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(71) Applicant: UNIVERSITY OF FLORIDA RESEARCH
FOUNDATION, INCORPORATED [US/US]; 223
Grinter Hall, Gainesville, FL 32611 (US).

(72) Inventors: BARBET, Anthony, F.; 8803 S.W. 138th
Street, Archer, FL 32618 (US). CROSBY, Francy, L.;
4680 NE 142 Ave., Williston, FL 32696 (US).

(74) Agents: EISENSCHENK, Frank, C. et al.; Saliwanchik,
Lloyd & Eisenschenk, P.O. Box 142950, Gainesville, FL
32614-2950 (US).

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(54) Title: VIRB10 FOR VACCINATION AGAINST GRAM NEGATIVE BACTERIA

(57) Abstract: The invention pertains to the use of VirB10 to immunize a host against an infection by a bacterium having T4SS. The invention provides a vaccine comprising VirB10, a fragment of VirB10, a polynucleotide encoding VirB10 or a polynucleotide encoding a fragment of VirB10 and a pharmaceutically acceptable carrier and/or adjuvant. The invention also provides a method of immunizing a host against an infection caused by a bacterium having T4SS, the method comprising administering to the host a vaccine of the invention. The vaccines and the methods of the invention can be used to immunize against infections caused by bacteria having T4SS in dogs, rabbits, cats, pigs, cattle, sheep, goats, deer, horses, rodents and humans.



DESCRIPTION

VIRB10 FOR VACCINATION AGAINST GRAM NEGATIVE BACTERIA

CROSS-REFERENCE TO RELATED APPLICATION

This application claims the benefit of U.S. Provisional Application Serial No. 62/180,245, filed June 16, 2015, the disclosure of which is hereby incorporated by reference in its entirety, including all figures, tables and amino acid or nucleic acid sequences.

This invention was made with government support under U54AI1057156 awarded by National Institutes of Health. The government has certain rights in the invention.

The Sequence Listing for this application is labeled "Seq-List.txt" which was created on June 10, 2016 and is 343 KB. The entire content of the sequence listing are incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

Type 4 secretion system (T4SS) is used by many gram negative bacteria, including certain pathogenic bacteria, to secrete effector proteins and DNA across cell membranes. The bacteria belonging to the genus *Rickettsia* and *Anaplasma* provide examples of the pathogenic bacteria having T4SS. Rickettsial diseases are present worldwide and pose the threat of use in a biological weapon. Vaccines currently available against diseases mediated by the bacteria having T4SS are inadequate.

BRIEF SUMMARY OF THE INVENTION

T4SS is typically formed from a macromolecular complex of about 12 proteins. One of the proteins of T4SS is VirB10. The invention provides for the use of VirB10 to immunize against an infection by a bacterium having T4SS. Accordingly, the invention provides a vaccine comprising VirB10, a fragment of VirB10, a polynucleotide encoding VirB10 or a polynucleotide encoding a fragment of VirB10 and a pharmaceutically acceptable carrier and/or an adjuvant.

The invention also provides a method of immunizing a host against an infection caused by a bacterium having T4SS, the method comprising administering to the host a vaccine comprising or consisting of VirB10, a fragment of VirB10, a polynucleotide encoding VirB10 or a polynucleotide encoding a fragment of VirB10 and a pharmaceutically

acceptable carrier and/or an adjuvant. The vaccines and the methods of the invention can be used to prevent infections caused by bacteria having T4SS in various hosts, for example, dogs, rabbits, cats, pigs, cattle, sheep, goats, deer, horses, rodents and humans.

BRIEF DESCRIPTION OF THE DRAWINGS

The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication, with color drawing(s), will be provided by the Office upon request and payment of the necessary fee.

Figures 1A-1F. SDS-PAGE and Western Blot analysis of the recombinant proteins VirB9-1, VirB9-2 and VirB10. SDS-PAGE analysis of recombinant VirB9-1 (A), VirB9-2 (C), and VirB10 (E). Lanes represent the different fractions analyzed during the purification procedure, 1 = Total crude protein, 2 = Supernatant fraction obtained after high speed centrifugation, 3 = Filtered supernatant, 4 = Nickel Column filtrate, 5 = Washed fraction, 6 = Eluted protein, 7 = Concentrated protein. Western Blot analysis using the monoclonal anti-His₆-tag antibody reacting with the recombinant protein (red arrows) VirB9-1 (33.5KDa) (B), VirB9-2 (31.6KDa) (D) and VirB10 (52.2KDa) (F) in different fractions.

Figure 2. *A. phagocytophilum* burden in mouse blood (different strains infected with HZ). *A. phagocytophilum* growth kinetics in blood were measured by determining GE/ μ l based on the single copy gene *msp5*. GE/ μ l calculations were normalized based on the volume of blood collected per animal at each time point.

Figures 3A-3B. *A. phagocytophilum* antibody responses in immunized C3H/HeN mice. A) *A. phagocytophilum* specific antibody responses in immunized mice were measured by IFA and titers expressed as the reciprocal of the highest dilution at which specific fluorescence was detected (B). Binding of antibodies specific to *A. phagocytophilum* was seen as defined red fluorescent inclusions (morulae) in infected HL-60 cells; in contrast similar fluorescent inclusions were not visualized using antibodies from negative control group V. Picture taken at 1:160 dilution of all antisera.

Figure 4. *A. phagocytophilum* burden in immunized/challenged C3H/HeN mice blood. *A. phagocytophilum* growth kinetics in blood were measured by determining GE/ μ l based on the single copy gene *msp5*. GE/ μ l calculations were normalized based on the volume of blood collected per animal at each time point.

Figure 5. Protection against *A. phagocytophilum* induced by immunization. *A. phagocytophilum* GE means plus standard deviations (error bars) at the peak of infection. 7

mice from each group were used for this analysis. The values that are significantly different from the values for the control group are indicated by asterisks *, $p < 0.05$.

Figure 6. 3 mice from the groups immunized with either VirB10 or ovalbumin control were sacrificed prior to challenge. Their antibody response was assayed by ELISA using intact *A. phagocytophilum* organisms attached to the ELISA plate. The values represent the average of duplicate readings from the three mice in either the VirB10 or control group. The data show the response of VirB10-immunized mice to whole organisms on the plate and suggest epitopes of VirB10 are surface-exposed and available to bind antibodies.

Figure 7. CLUSTAL format alignment of VirB10s from multiple organisms by MAFFT (v7.220) (Amarginale_Virb, SEQ ID NO: 123; Aphagocytophilu, SEQ ID NO: 124; Echaffeenis_Vi, SEQ ID NO: 125; Eruminantium_Vi, SEQ ID NO: 126; Rtyphi_Virb10_C, SEQ ID NO: 127; RproWazekii_Vir, SEQ ID NO: 128; Rconorii_VirB10, SEQ ID NO: 129; Rrickettsii_Vir, SEQ ID NO: 130; Otsutsugamushi_, SEQ ID NO: 131; and Ecoli_VirB10_3J, SEQ ID NO: 132).

BRIEF DESCRIPTION OF THE SEQUENCES

SEQ ID NO: 1: Sequence of VirB10 from *A. phagocytophilum* strain HZ.

SEQ ID NO: 2: Sequence of VirB9-1 protein from *A. phagocytophilum* strain HZ.

SEQ ID NO: 3: Sequence of VirB9-2 protein from *A. phagocytophilum* strain HZ.

SEQ ID NO: 4: Forward primer for amplification of DNA encoding Vir9B-1 protein.

SEQ ID NO: 5: Reverse primer for amplification of DNA encoding Vir9B-1 protein.

SEQ ID NO: 6: Forward primer for amplification of DNA encoding Vir9B-2 protein.

SEQ ID NO: 7: Reverse primer for amplification of DNA encoding Vir9B-2 protein.

SEQ ID NO: 8: Forward primer for amplification of DNA encoding VirB10.

SEQ ID NO: 9: Reverse primer for amplification of DNA encoding VirB10.

SEQ ID NO: 10: Forward primer for amplification of *msp5* gene from *A. phagocytophilum* strain HZ for qPCR.

SEQ ID NO: 11: Reverse primer for amplification of *msp5* gene from *A. phagocytophilum* strain HZ for qPCR.

SEQ ID NO: 12: Sequence of the qPCR probe for *msp5* gene from *A. phagocytophilum* strain HZ.

SEQ ID NOs: 13 to 27: Sequences of antigenic peptides of VirB10.

SEQ ID NOs: 28-113: VirB10 sequences obtained from UniProt (web site: uniprot.org/uniprot).

SEQ ID NOs: 114-122: Sequences of PCR primers used to amplify VirB9-1, VirB9-2 and VirB10 and Taqman qPCR primers and probes used to quantify *A. phagocytophilum* load.

SEQ ID NOs: 123-132: Sequences of VirB10s from multiple organisms.

DETAILED DISCLOSURE OF THE INVENTION

Human granulocytic anaplasmosis (HGA) is a tick-borne disease caused by the etiologic agent *A. phagocytophilum*, a bacterium containing T4SS. HGA was designated as a nationally notifiable disease in the United States in 1998. HGA is described as a zoonosis since the pathogen infects humans as well as animals including dogs, cattle, sheep, deer, horses and rodents. Vaccines against HGA are not currently available and the currently available vaccines against other bacteria containing T4SS are not adequate. Thus, the subject application provides a component of T4SS as a vaccine against diseases caused by bacteria having T4SS, for example, *A. phagocytophilum* and methods of immunizing a host against an infection by a bacterium having T4SS. In some embodiments, outer membranes and fragments thereof that are obtained from bacteria having T4SS and which contain VirB10 are excluded as vaccine components in the methods of immunizing a host against infection by a bacterium having T4SS or as components of a vaccine.

In one embodiment, the invention provides a protein present in T4SS, for example, VirB10 or a fragment of VirB10, to immunize against an infection by a bacterium having T4SS, for example, *A. phagocytophilum*. In another embodiment, the invention provides a polynucleotide encoding VirB10 or a polynucleotide encoding a fragment of VirB10 to immunize against an infection by a bacterium having T4SS, for example, *A. phagocytophilum*. In particular embodiments, the VirB10 polypeptide comprises SEQ ID NO: 1.

Typically, T4SS is present in gram negative bacteria, such as *Rickettsia* spp., *Ehrlichia* spp., *Helicobacter* spp., *Legionella* spp., *Bartonella* spp., *Brucella* spp., and *Anaplasma* spp.

Non-limiting examples of the bacterial species containing endogenous T4SS include: *Rickettsia typhi*, *Rickettsia prowazekii*, *Rickettsia rickettsia*, *Rickettsia conorii*, *Ehrlichia chaffeensis*, *Helicobacter pylori*, *Legionella pneumophila*, *Bartonella species*, *Anaplasma marginale*, *Anaplasma phagocytophilum*, *Ehrlichia ruminantium*, *Brucella species* and *Ehrlichia canis*. Additional examples of bacterial species having T4SS and in which the invention can be practiced are well known to a person of ordinary skill in the art and such embodiments are within the purview of the invention. The subject invention can also provide protection against bacterial species that lack an endogenous T4SS system and which have been genetically manipulated to express T4SS.

In one embodiment, VirB10 of *Anaplasma phagocytophilum* or a fragment of VirB10 can be used to immunize a host against *A. phagocytophilum* infection. VirB10 in bacteria other than *A. phagocytophilum* can be identified based on sequence homology and such VirB10 or their fragments can be used in a vaccine to immunize against the infection by bacteria having T4SS. The following list provides non-limiting examples of UniProt entries for VirB10 proteins (each of which is hereby incorporated by reference in its entirety) in various bacteria having T4SS: A0A011QMA5 (SEQ ID NO: 28), A0A011TLZ5 (SEQ ID NO: 29), A0A021WBD5 (SEQ ID NO: 30), A0A021XC05 (SEQ ID NO: 31), A0A059FPP8 (SEQ ID NO: 32), A0A061Q3H1 (SEQ ID NO: 33), A0A063X2U5 (SEQ ID NO: 34), A0A068HFV0 (SEQ ID NO: 35), A0A069HMU4 (SEQ ID NO: 36), A0A070A750 (SEQ ID NO: 37), A0A071M5U1 (SEQ ID NO: 38), A0A073IY47 (SEQ ID NO: 39), A0A074MLS9 (SEQ ID NO: 40), A0A074TCY6 (SEQ ID NO: 41), A0A076G4V3 (SEQ ID NO: 42), A0A085AA72 (SEQ ID NO: 43), A0A085IIV0 (SEQ ID NO: 44), A0A090MT70 (SEQ ID NO: 45), A0A095CKP1 (SEQ ID NO: 46), A0A099QA58 (SEQ ID NO: 47), A0A0A0XLH7 (SEQ ID NO: 48), A0A0A1FHZ1 (SEQ ID NO: 49), A0A0A1IXK7 (SEQ ID NO: 50), A0A0A1PDK4 (SEQ ID NO: 51), A0A0A6W9W5 (SEQ ID NO: 52), A0A0B2BVJ6 (SEQ ID NO: 53), A0A0B2C1C1 (SEQ ID NO: 54), A0A0B5E2C6 (SEQ ID NO: 55), A0A0B7J1D9 (SEQ ID NO: 56), A0A0B7MV56 (SEQ ID NO: 57), A0A0C1ELG7 (SEQ ID NO: 58), A0A0C1MQK6 (SEQ ID NO: 59), A0A0D6GL13 (SEQ ID NO: 60), A0A0D6PK86 (SEQ ID NO: 61), A0A0D6QEJ3 (SEQ ID NO: 62), A1YBN8 (SEQ ID NO: 63), A3U3E8 (SEQ ID NO: 64), A3UHL6 (SEQ ID NO: 65), A3VIX9 (SEQ ID NO: 66), A3XDX0 (SEQ ID NO: 67), A5CFI6 (SEQ ID NO: 68), A7FCK5 (SEQ ID NO: 69), B2FJH5 (SEQ ID NO: 70), B4RHY3 (SEQ ID NO: 71), B6JK51 (SEQ ID NO: 72), B9JE62 (SEQ ID NO: 73), C3KFT1 (SEQ ID NO: 74), C6V5M2 (SEQ ID NO: 75), D3NTS2 (SEQ

ID NO: 76), D5T6H3 (SEQ ID NO: 77), E0SJI8 (SEQ ID NO: 78), E5Y6Z7 (SEQ ID NO: 79), F0J7S0 (SEQ ID NO: 80), F4GMM5 (SEQ ID NO: 81), F7XVQ3 (SEQ ID NO: 82), H2ERZ5 (SEQ ID NO: 83), H9AY00 (SEQ ID NO: 84), I0EPK5 (SEQ ID NO: 85), I4MQG7 (SEQ ID NO: 86), I7F101 (SEQ ID NO: 87), J0B7G3 (SEQ ID NO: 88), J1IY73 (SEQ ID NO: 89), J8TK55 (SEQ ID NO: 90), K9P1S4 (SEQ ID NO: 91), L7SYL6 (SEQ ID NO: 92), N1MFN1 (SEQ ID NO: 93), Q0FXR1 (SEQ ID NO: 94), Q1LN34 (SEQ ID NO: 95), Q2K2E3 (SEQ ID NO: 96), Q2YJ81 (SEQ ID NO: 97), Q52SK2 (SEQ ID NO: 98), Q5EPB9 (SEQ ID NO: 99), Q69BE6 (SEQ ID NO: 100), Q8RPM1 (SEQ ID NO: 101), Q9A5M5 (SEQ ID NO: 102), Q9AGG7 (SEQ ID NO: 103), S2WS02 (SEQ ID NO: 104), S5YJB5 (SEQ ID NO: 105), S9QA92 (SEQ ID NO: 106), T0HQB6 (SEQ ID NO: 107), T0QH67 (SEQ ID NO: 108), T1XMT8 (SEQ ID NO: 109), U1H6S8 (SEQ ID NO: 110), V8QMP0 (SEQ ID NO: 111), X6JZV3 (SEQ ID NO: 112) and X7EE79 (SEQ ID NO: 113). A person of ordinary skill in the art can identify VirB10 in additional bacteria having T4SS and such embodiments are within the purview of the invention.

Accordingly, an embodiment of the invention provides a vaccine comprising an immunologically effective amount of VirB10 or a fragment of VirB10 and a pharmaceutically acceptable carrier and/or an adjuvant. Another embodiment of the invention provides a vaccine comprising an immunologically effective amount of a polynucleotide encoding VirB10 or a polynucleotide encoding a fragment of VirB10 and a pharmaceutically acceptable carrier and/or an adjuvant. For the purposes of this invention the term “immunologically effective amount” refers to the amount of VirB10 or a fragment of the VirB10 which elicits immune response in a host so that the host is protected from future infection caused by the bacterium in which VirB10 is present naturally (endogenously) or a bacterium genetically modified to express VirB10.

The term “endogenous” (and grammatical variations thereof) or the phrase “the bacterium in which VirB10 is present naturally” indicates that a particular VirB10 is a part of the T4SS present in the bacterium as the bacterium exists in nature, *i.e.*, in the wild type bacterium.

In one embodiment VirB10, a fragment of VirB10, a polynucleotide encoding VirB10 or a polynucleotide encoding a fragment of VirB10 is labeled. The label can be designed for *in vivo* visualization of the protein, peptide or polynucleotide, for targeting the protein, peptide or polynucleotide to a specific tissue, organ or cell of the host, to increase the *in vivo*

stability of the protein, peptide or polynucleotide or to increase immunogenicity of the protein or peptide.

Non-limiting examples of a label designed to visualize VirB10, a fragment of VirB10, a polynucleotide encoding VirB10 or a polynucleotide encoding a fragment of VirB10 include a fluorescent label, an enzyme label and a chromophore label. Additional examples of labels designed to visualize VirB10, a fragment of VirB10, a polynucleotide encoding VirB10 or a polynucleotide encoding a fragment of VirB10 are well known to a person of ordinary skill in the art and such embodiments are within the purview of the invention.

A label designed to target VirB10, a fragment of VirB10, a polynucleotide encoding VirB10 or a polynucleotide encoding a fragment of VirB10 to a tissue, organ or cell includes antibodies or fragments of antibodies or other biomolecules which specifically bind to one or more surface biomolecules present on the target tissue, organ or cell. The label can be designed to target, for example, Fc receptors, C-type lectins, complement receptors, major histocompatibility proteins, or other receptors present on the surface of dendritic cells or antigen presenting cells. Additional examples of suitable target biomolecules and corresponding binding biomolecules are well known to a person of ordinary skill in the art and such embodiments are within the purview of the invention.

A label designed to increase the *in vivo* stability or immunogenicity of VirB10 or a fragment of VirB10 includes tripalmitoyl-S-glyceryl cysteine, polylysine core, carbohydrate, N-pyroglutamate, amide group on the C terminus, acetyl group, glycosyl group, lipid group, unnatural amino acids, peptidomimetics, peptide carriers and polyethylene glycol (PEG). Non-limiting examples of labels that increase the *in vivo* stability or immunogenicity of VirB10 or a fragment of VirB10 are discussed in Goodwin *et al.* The contents of Goodwin *et al.* are herein incorporated by reference in their entirety. Additional examples of labels which can increase the *in vivo* stability or immunogenicity of VirB10 or a fragment of VirB10 are well known to a person of ordinary skill in the art and such embodiments are within the purview of the invention.

In one embodiment, VirB10 or a fragment of VirB10 is modified to increase its *in vivo* stability. Non-limiting examples of modifications which increase the *in vivo* stability of VirB10 or a fragment of VirB10 include incorporation of non-natural amino acids, pseudo-peptide bonds and cyclization. Additional examples of modifications that increase the *in vivo* stability of VirB10 or a fragment of VirB10 are also described in Goodwin *et al.* and Gentilucci *et al.* The contents Goodwin *et al.* and Gentilucci *et al.* are herein incorporated by

reference in their entirety. Additional examples of modifications which can increase the *in vivo* stability of VirB10 or a fragment of VirB10 are well known to a person of ordinary skill in the art and such embodiments are within the purview of the invention.

An embodiment of the invention provides a vaccine comprising a fragment of VirB10 or a polynucleotide encoding a fragment of VirB10 and a pharmaceutically acceptable carrier and/or an adjuvant, wherein the fragment elicits an immune response in a host and the host is immune to a future infection caused by the bacterium in which the particular VirB10 is present naturally. The fragment of VirB10 used in the vaccines of the invention can comprise about 5 to about 50, about 10 to about 40, about 15 to about 30, about 20, about 10 or about 5 amino acids.

In one embodiment, the fragment of VirB10 is selected from the fragments provided in Table 1. In another embodiment, the VirB10 fragment or VirB10 polypeptide can be fused to a heterologous sequence, such as a carrier protein (e.g., bovine serum albumin, keyhole limpet hemocyanin, ovalbumin or other carrier protein used to stimulate an immune response to peptides).

Table 1. Examples of fragments of VirB10 protein used in the vaccines of the invention.

SEQ ID NO	Start Position in SEQ ID NO: 1	Sequence	End Position in SEQ ID NO: 1
13	16	DNVNVVGVAKSKKLFVIIIVLIATGLMYYFF	46
14	85	APRILTPPPRLPELPPLVMPTVPDIPVVTKLLKP	118
15	147	EISLPLPYK	155
16	214	QSPSVRA	220
17	227	RYIILQG	233
18	235	MIDAVLET	242
19	247	DISGVLRAVVS RDVYASSGDAVVIPKG	273
20	275	RLIGSYFF	282
21	289	VRVDVNWSRVILPHGV DIQIA	309
22	321	ISGVVDN	327
23	329	VGSILTSTIFLAGISLGTAYVTEQIPS	355
24	357	RTETVKVET	365
25	376	TSSSLSTKIVSD	387
26	407	TPTVYVDQ	414
27	416	TVMKV FVNQDVVFP	429

The vaccine of the invention can be formulated using adjuvants, emulsifiers, pharmaceutically-acceptable carriers or other ingredients routinely provided in a vaccine. Optimum formulations can be readily designed by one of ordinary skill in the art and can include formulations for immediate release and/or for sustained release, and for induction of systemic immunity and/or induction of localized mucosal immunity (*e.g.*, the formulation can be designed for intranasal, intravaginal or intrarectal administration).

Guidelines for designing optimal vaccines can be found in Brito *et al.*, Avanti and Li *et al.* The contents of Brito *et al.* are herein incorporated by reference in their entirety, particularly, page 132, Table 1; page 133 under immune potentiator adjuvants; page 133-136 under aluminum salt adjuvants; page 136-139 under emulsions; 139-140 under liposomes as adjuvants; page 140-141 under PLG particulate delivery systems; and page 141 under alternate particulate systems. The contents of Avanti *et al.* are also herein incorporated by reference in their entirety, particularly Chapters 1, 2 and 7. Further, the contents of Li *et al.* are herein incorporated by reference in their entirety, particularly pages 521-527 under Particulate Peptide Vaccine Delivery Methods.

The vaccine disclosed herein can be formed with a pharmaceutically acceptable carrier such as a phosphate buffered saline, a bicarbonate solution, or an adjuvant to produce a pharmaceutical composition. The carrier must be “acceptable” in the sense that it is compatible with the active ingredient of the composition, and preferably capable of stabilizing the active ingredient and not deleterious to the subject to be treated. The carrier is selected on the basis of the mode and route of administration and standard pharmaceutical practice. Suitable pharmaceutical carriers and diluents, as well as pharmaceutical necessities for their use, are described in Remington’s Pharmaceutical Sciences.

In one embodiment, the antigen is mixed with an adjuvant to form a composition useful for immune modulation. This composition may be prepared as injectable, as liquid solutions or as emulsions. See U.S. Pat. Nos. 4,601,903; 4,599,231; 4,599,230; and 4,596,792. An “adjuvant” refers to a substance added to an immunogenic composition, such as a vaccine, that, while not having any specific antigenic effect in itself, can stimulate the immune system and increase the immune response to the immunogenic composition. Examples of adjuvants include, but are not limited to, alum, alum-precipitate, Freund’s complete adjuvant, Freund’s incomplete adjuvant, monophosphoryl-lipid A/trehalose dicorynomycolate adjuvant and water in oil emulsions.

A further embodiment of the invention provides a method of immunizing a host against an infection by a bacterium having T4SS, the method comprising administering to the host a vaccine comprising a pharmaceutically effective amount of VirB10, a fragment of VirB10, a polynucleotide encoding VirB10 or a polynucleotide encoding the fragment of VirB10 and a pharmaceutically acceptable carrier and/or an adjuvant.

The method of the invention can be used to immunize a host, for example, a mammal, against an infection by a bacterium having the T4SS. Non-limiting examples of mammals in which the methods of the invention can be practiced include dogs, cats, pigs, cattle, rabbits, sheep, goats, deer, horses, rodents and humans. Additional examples of hosts in which the methods of the invention can be practiced are well known to a person of ordinary skill in the art and such embodiments are within the purview of the invention.

In one embodiment, the invention provides a method of immunizing a host against an infection by a bacterium containing T4SS, wherein the method comprises administering to the host a vaccine comprising VirB10, a fragment of VirB10, a polynucleotide encoding VirB10 or a polynucleotide encoding the fragment of VirB10 and a pharmaceutically acceptable carrier and/or an adjuvant, and wherein the bacterium is a member of *Rickettsia* spp., *Ehrlichia* spp., *Helicobacter* spp., *Legionella* spp., *Bartonella* spp., *Brucella* spp., and/or *Anaplasma* spp. In certain embodiments, the bacterium is *Rickettsia prowazekii*, *Rickettsia rickettsia*, *Rickettsia conorii*, *Ehrlichia chaffeensis*, *Helicobacter pylori*, *Legionella pneumophila*, a *Bartonella* species, a *Brucella* species (e.g., *Brucella abortus*), *Anaplasma marginale*, *Anaplasma phagocytophilum*, *Ehrlichia ruminantium* or *Ehrlichia canis*.

The vaccine of the invention can be administered by any convenient route including subcutaneous, intradermal, intranasal, oral, intramuscular, intraperitoneal, or other parenteral or enteral route. A person of ordinary skill in the art can identify a particular route of administration suitable for a particular host and a given bacterium and such embodiments are within the purview of the invention.

VirB10, a fragment of VirB10, a polynucleotide encoding VirB10 or a polynucleotide encoding a fragment of VirB10 can be administered as a single dose or multiple doses. Optimum immunization schedules can be readily determined by the ordinarily skilled artisan and can vary with parameters, for example, age, weight and species of the host, the type of vaccine composition and the bacterium against which immunization is desired and such embodiments are within the purview of the invention.

An embodiment of the invention provides a method of immunizing a host against an infection by a bacterium having T4SS, wherein the immunization is performed according to prime boost immunization. For the purpose of this invention, the phrase “prime boost immunization” indicates that the VirB10, a fragment of VirB10, a polynucleotide encoding VirB10 or a polynucleotide encoding a fragment of VirB10 is administered in a plurality of doses. As such, the immune system of a host encounters multiple exposures to VirB10, a fragment of VirB10, a polynucleotide encoding VirB10 or a polynucleotide encoding a fragment of VirB10, which results in a stronger immune response compared to single administration of the vaccine. Certain examples of prime boost immunizations are discussed in Woodland *et al.*, the contents of which are herein incorporated by reference in their entirety.

In one embodiment, the prime boost immunization comprises administering a pharmaceutically effective amount of a polynucleotide encoding VirB10 or a polynucleotide encoding a fragment of VirB10 followed by administering a pharmaceutically effective amount of VirB10 or a fragment of VirB10. In another embodiment, the prime boost immunization comprises administering a pharmaceutically effective amount of VirB10 or a fragment of VirB10 followed by administering a polynucleotide encoding VirB10 or a polynucleotide encoding a fragment of VirB10.

In certain embodiments, the interval between the administration of the polynucleotide and the protein or a fragment of the protein is about 1 week to about 4 weeks, about 2 weeks to 3 weeks, 2 to 4 weeks, or about 2 weeks. In another embodiment of the invention, the vaccine is administered in the form of a “cocktail” that contains at least two polynucleotides or at least two peptides. The cocktail can also contain a mixture of a polynucleotide and a peptide.

All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety, including all figures and tables, to the extent they are not inconsistent with the explicit teachings of this specification.

Following are examples which illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

EXAMPLE 1 – PREPARATION OF SOLUBLE RECOMBINANT VIRB9-1, VIRB9-2 AND VIRB10S

The open reading frames of *A. phagocytophilum* virB9-1, virB9-2 and virB10 genes were amplified by PCR (Table 2) and the purified products cloned into a DNA vaccine vector and the pET101/D-TOPO directional expression system. Recombinant constructs were purified and sequences were confirmed by PCR and DNA sequencing. The pET101 constructs were re-transformed into *E. coli* BL21 Star (DE3) cells and induced to express with 0.5 mM IPTG and growth in M9 minimal media supplemented with 50 µg/ml carbenicillin and 1% glucose. Expression of recombinant VirB9-1, Vir9-2 and VirB10 was verified by SDS-PAGE followed by Western blot analyses using His₆-tagged monoclonal antibody (Fig. 1). 500 ml of IPTG-induced cultures were processed and soluble fractions containing the recombinant His₆-tagged fusion proteins were purified using low-density Nickel agarose bead columns. Eluted protein was concentrated using Centricon Plus-70 centrifugal filter units, and concentration determined using a Qubit protein assay kit. The recombinant proteins were run on a SDS-PAGE gel to confirm purity.

For preparation of the DNA vaccine, the VirB9-1, VirB9-2, and VirB10 were separately amplified by PCR and cloned into the vector pcDNA3.1/CT-GFP-TOPO (Invitrogen). This vector provides the CMV (cytomegalovirus) promoter 5' to the inserted gene for constitutive expression in mammalian cells and the GFP (Green Fluorescent Protein) gene 3' to the inserted gene. The validity of the constructs was checked prior to use in immunizations by sequencing the gene insert and junction regions and by transfecting RF6A endothelial cells with isolated plasmid DNA to verify GFP expression by fluorescence. For preparation of immunizing plasmid DNA, bacteria were grown in LB/Ampicillin 100 µg/ml. The ZR Plasmid Gigaprep kit (Zymo Research) was used to isolate endotoxin-free plasmid DNA from the bacterial pellets. The yields of DNA obtained were: TOPO-GFP (control, no inserted gene): 6 liters of culture gave ~6.5mg; VirB 9-1 plasmid: 6 liters of culture gave ~ 29mg; VirB 9-2 plasmid: 6 liters of culture gave ~19mg; and VirB10 plasmid: 4 liters of culture gave ~23mg.

The culture and purification conditions described above were optimized to produce adequate yields of soluble recombinant VirB9-1, VirB9-2 and VirB10. For protein expression the temperature was reduced to 4°C to avoid protein aggregation and reduction of heat shock proteases that could promote inclusion body formation, cellular toxicity and high levels of protein degradation. Additionally, modification of nutrient media was employed to

avoid excess bacterial growth and depletion of substrates and cofactors. This resulted in the isolation of soluble recombinant VirB9-1, VirB9-2 and VirB10s (Fig. 1) suitable for use in the immunization experiments.

5 Table 2. PCR primers used to amplify VirB9-1, VirB9-2 and VirB10 and Taqman qPCR primers and probes used to quantify *A. phagocytophilum* load.

PCR		Target	Size
AB1703	CACCATGAGCACAAATATTGGCGTACCAG (SEQ ID NO: 114)	virB9-1	769bp
AB1704	ACTAAGAGCCTGATTCACTTCTACACTCCTGC (SEQ ID NO: 115)		
AB1705	CACCATGGCTGATGATCACATTAAGACCTTGAAC (SEQ ID NO: 116)	virB9-2	736bp
AB1706	TTTCCGGCGTCTTTCAGCACCTTC (SEQ ID NO: 117)		
AB1707	CACCATGGCTGACGAAATAAGGGGTTCTAG (SEQ ID NO: 118)	virB10	1306bp
AB1708	CCTCACCGCATCACGAGGAAATACTACG (SEQ ID NO: 119)		
qPCR			
AB1334	AGATGCTGACTGGGGATGAG (SEQ ID NO: 120)	msp5	125bp
AB1335	TCGGCATCAACCAAGTACAA (SEQ ID NO: 121)		
*AB1336	CGTAGGTGAGTCTGATAGTGAAGG (SEQ ID NO: 122)		
* Oligonucleotide labeled with Hexachloro-fluorescein (HEX) at the 5' end and Tetramethylrhodamine TAMRA at the 3' end			

EXAMPLE 2 – *A. PHAGOCYTOPHILUM* INFECTION KINETICS IN DIFFERENT MOUSE STRAINS

10 Three different mouse strains (C57BL/6, C3H/HeN, and Balb/C) were tested to evaluate *A. phagocytophilum* infection kinetics and to select an appropriate mouse strain for *A. phagocytophilum* infection for immunization and challenge experiments. Two C57BL/6, two C3H/HeN and two Balb/C mice were inoculated with isolated organisms from 5.22×10^5 infected HL-60 cells (> 90% infection). DNA extracted from blood collected every other day

15 over 24 days was used to determine the *A. phagocytophilum* load by measuring the increase in the number of genome equivalents (GE) using qPCR with primers and probes that target the single copy gene *msp5* (Table 2). Ten-fold serial dilutions of the NY18E2/pCR-TOPO

plasmid carrying the *msp5* gene were used for standard curve preparation, and the *msp5* gene copy numbers were calculated based on the standard curve.

Results showed that C3H/HeN and Balb/C mice are susceptible to *A. phagocytophilum* (human HZ strain) infection as shown by an increase of GE in blood at 8 days post-infection for C3H/HeN mice (average of 705 GE/ μ l of blood) and between 6 and 8 days post-infection in Balb/C mice (average of 636 GE/ μ l of blood) (Fig. 2). In contrast, in C57BL/6 mice, only mouse # 2 presented a minor increase of up to 150 GE/ μ l of blood at day 6 post-infection indicating that this strain better controlled *A. phagocytophilum* infection. During the course of infection, a relapse peak of infection was detected after 16 days post-infection in both C3H/HeN and Balb/C strains suggesting a possible chronic infection. C3H/HeN strain was selected to determine mouse response to immunization with DNA vaccine followed by a recombinant protein vaccine encoding VirB9-1, VirB9-2 and VirB10 because the results presented here support previous work which indicate that C3H/HeN is a good model for *A. phagocytophilum* infection.

Immunization and challenge of C3H/HeN

5 groups of 10 mice were vaccinated in a prime boost fashion with plasmid DNA immunization followed by recombinant proteins as described in Table 3.

Table 3. C3H/HeN mice immunization schedule.

Group	Immunogen	Dosage	Amount	
I	<i>virB9-1</i> plasmid	2	100 μ g	2 week intervals
	<i>rVirB9-1</i> protein	2	100 μ g	2 week intervals
II	<i>virB9-2</i> plasmid	2	100 μ g	2 week intervals
	<i>rVirB9-2</i> protein	2	100 μ g	2 week intervals
III	<i>virB10</i> plasmid	2	100 μ g	2 week intervals
	<i>rVirB102</i> protein	2	100 μ g	2 week intervals
	<i>virB9-1</i> plasmid, <i>virB9-2</i> plasmid, <i>virB10</i> plasmid	2	100 μ g/plasmid	2 week intervals
IV	<i>rVirB9-1</i> , <i>rVirB9-2</i> , <i>rVirB10</i>	2	100 μ g/protein	2 week intervals
	<i>Empty TOPO-GFP</i>	2	100 μ g	2 week intervals
V	Ovalbumin	2	100 μ g	2 week intervals

Two weeks after the last immunization, three mice were randomly selected for serum collection and for isolation of spleen and lymph nodes from each group. Preliminary serological work was performed using immunofluorescence assay (IFA) to determine how the immunized mice reacted to whole *A. phagocytophilum* organisms and to measure antibody titers. Antigen slides were prepared from *A. phagocytophilum*/HZ infected HL-60

cells. Sera from the three mice in each group were combined and serially diluted starting from 1:80 up to 1:81920. Ten microliters of serum was applied to each well with duplicates for each dilution and incubated for 1 hour at room temperature in a humidified chamber. After incubation the serum was removed and the slides washed five times for 5 minutes. Ten
 5 microliters of Alexa fluor 568-Goat anti-mouse IgG antibody at a dilution of 1:1600 was applied to each well and incubated for 1 hour at room temperature. The slides then were washed as described above and mounted with ProLong Gold antifade reagent with DAPI (4',6-diamidino-2-phenylindole) (Fig. 3). IFA analysis showed that higher titers of antibodies against *A. phagocytophilum* were detected in the serum of vaccinated animals (Groups I
 10 through IV), compared to negative control mice (Group V) vaccinated with *Empty TOPO-GFP/Ovalbumin* (Fig. 3).

Protection against *A. phagocytophilum* induced by *virB9*/VirB9, *virB9-2*/VirB9-2, and *virB10*/VirB10 was evaluated. 35 immunized mice (7 mice/group) were challenged with one dose of isolated organisms from 5.63×10^5 HL-60 infected cells (> 90% infected) and the
 15 bacterial burden in the blood of each mouse was measured by real time qPCR targeting the single copy gene *msp5* to determine the number of *A. phagocytophilum* GE as described above. On day 8, the bacterial load in the blood ranged from 451 GE/ μ l up to 1267 GE/ μ l in the mice in Group I, from 358 GE/ μ l up to 2188 GE/ μ l in Group II, from 396 GE/ μ l up to 980 GE/ μ l in Group III, from 118 GE/ μ l up to 2225 GE/ μ l in Group IV and from 607 GE/ μ l
 20 up to 2988 GE/ μ l in negative control Group V (Fig. 4). However real time qPCR did not detect any *A. phagocytophilum* GE in one mouse from Group I, or in two mice from Group III. Although the bacterial load in Groups I, II and IV was lower than in Group V, these differences were not significant. In contrast, there was a significant difference between the bacterial load in Group III when compared with Group V ($p = 0.032$).

25 These results indicate that immunized mice (pre-challenge) with VirB9, VirB9-2, VirB10 and the mixture of VirB9-1, VirB9-2 and VirB10 developed antibodies that reacted with *A. phagocytophilum*. However real-time qPCR of DNA extracted from the blood of the challenged mice showed that the bacterial load was significantly lower only in Group III when compared to the negative control mice in Group V. Such an result was not expected in
 30 view of the antibody titers observed in the Group II and Group IV mice and in view of the Group IV animals that were immunized (primed and boosted) with a mixture of VirB9-1, VirB9-2 and VirB10 (see Table 3 and Figure 3).

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims. In addition, any elements or limitations of any invention or embodiment thereof disclosed herein can be combined with any and/or all other elements or limitations (individually or in any combination) or any other invention or embodiment thereof disclosed herein, and all such combinations are contemplated within the scope of the invention without limitation thereto.

5

REFERENCES

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4. Woodland *et al.* (2004), Jump-starting the immune system: prime-boosting comes of age, *TRENDS in Immunology*, 25(2):98-104.
- 15 5. Goodwin *et al.* (2009), Peptides as therapeutics with enhanced bioactivity, *Current Medicinal Chemistry*, 19:4451-4461.
6. Yang *et al.* (2009), An introduction to epitope prediction methods and software, *Reviews in Medical Virology*, 19: 77-96.
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CLAIMS

We claim:

1. A vaccine comprising an immunologically effective amount of VirB10 or a fragment of VirB10 and a pharmaceutically acceptable carrier and/or an adjuvant.
2. The vaccine of claim 1, wherein VirB10 or the fragment of VirB10 is conjugated to a carrier protein.
3. The vaccine of claim 2, wherein the carrier protein is keyhole limpet hemocyanin, ovalbumin or bovine serum albumin.
4. The vaccine of claim 1, wherein VirB10 or the fragment of VirB10 is cyclized or comprises SEQ ID NO: 1.
5. The vaccine of claims 1-4, wherein the fragment of VirB10 comprises about 5 to about 50, about 10 to about 40, about 15 to about 30, about 20, about 10 or about 5 amino acids.
6. The vaccine of claim 1, wherein the fragment of VirB10 is selected from SEQ ID NO: 13 to 27.
7. A vaccine comprising an immunologically effective amount of a polynucleotide encoding VirB10 or a polynucleotide encoding a fragment of VirB10 and a pharmaceutically acceptable carrier and/or an adjuvant, said polynucleotide being operably linked to a promoter in a vector.
8. A method of immunizing a host against an infection by a bacterium having Type 4 secretory system (T4SS), the method comprising administering to the host the vaccine of any of claims 1-4, 6 or 7.
9. The method of claim 8, wherein the host is a mammal.

10. The method of claim 9, wherein the mammal is a dog, cat, pig, bovine, rabbit, sheep, goat, deer, horse, rodent or human.

11. The method of claims 9-10, wherein the bacterium having T4SS is a *Rickettsia* spp., *Ehrlichia* spp., *Helicobacter* spp., *Legionella* spp., *Bartonella* spp., *Brucella* spp. or *Anaplasma* spp.

12. The method of claim 11, wherein the bacterium is *Rickettsia typhi*, *Rickettsia prowazekii*, *Rickettsia rickettsia*, *Rickettsia conorii*, *Ehrlichia chaffeensis*, *Helicobacter pylori*, *Legionella pneumophila*, *Bartonella species*, *Brucella abortus*, *Anaplasma marginale*, *Anaplasma phagocytophilum*, *Ehrlichia ruminantium* or *Ehrlichia canis*.

13. The method of claims 8-12 or 16-18, wherein the vaccine is administered via a subcutaneous, intradermal, intranasal, oral, intramuscular or intraperitoneal route.

14. The method of claims 8-13 or 16-18, wherein the vaccine is administered in a single dose or multiple doses.

15. The method of claims 8-14 or 16-18, wherein the vaccine is administered according to prime boost immunization.

16. A method of immunizing a host against an infection by a bacterium having Type 4 secretory system (T4SS), the method comprising administering to the host the vaccine of claim 5.

17. The method of claim 8, wherein the bacterium having T4SS is a *Rickettsia* spp., *Ehrlichia* spp., *Helicobacter* spp., *Legionella* spp., *Bartonella* spp., *Brucella* spp. or *Anaplasma* spp.

18. The method of claim 17, wherein the bacterium is *Rickettsia typhi*, *Rickettsia prowazekii*, *Rickettsia rickettsia*, *Rickettsia conorii*, *Ehrlichia chaffeensis*, *Helicobacter pylori*, *Legionella pneumophila*, *Bartonella species*, *Brucella abortus*, *Anaplasma marginale*, *Anaplasma phagocytophilum*, *Ehrlichia ruminantium* or *Ehrlichia canis*.

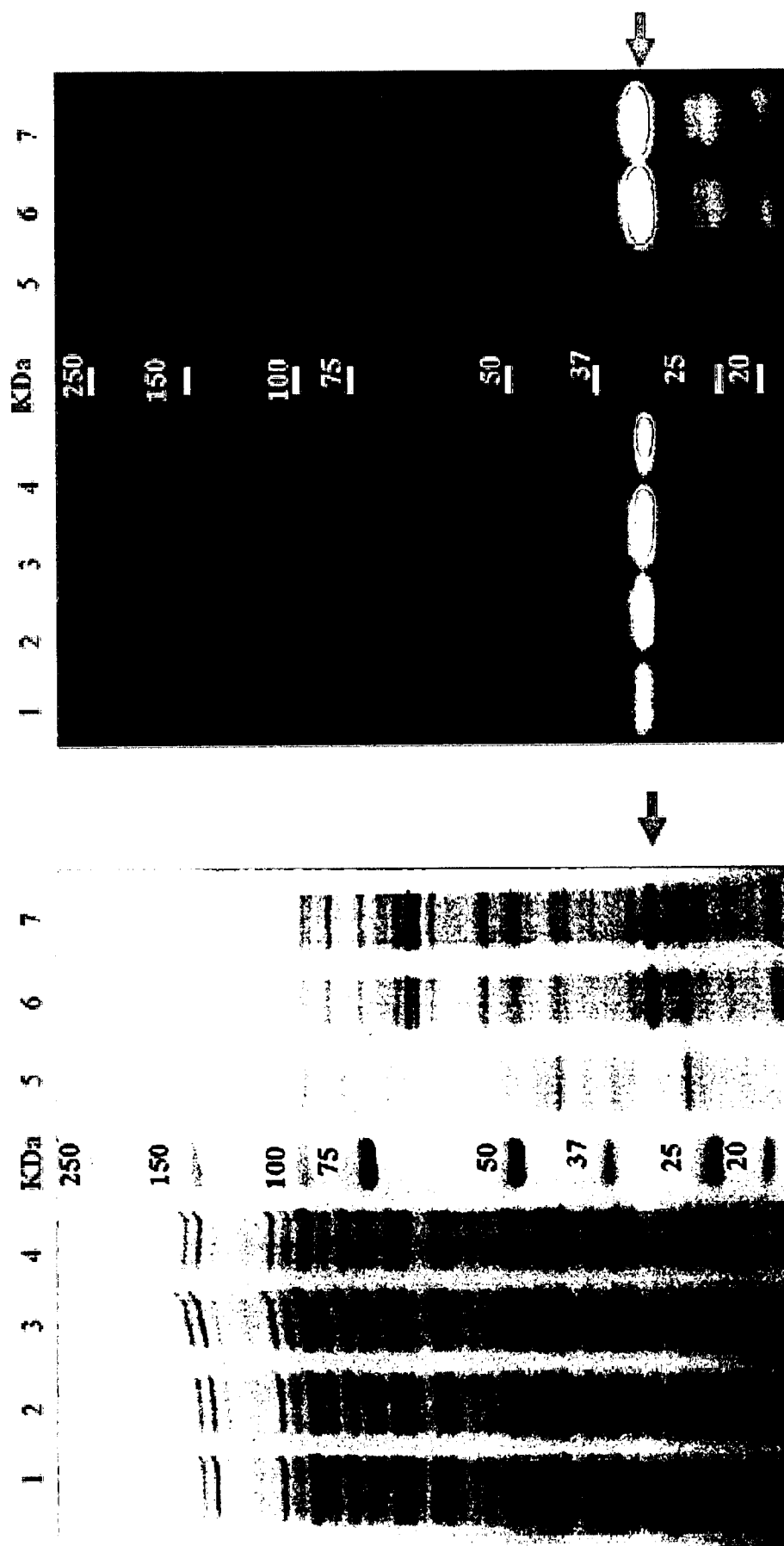
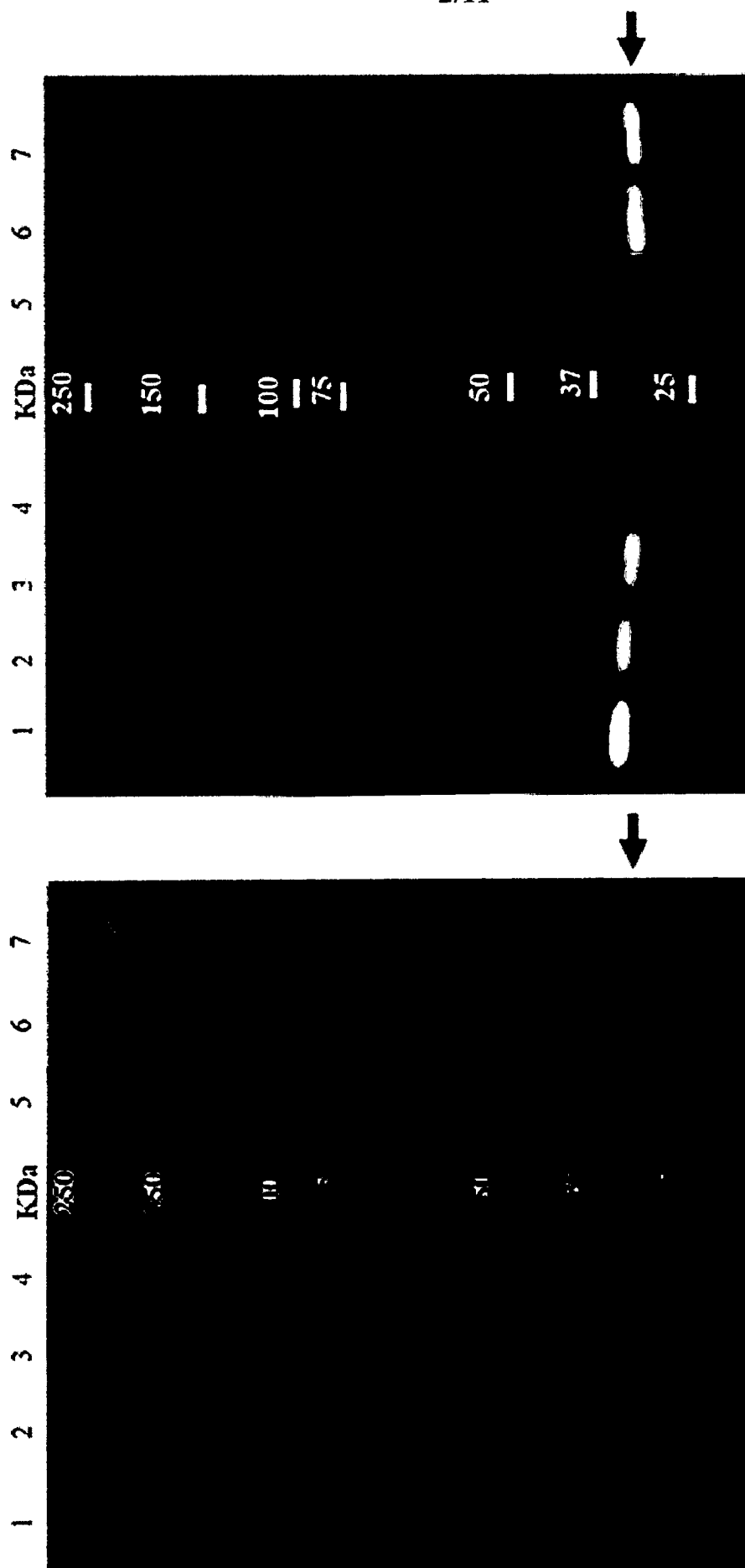


Figure 1B

Recombinant VirB9-1

Figure 1A

2/11



Recombinant VirB9-2

Figure 1D

Figure 1C

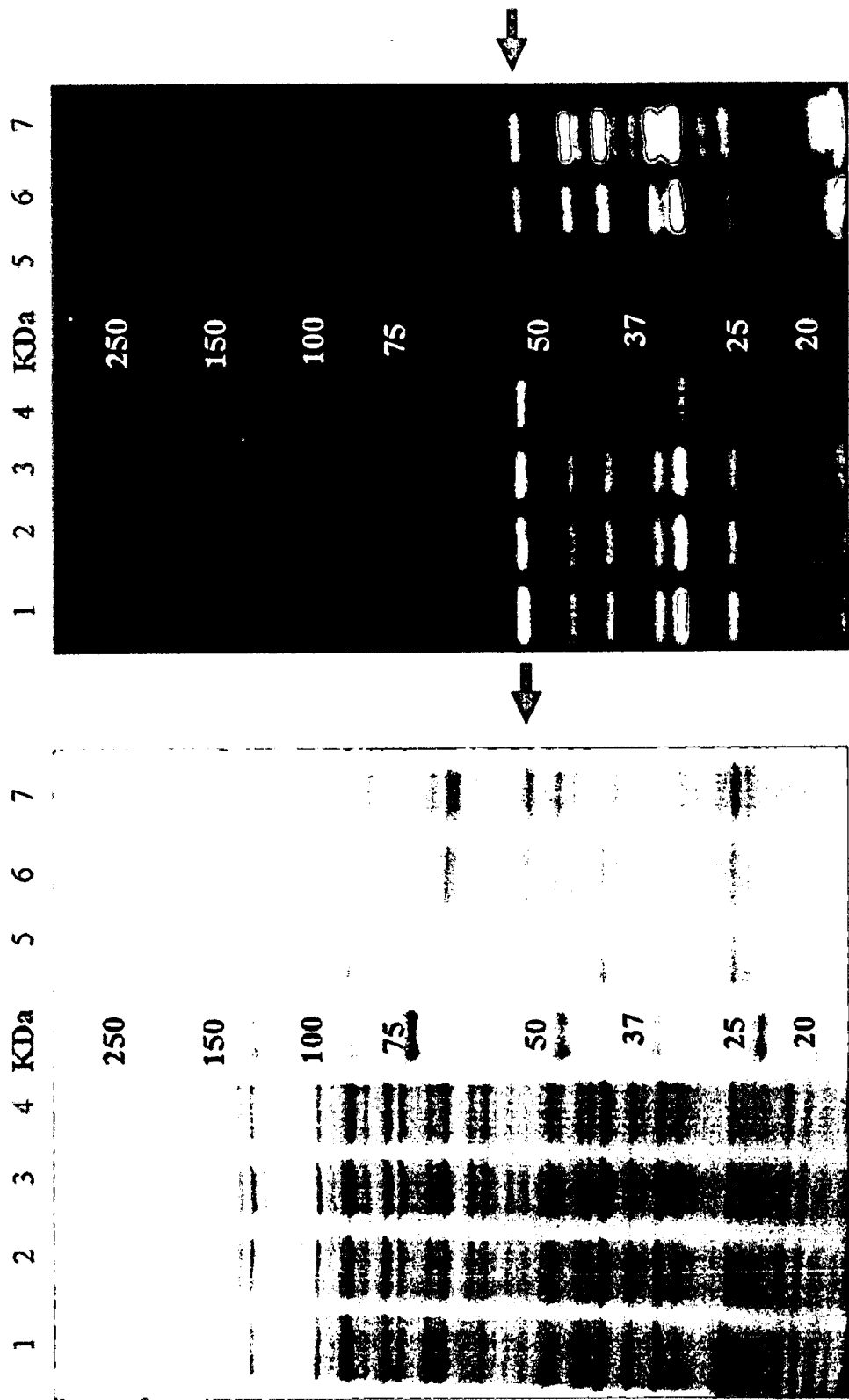


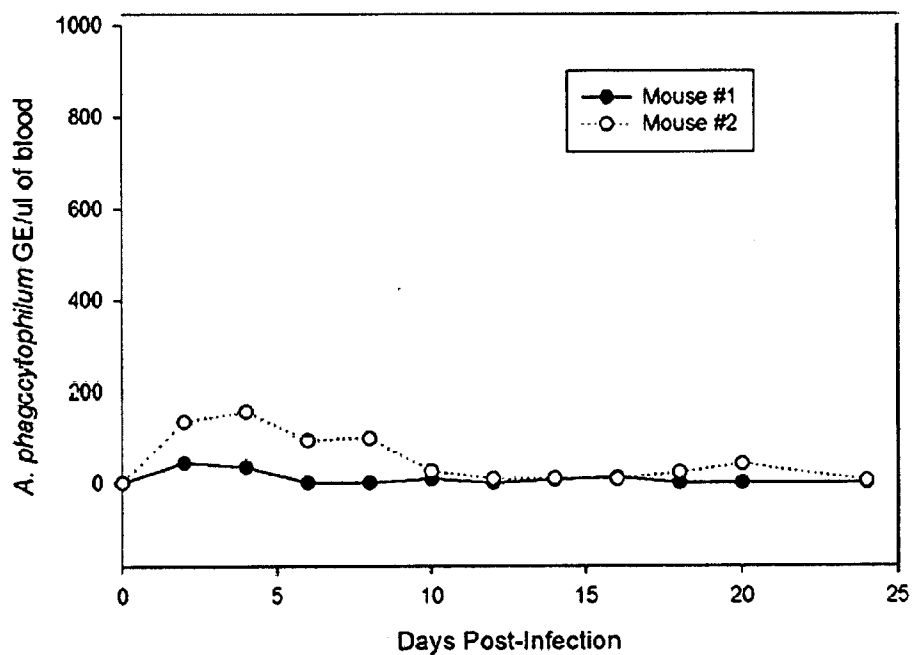
Figure 1F

Recombinant VirB10

Figure 1E

4/11

C57BL/6 Mice



C3H/HeN Mice

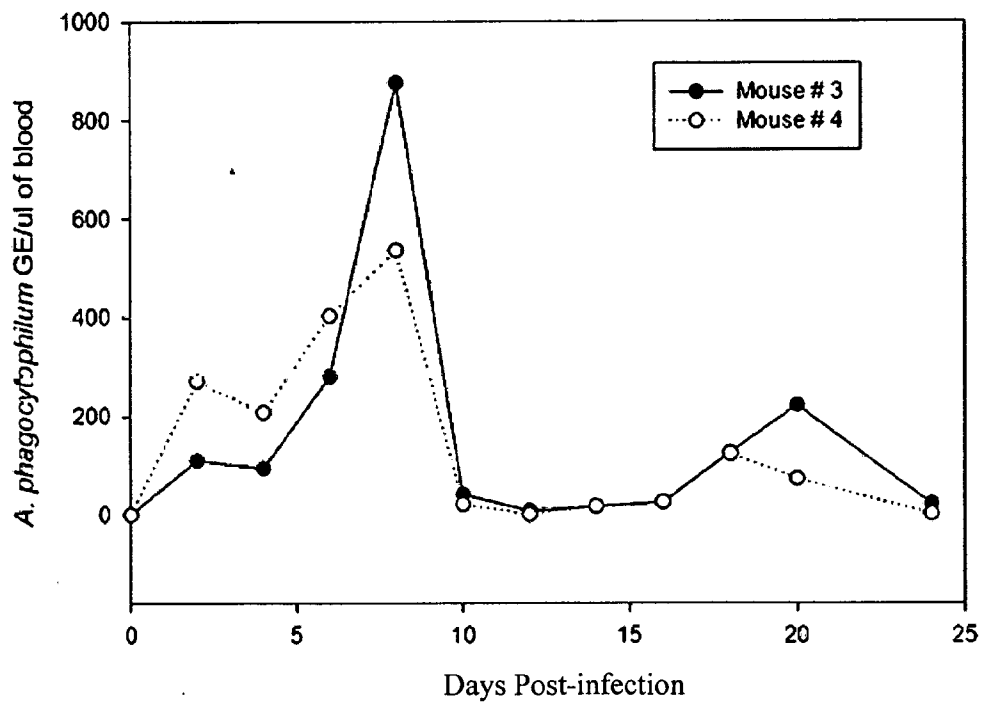


Figure 2

5/11

Balb/C Mice

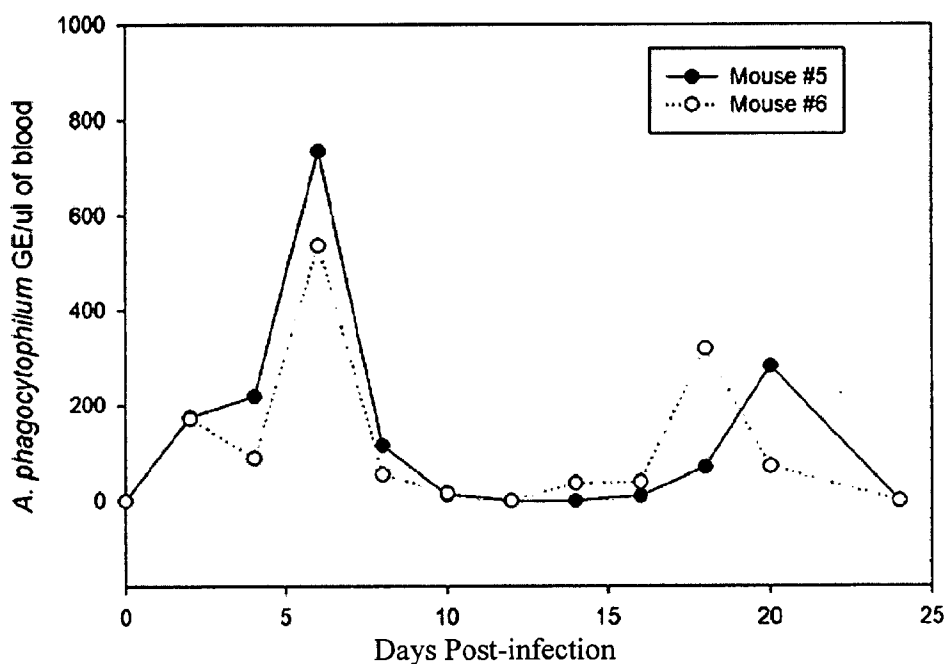


Figure 2 (continued)

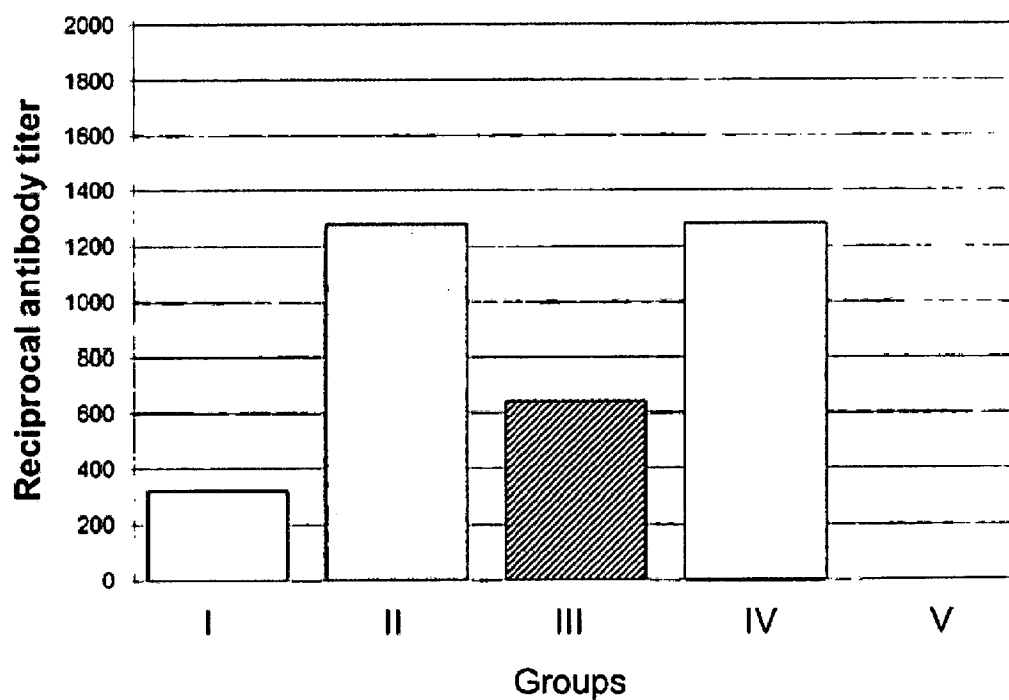
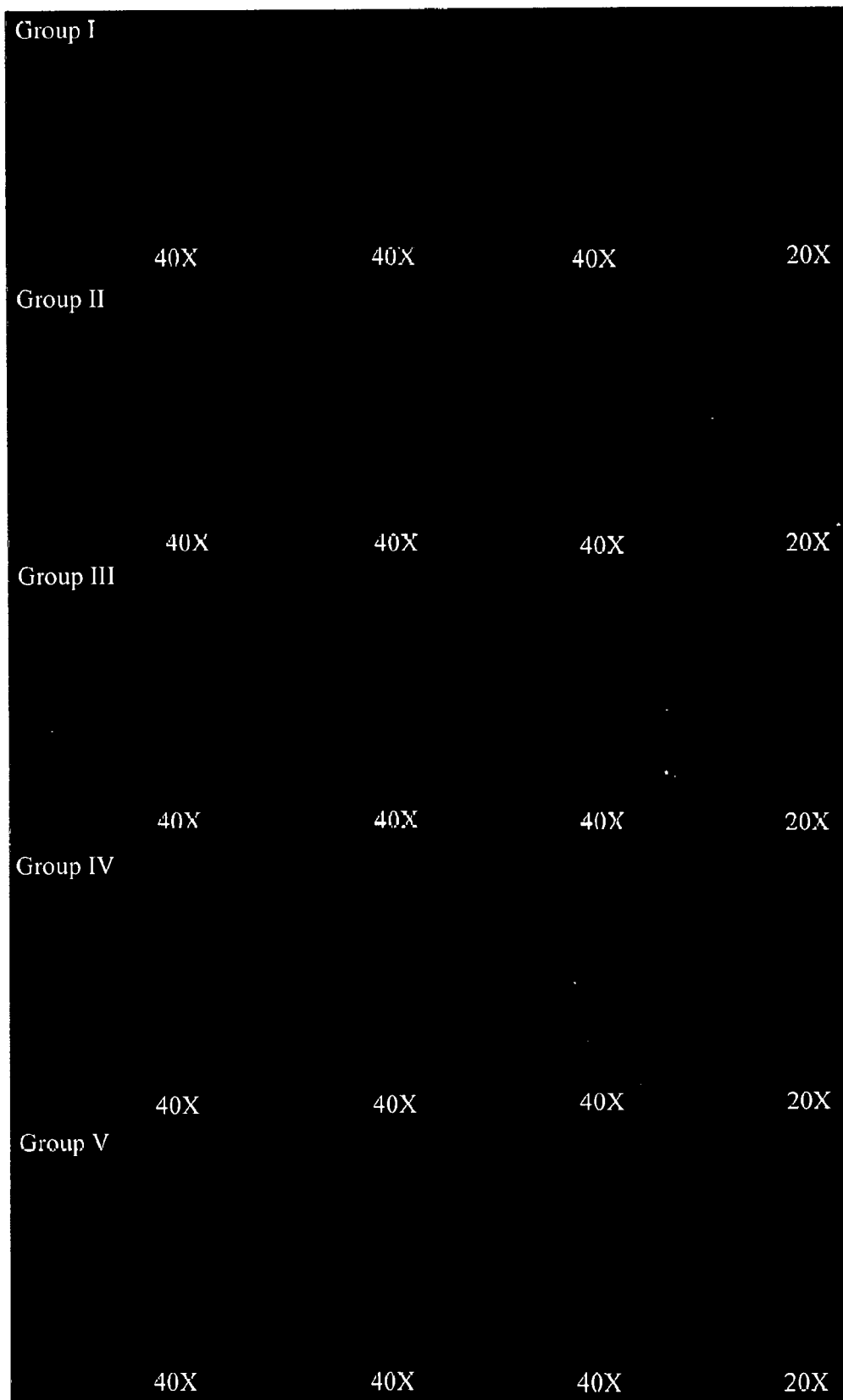


Figure 3A

6/11

**Figure 3B**

7/11

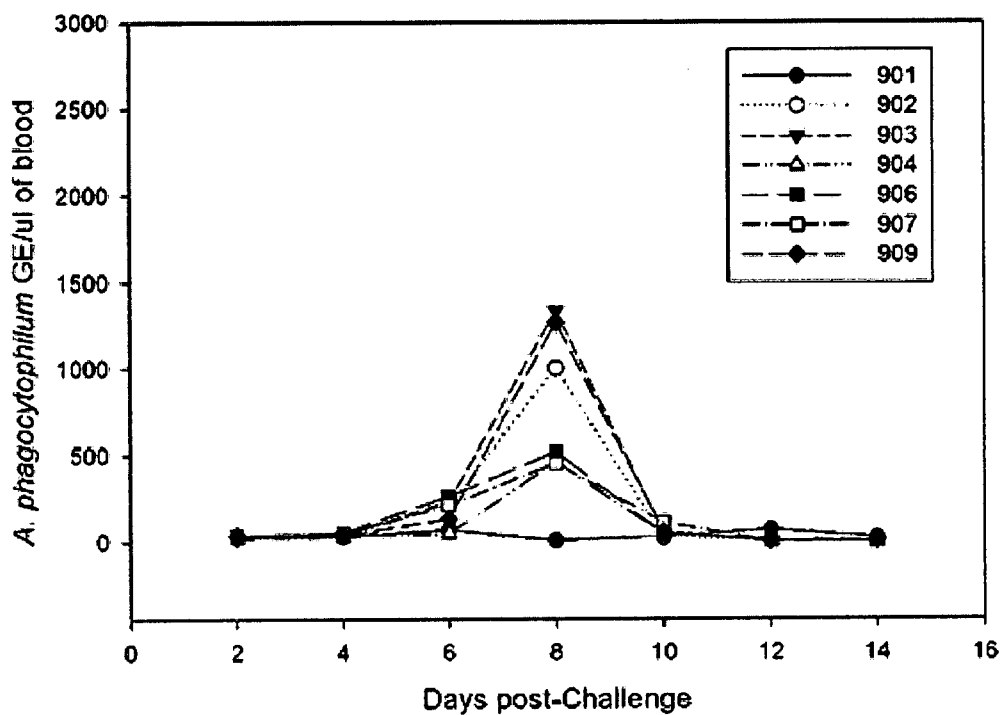
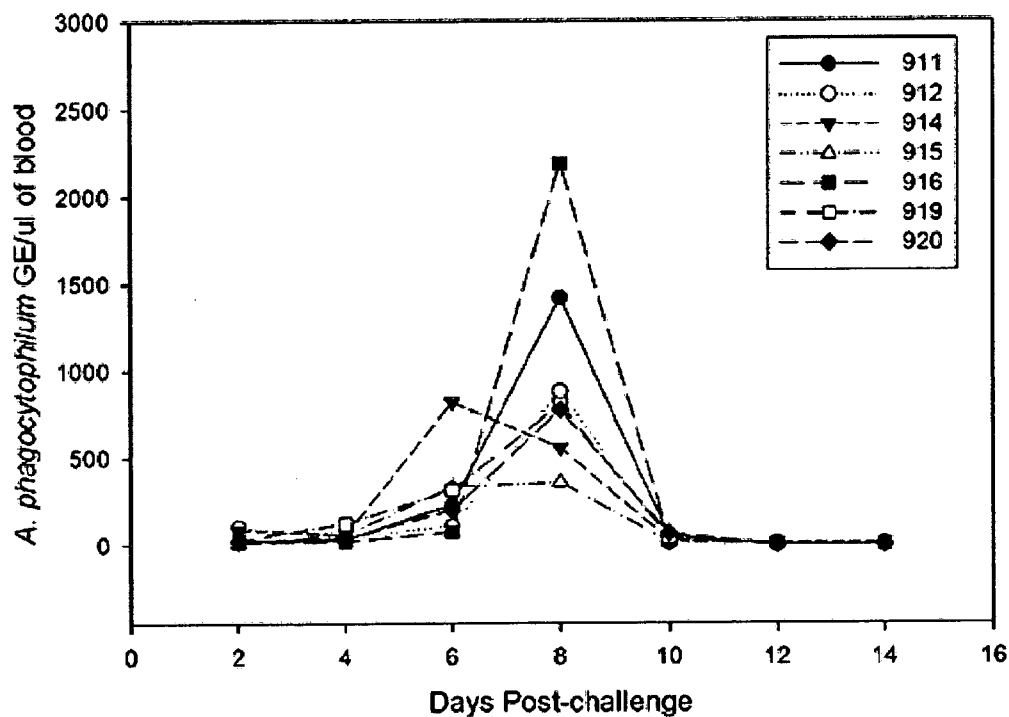
Group #1 (*pvirB9-1/rVirB9-1*)Group #2 (*pvirB9-2/rVirB9-2*)

Figure 4

8/11

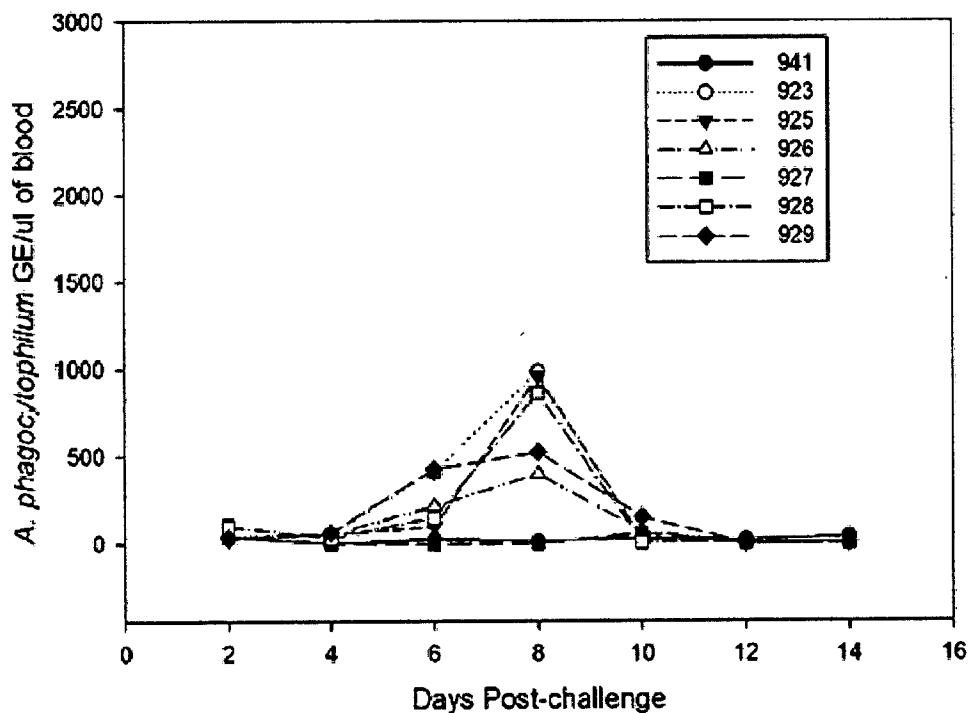
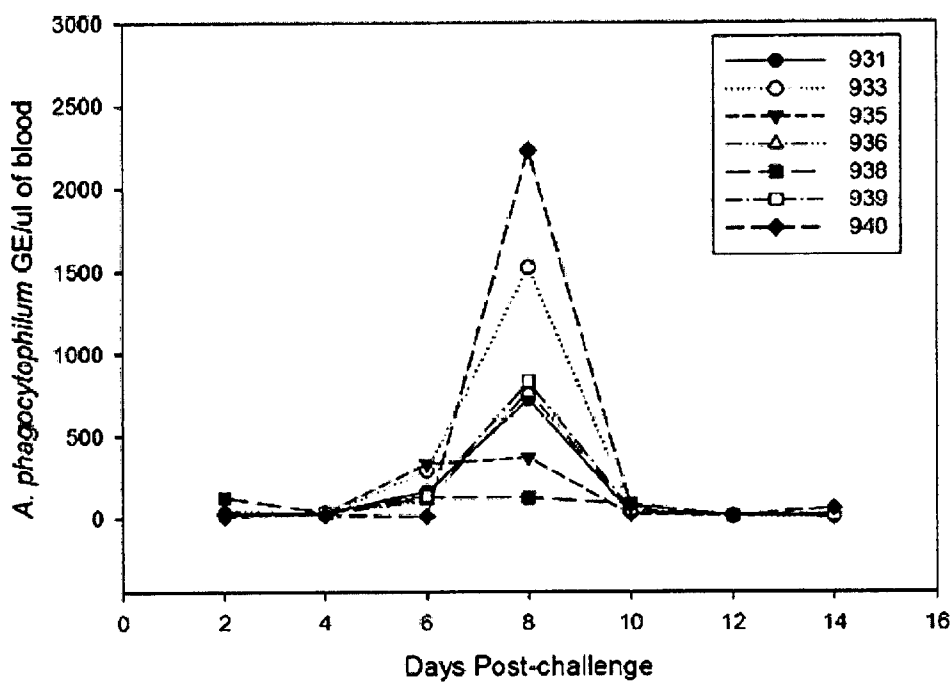
Group #3 (*pvirB10/rVirB10*)Group #4 (*pvirB9-1, pvirB9-2, pvirB10/rVirB9-1, rVirB9-2, rVirB10*)

Figure 4 (continued)

9/11

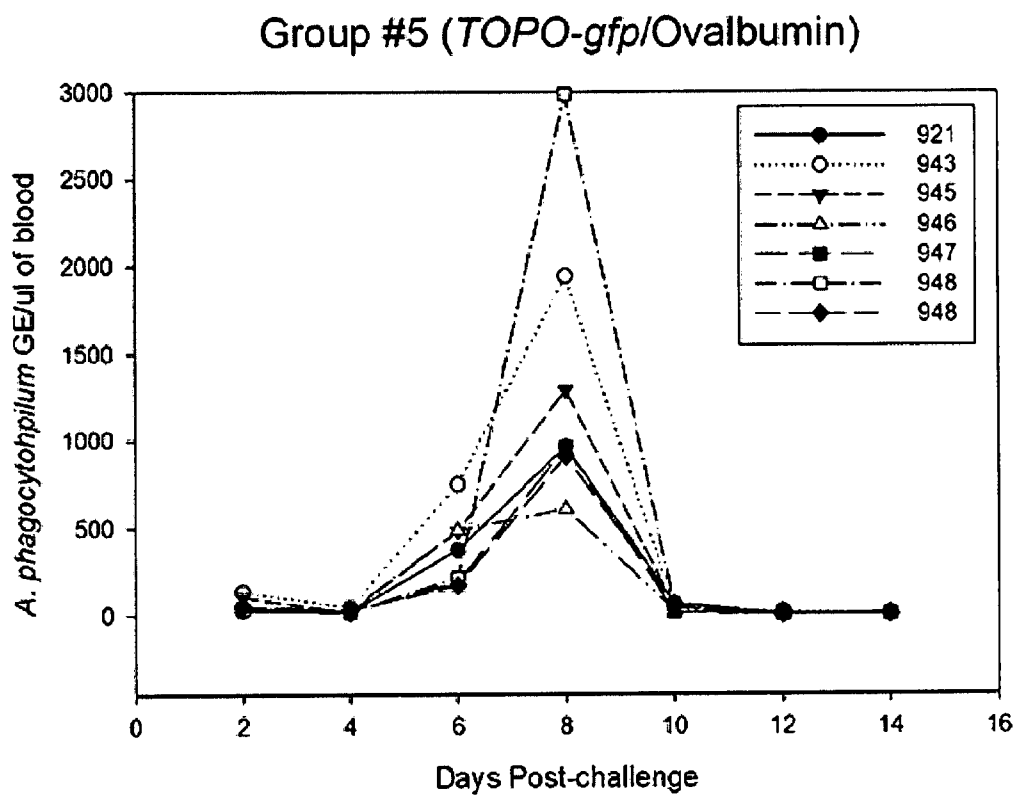


Figure 4 (continued)

10/11

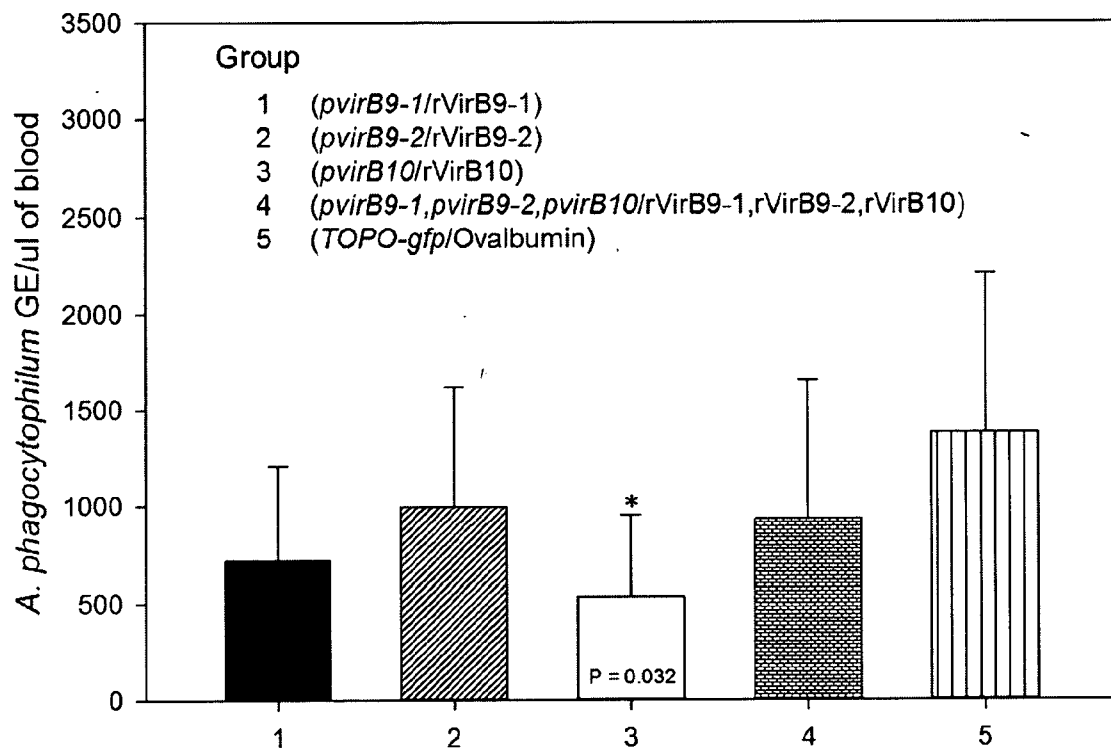


Figure 5

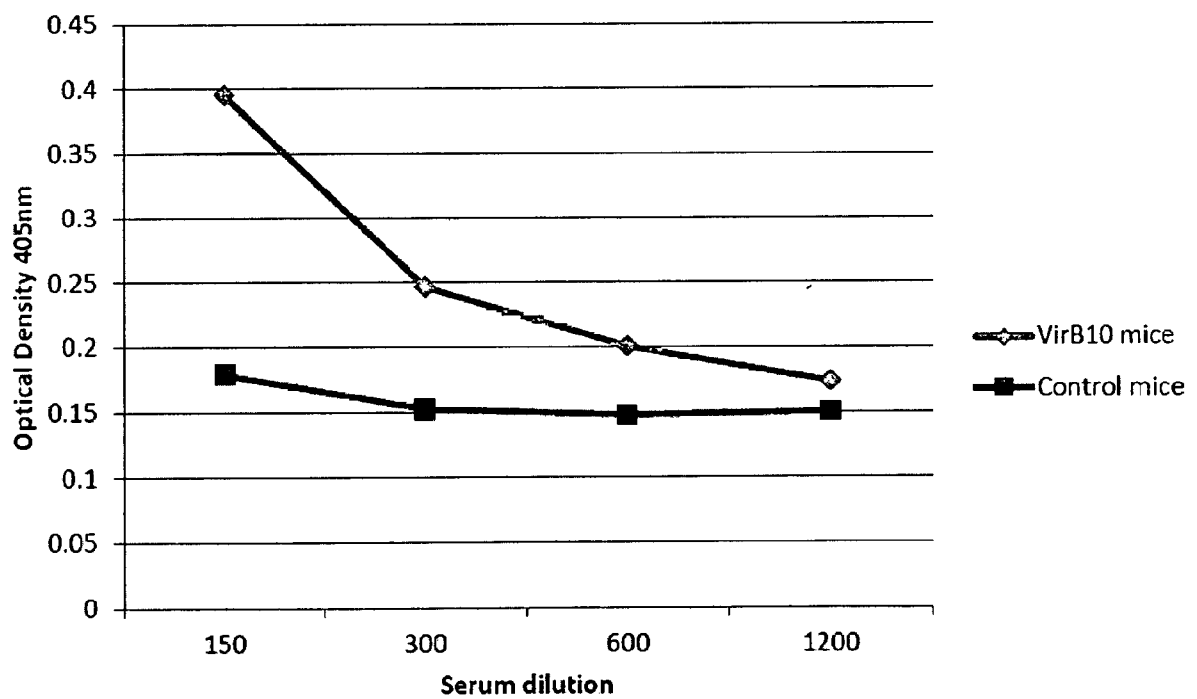


Figure 6

11/11

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Amarginale_Virb -----DGTSSP-----SVKATRVGDPGYVILQGHMIDAVLETAINS
Aphagocytophilu -----EGTQSP-----SVRATRVGDTRYIILQGHMIDAVLETAINS
Echaffeensis_Vi -----QTTSSP-----NVVATKVSNNLELTILQGGKIIDVVLETAINS
Eruminantium_Vi -----TSSP-----NVVATKINNLELTILQGGKIIDVVLETAINS
Rtyphi_Virb10_C -----PEQITE-----AVTFKDRGDMFLLLRGKLIDAVLETGINS
Rprowazekii_Vir -----PEQMT-----AVTFKDRGDMFLLLRGKLIDAVLETGINS
Rconorii_VirB10 -----PEQITA-----ETTFKDRGDMSLVLRGKLIDAVLETAINS
Rrickettsii_Vir -----PEQITA-----ETTFKDRGDMSLVLRGKLIDAVLETAINS
Otsutsugamushi_ -----EEMEQ-----IRNFTDAGELRYLLGRGTVIDAVIISAVNS
Ecoli_VirB10_3J SNSPGAQPQDNETSEGSSALAKNLTTPARLKASRAGVXANPSLTVPKGKXIPC GTGTELDT
: : : * * : : :

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Amarginale_Virb DIPGVLRAIVSRDVYAEAGNMVMIPKGSRLIGSY-FFDASGMNTRVTVSWSRVILP-HGI
Aphagocytophilu DISGVLRAVVS RDVYASSGDAVVI PKGSRLIGSY-FFDSAGNNVRVDVNNWSRVILP-HGV
Echaffeensis_Vi DLQGALRGIVARDVYAEASNTVMI PKGSRLIGSY-SFDASPGKTRVQISWNRVILP-HGI
Eruminantium_Vi DLQGTLRGIVARDVYSEAGNVMI PKGSRLIGNY-SFNASPGKTRVQISWNRVILP-HGV
Rtyphi_Virb10_C DLGGEIRAIISRDFSEKGVILIPKGSKIFGKYATSTSSDSYGRVSI IWDRLDT-NGY
Rprowazekii_Vir DLGGEIRAIISRDFSEKGVILIPKGSKIFGKYATSTSSDSYGRVSI IWDRLDT-NGY
Rconorii_VirB10 DLGGEIRAIISRDFSEKDKVILIPKGSKIFGKYATSTSSDSYGRVSI IWDRLDT-SGY
Rrickettsii_Vir ELGGEIRAIISRDFSEKDKVILIPKGSKIFGKYATSTSSDSYGRVSI IWDRLDT-SGY
Otsutsugamushi_ DFIGEIVAMVSRDVYSQEGKTILIPRGSKIFGYP-SVLDNAAVGRMMITWSEVQIAGSQY
Ecoli_VirB10_3J TVPGQVSCRVSQDVYSADGLVRLIDKGSWVDGQI-TGGIKDQARVFLWERIRNDQDGT
: * : : : * : : * : : * : :

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Amarginale_Virb DIQINSAGTDELGRNGSAGFIDTKMGNVLTSTILLAGVSMGTAFTVTSKI--PALQSEI--
Aphagocytophilu DIQIASSGTDELGRNGISGVVDNKGVSILTSTIFLAGISLGTAYVTEQI--PSLRTE TV-
Echaffeensis_Vi DITLDSNGTDELGRQAGSVVDTKIGNILTSTILLAGVSIATSYATSKI--PEINNYFIL
Eruminantium_Vi DIALDSTGTDELGRQAGSVVDTKVGSILTSTILLAGVSIATSYVTSKI--PEINDHP II
Rtyphi_Virb10_C TIEFDSPAVDNLGRPGQLGRVDN KYEQFANAVLQSGFNI GLAKILD KLVPPIIDSQAAA
Rprowazekii_Vir TIEFDSPAVDNLGRPGQLGRVDN KYEQFANAVLQSGFNI GLAKILD KLVPPIIDSQAAA
Rconorii_VirB10 TIEFDSPAVDNLGRPGQLGRVDN KYEQFANAVLQSGFNI GLAKVLD KLVPPIIASQAAA
Rrickettsii_Vir TIEFDSPAVDNLGRPGQLGRVDN KYEQFANAVLQSGFNI GLAKVLD KLVPPIIASQAAA
Otsutsugamushi_ KLNLSAPTIDRLGKIGVAGKVNNKNLARIMHAVLVS AVNIGAFTLDKV--VALQQQMAN
Ecoli_VirB10_3J IVNIDSAGTNSLGSAGIPGQVDAH XWERLGAIXISLFSDTLTALVNQTQSNNIQYNSTE
: : : : * * * : : : : : : : :

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Amarginale_Virb -----KDTTEEKGE-----KKKEKSSTL-----
Aphagocytophilu -----KVETPADGK-----DGKKTSSSL-----
Echaffeensis_Vi ESDSKEK-----KDKEKDDTG-----DKSKSTKTTL-----
Eruminantium_Vi ESKSDDKD-----KDKDKDKDK-----DKDK-TKTTL-----
Rtyphi_Virb10_C TNSATATQILNIAQTISSNTAIDVNTRIVTICTNIIAAITDKTSIAYTTMTQACATAQNA
Rprowazekii_Vir TNSATATQILNVAQTISSNTAMDVNTRIVTICTNIIAAITDKTSIAYATMTQACTTAQNA
Rconorii_VirB10 TNSATAKQLLNIAQTIASNTAMDANTRIVTICTNIIAAITDKTSTAYTTMTQACTTAQTA
Rrickettsii_Vir TNSATAKQLLNIAQTIASNTAMDANTRIVTICTNIIAAITDKTSTAYTTMTQACTTAQTA
Otsutsugamushi_ TTSGLTGTLIK NAMNINND SVKSDSQKITEICGMSRSLITOKSSSVYTTINKKCMIEITS
Ecoli_VirB10_3J NSGG-----

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Amarginale_Virb -----PVKIVSDAVKDFSESMKALIKKY
Aphagocytophilu -----STKIVSDAIKDFSDSMKEIVNKY
Echaffeensis_Vi -----PVKILSQAVDDFSNSIKDIIKKY
Eruminantium_Vi -----PVKILSKAVDDFSQSIKDIIQKY
Rtyphi_Virb10_C SSANTSE-QRLQTLVQAVNTAASNLLTTTSIASTPTQAAQASTQAFDTVNVVNMTIQ-
Rprowazekii_Vir SSAHTSE-QRLQTLVQAVNTAASNLLTTTSIASTPTQAAQASTQAFDTVNVVNMTIQ-
Rconorii_VirB10 SSANTAE-QRLQTLVQAVNTAASNLLTTTSIASTPTQAAQASTQAFDTVNVVNMTIQ-
Rrickettsii_Vir SSANTAE-QRLQTLVQAVNTAASNLLTTTSIASTPTQAAQASTQAFDTVNVVNMTIQ-
Otsutsugamushi_ IIANVDDKQKLSSLINTIIN----ISNSTANNSNSNTQKATDTALNDVNAIKKIIPS-
Ecoli_VirB10_3J -----QLASEAL-----RSY
: * .

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Amarginale_Virb VDTSKPTIYVDQGTVMKVFNQDIVFPREAVR-----
Aphagocytophilu SNR-TPTVYVDQGTVMKVFNQDVVFPREAVR-----
Echaffeensis_Vi SNN-NPTVYVDQGTLLKVFNKDIVFPKSAVR-----
Eruminantium_Vi TNN-NPTVYVDQGTLLKVFNKDIVFPKEAVR-----
Rtyphi_Virb10_C QHF-KPTTTVNQGTPIRIYVNKDYKFPRTVLLKSKVMK
Rprowazekii_Vir QHF-KPTTTVNQGTPIRIYVNKDYKFPKTVLLKSKVMK
Rconorii_VirB10 QQF-KPTTTVNQGTPVRIYVNKDYKFPKAVLLKSKVM-
Rrickettsii_Vir EQF-KPTTTVNQGTPVRIYVNKDYKFPKAVLLKSKVMK
Otsutsugamushi_ QEF-NRTITLNGQGNIKIYVNKDYLFPTKAVSGIKVL-
Ecoli_VirB10_3J XSI-PPTLYDQGDVAISIFVARDLDFSGVYTLADN---
: * : : * : : * .

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Figure 7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/037728

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

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

a. ☒ forming part of the international application as filed:

☒ in the form of an Annex C/ST.25 text file.

☐ on paper or in the form of an image file.

b. ☐ furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

☐ in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).

☐ on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).

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

3. Additional comments:

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.: 8-18
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 8-18 pertain to a method for treatment of the human body by therapy or surgery, and thus relate to a subject matter which this International Searching Authority is not required, under PCT Article 17(2)(a)(i) and PCT Rule 39.1(iv), to search.
2. ☒ Claims Nos.: 12, 16
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:
Claims 12 and 16 refer to the multiple dependent claim which does not comply with PCT Rule 6.4(a).
3. ☒ Claims Nos.: 5, 11, 13-15
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of any 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.:

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/US2016/037728**A. CLASSIFICATION OF SUBJECT MATTER****A61K 39/02(2006.01)i, A61K 39/39(2006.01)i, A61K 39/00(2006.01)i**

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K 39/02; A61K 39/00; G01N 33/53; C07K 14/195; C40B 30/04; A61K 39/39

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

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

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

eKOMPASS(KIPO internal) & keywords: VirB 10, vaccine, T4SS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MORSE, K. et al., "Association and evidence for linked recognition of type IV secretion system proteins VirB9-1, VirB9-2, and VirB10 in <i>Anaplasma marginale</i> ", <i>Infection and Immunity</i> , 2012, Vol. 80, No. 1, pp. 215-227 See abstract; and page 225.	1,7
Y		2-4,6
Y	US 2009-0280137 A1 (SAMOYLOVA, T. I. et al.) 12 November 2009 See abstract; and paragraphs [0007], [0043], [0057].	2,3
Y	US 2011-0143377 A1 (HOEY, J. G. et al.) 16 June 2011 See abstract; SEQ ID NO: 7; paragraphs [0011], [0129]; claims 1-38; and table 2.	4,6
X	ARAUJO, F. R. et al., "IgG and IgG2 antibodies from cattle naturally infected with <i>Anaplasma marginale</i> recognize the recombinant vaccine candidate antigens VirB9, VirB10, and elongation factor-Tu", <i>Memorias do Instituto OswaldorCruz</i> , 2008, Vol. 103, No. 2, pp. 186-190 See abstract; and page 189.	1,7

☒ 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

21 October 2016 (21.10.2016)

Date of mailing of the international search report

21 October 2016 (21.10.2016)

Name and mailing address of the ISA/KR

International Application Division

Korean Intellectual Property Office

189 Cheongsa-ro, Seo-gu, Daejeon, 35208, Republic of Korea

Facsimile No. +82-42-481-8578

Authorized officer

CHO, KI YUN

Telephone No. +82-42-481-5655



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2016/037728

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CASTELAO, A. B. C. et al., "Characterization of the immunogenicity of recombinant proteins VirB9, VirB10 and Elongation Factor Tu of Anaplasma marginale in mice", Brazilian Journal of Veterinary Research and Animal Science, 2012, Vol. 49, No. 5, pp. 377-385 See abstract.	1,7

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2016/037728Patent document
cited in search reportPublication
datePatent family
member(s)Publication
date

US 2009-0280137 A1

12/11/2009

US 8057992 B2

15/11/2011

WO 2009-062034 A1

14/05/2009

US 2011-0143377 A1

16/06/2011

US 2012-0183979 A1

19/07/2012

US 8283130 B2

09/10/2012

US 8389678 B2

05/03/2013