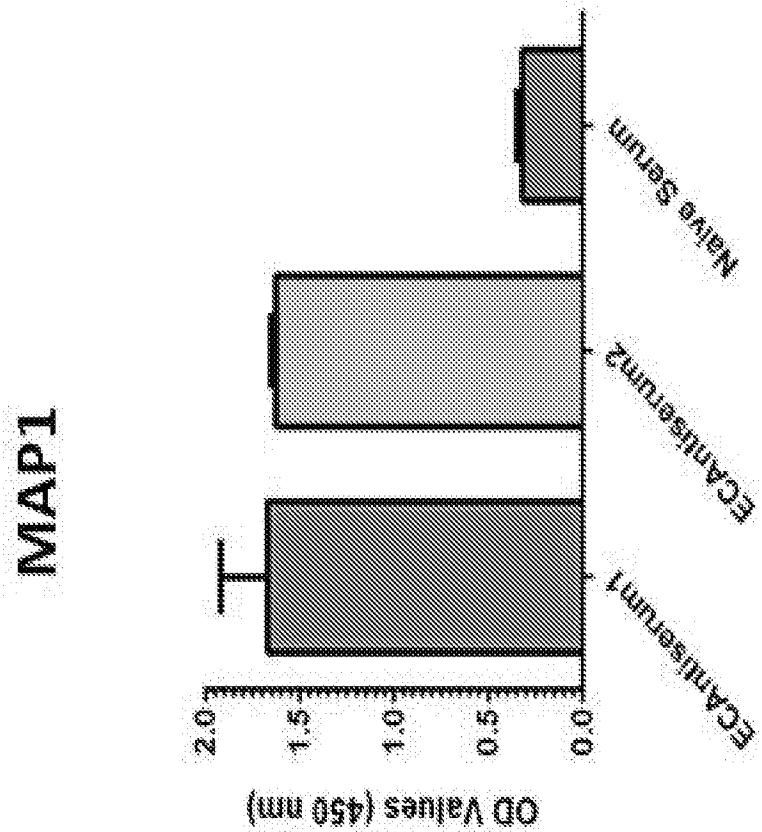


Fig. 10C



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 18/41863

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 39/00, A61K 39/02, A61K 39/395, C12N 15/11 (2018.01)

CPC - A61K 39/00, A61K 39/02, A61K 39/40

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)

See Search History Document

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

See Search History Document

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

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ----- Y	US 2009/0075368 A1 (RIKIHISA et al.) 19 March 2009 (19.03.2009) abstract; para [0008]; [0012]; [0045]; [0052]; [0065]; [0070]; [0073]; [0095]; [0097]; SEQ ID NO: 69.	1-9 ----- 10
Y	WO 1995/22603 A1 (THE UNIVERSITY OF MELBOURNE) 24 August 1995 (24.08.1995) p. 11, ln 25-29; p. 12, ln 5-13.	10



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

26 October 2018

Date of mailing of the international search report

20 NOV 2018

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents

P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 18/41863

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

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

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

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

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

Box No. 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:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-10, directed to a composition.

Group II, claims 11-19, directed to a method for reducing the incidence of and/or severity of at least one clinical sign caused by infection from a tick-borne pathogen.

Group III, claims 20-27, directed to a method of making a glycosylated recombinant antigenic protein subunit of a tick-borne pathogen.

The inventions listed as Groups I-III do not relate to a single special technical feature under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:
--continued on first extra sheet--

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-10

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.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 18/41863

--continued from Box No III: Observations where unity of invention is lacking--

Special technical features:

Group I has the special technical feature of a composition comprising a recombinant antigenic protein subunit, that is not required by Group II or III.

Group II has the special technical feature of treating an animal by administering a composition to an animal in need thereof, that is not required by Group I or III.

Group III has the special technical feature of inserting nucleotide sequences into an appropriate expression system, causing the expression system to express the nucleotides, and recovering the expressed recombinant protein, that is not required by Group I or II.

Common technical features:

Groups I-III share the common technical feature of a recombinant antigenic protein subunit from a tick-borne pathogen, wherein the subunit is glycosylated. However, this shared technical feature does not represent a contribution over prior art, because this shared technical feature is taught by US 2009/0075368 A1 to Rikihisa et al., (hereinafter Rikihisa).

Rikihisa teaches a recombinant antigenic protein subunit from a tick-borne pathogen *Ehrlichia chaffeensis* (para [0002] "*Ehrlichia chaffeensis* infects the monocytes and macrophages in humans and causes human monocytic ehrlichiosis...Most patients have a history of tick exposure"; [0008] "The outer membrane proteins from *E. chaffeensis*, particularly a recombinant form of OMP-1, are immunogenic...useful in a vaccine for protecting against infection with *E. chaffeensis*"), wherein the subunit is glycosylated (para [0052] "The present invention also encompasses OMP proteins and P30F proteins in which one or more amino acids, preferably no more than 10 amino acids, in the respective OMP protein or P30F are altered by posttranslational processes or synthetic methods. Examples of such modifications include...glycosylation").

As the technical features were known in the art at the time of the invention, they cannot be considered special technical features that would otherwise unify the groups.

Therefore, Group I-III lack unity under PCT Rule 13 because they do not share the same or corresponding special technical feature.