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— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

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(54) Title: AN ORAL VACCINE TO PROTECT ANIMALS AGAINST PLAGUE

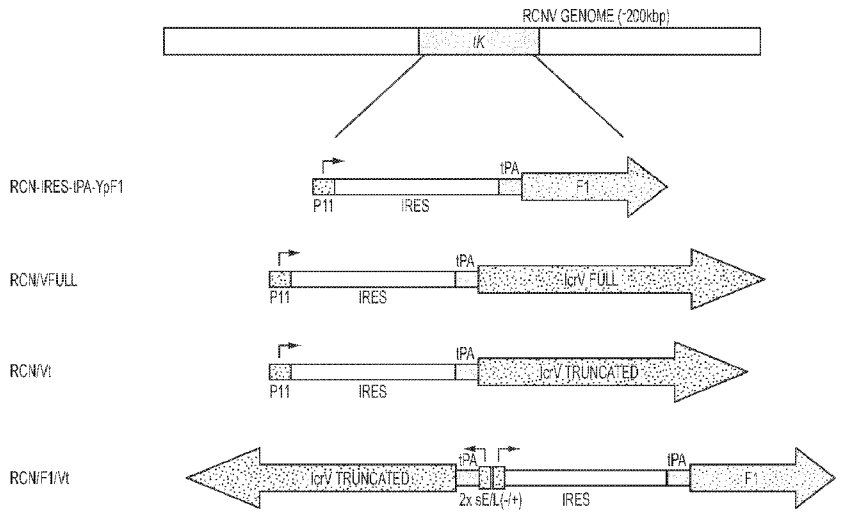

FIG. 1

(57) Abstract: A vaccine against plague for animals is disclosed comprising a recombinant raccoon poxvirus having at least one heterologous nucleic acid sequence translating for a protein antigenic for Y. pestis. The heterologous nucleic acid sequence of the vaccine comprises a novel truncated form of the V gene of Y. pestis, or some combination the novel sequence and the sequence translating for the F1-antigen of Y. pestis. We further disclose an oral vaccine compromising a recombinant raccoon poxvirus suitable for administration as bait or feed to animals in the wild.

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TITLE

AN ORAL VACCINE TO PROTECT ANIMALS AGAINST PLAGUE

RIGHTS IN THE INVENTION

[0001] This invention was made with support from the United States Government, specifically the United States Geological Survey, and the United States government has certain rights in this invention.

CROSS REFERENCE TO RELATED APPLICATION

[0002] This application claims the benefit of U.S. Provisional Application No. 61/281,310, filed November 3, 2009, which is hereby incorporated by reference in its entirety.

FIELD AND BACKGROUND OF THE INVENTION

[0003] The present invention relates to novel recombinant raccoon poxvirus vaccines against plague having improved efficacy that may be orally administered and used for large scale vaccination. The present invention further relates to a novel truncated form of the V gene of *Y. pestis* (V307), and a recombinant raccoon poxvirus construct containing V307 therein (RCN-V307). Vaccines of the present invention may include either (1) RCN-V307 administered with a recombinant raccoon poxvirus construct containing F1 therein – designated herein as ((RCN-F1) + (RCN-V307)), or (2) a single recombinant raccoon poxvirus construct containing both F1 and V307 therein (designated

herein as RCN-(F1+V307)). The vaccines may be administered orally, and preferably in the form of a bait.

[0004] Plague, caused by Y. pestis, is a zoonotic disease transmitted primarily by fleas that has recently re-emerged in numerous parts of the world. To reduce this public health threat and to protect against the potential use of Y. pestis as a bioweapon, development of novel plague vaccines for both humans and animals has been the focus of extensive research in recent years. [1-3]

[0005] The capsular F1 antigen (17.5 kDa) and the secreted V antigen (35 kDa) are natural virulence factors produced by *Y. pestis* [4, 5] and have been shown to be highly immunogenic, conferring a degree of protection equivalent to that of live plague vaccines such as the EV76 vaccine [6]. Vaccines based on these two antigens and a recombinant protein fusion of F1 and V have been developed [7], providing distinct advantages in comparison to the previous bacterial vaccines. However, like most proteins, they are usually weakly immunogenic when administered in the absence of an appropriate adjuvant or when administered via the oral route.

[0006] Prairie dogs (*Cynomys* spp.) are highly susceptible to sylvatic plague, caused by *Y. pestis*, and this zoonotic disease often decimates entire colonies of prairie dogs, causing local extinctions or regional reductions of their populations (Cully et al. 1997 [37]; Roach et al. 2001 [39]). Along with other wild

rodents, prairie dogs are also considered a significant reservoir of plague for other wildlife, domestic animals, and humans in the western United States. Arresting the spread of plague in prairie dogs is, therefore, desirable to reduce public health risks as well as protect the animal from local extinction. Current efforts to halt the spread of plague in prairie dog colonies typically rely on dusting individual prairie dog burrows with pesticides that kill plague-infected fleas after a plague outbreak has begun. However, pesticide application is labor intensive, costly and difficult to sustain over time. Most importantly, such programs are often initiated too late to substantially affect colony survival.

[0007] Prevention of plague in wild rodents by vaccination could reduce outbreaks of the disease. However, practicable large-scale vaccination of free-ranging wildlife populations can only be achieved through voluntary consumption of vaccine. Reduction of plague outbreaks in prairie dogs through oral vaccination in targeted locations could minimize the risk of disease transfer to other animals, particularly the endangered black-footed ferret, which relies exclusively on prairie dogs for food and habitat. Reduction of plague outbreaks in prairie dogs would enhance prairie dog and ferret conservation, while also contributing to the protection of public health.

[0008] With some exceptions, most of the plague vaccines formulated to date represent poor candidates for oral vaccination as they cannot withstand the alimentary tract or do not illicit a strong mucosal immune response (e.g. Thomas

et al. 1992 – [40]). Oral vaccination against plague using a live avirulent *Y. pseudotuberculosis* strain (Blisnick et al. 2008 [35]) or *Salmonella*-vectored vaccines expressing plague antigens (Yang et al. 2007 [42]) has been shown to protect laboratory mice, although the stability and feasibility of these products in baits used to deliver vaccines to wildlife has never been demonstrated.

[0009] Poxviruses are good candidates for the development of wildlife vaccines because of their ability to infect mucosal tissue and their stability in baits distributed in the environment. Raccoon poxvirus (RCN) was first isolated from the upper respiratory tract tissue of 2 of 92 apparently healthy raccoons captured in 1961-1962 during a survey of wildlife at Aberdeen Proving Grounds, Maryland [8]. Like vaccinia and canary poxviruses, RCN has been used previously as an experimental vaccine vector [9]. A significant advantage of RCN compared to other poxvirus vectors approved for veterinary applications is its ability to induce immune responses when delivered by mucosal routes [10]. Recombinant RCN (rRCN) vaccines have been administered to a variety of mammalian species, including mice, rats, rabbits, raccoons, skunks, bobcats, cats, dogs and sheep [9-12], prairie dogs [13] and black-footed ferrets (Rocke, unpublished data) with no reported side effects. rRCN vaccines have induced protective immune responses against rabies in raccoons, dogs, cotton rats, rabbits, bobcats, and foxes [9, 12, 14], and protective immune responses in domestic cats against feline panleukopenia virus, feline caliciviruses, and feline infectious peritonitis [11, 15].

[0010] We have demonstrated that a vaccine with several molecular elements, such as the encephalomyocarditis virus (EMCV) internal ribosomal entry site (IRES) and tissue plasminogen activator (tPA), significantly enhanced the expression levels of F1 in a recombinant RCN-based vaccine [16]. This vaccine, designated RCN-F1, and administered via intramuscular injection, was shown to fully protect mice against intradermal (i.d.) challenge with *Y. pestis* [16] at challenge doses as high as 4.8×10^3 LD_{50}.

[0011] In previous experiments (Mencher et al. 2004 [13], Rocke et al. 2008b [31]), recombinant raccoon pox virus (RCN) was demonstrated to be an effective oral delivery system for the fraction 1 (F1) capsular antigen of *Y. pestis* – RCN-F1. Approximately 50% of black-tailed prairie dogs that voluntarily consumed oral baits containing RCN-F1 survived subsequent challenge with *Y. pestis* at dosages of 70,000-130,000 cfu, which applicants believe represents numerous and repeated flea bites. A recent study showed that the oriental rat flea *(Xenopsylla cheopis)* transmitted from 2 – 4,970 cfu of *Y. pestis* per bite, an average of 636 + or – 1424 (Lorange et al. 2005 – [38]); unfortunately, similar estimates for prairie dog fleas are not available.

[0012] The prior art teaches an orally administered RCN-F1 vaccine, administered as a bait, as having limited efficacy against *Y. pestis* challenge. The need exists for an improved and effective, oral vaccine that provides rapid

protection against plague exposure for ultimate use in vaccinating animals in the wild and other animals susceptible to plague. The present invention provides for such a vaccine.

SUMMARY OF THE INVENTION

[0013] The present invention relates to novel recombinant raccoon poxvirus vaccines against plague having improved efficacy that may be orally administered, and may be suitably used for large-scale vaccination of free-ranging wildlife populations, and in particular black-tailed prairie dogs and black-footed ferrets.

[0014] The present invention includes a novel truncated form of the V gene of *Yersinia pestis (Y. pestis)* (V307), and an antigen expressed by V307 or an epitope thereof that is capable of eliciting the desired immune response. The present invention further includes a recombinant raccoon poxvirus construct having the truncated form of the V gene of *Y. pestis* inserted therein (RCN-V307). The RCN-V307 is used in combination with a recombinant raccoon poxvirus construct that expresses the F1-antigen of *Y. pestis* (RCN-F1) to form a recombinant raccoon poxvirus vaccine suitable for oral administration – ((RCN-F1) + (RCN-V307)).

[0015] The present invention further includes a single recombinant raccoon poxvirus construct having both the truncated form of the V gene of *Y.*

pestis (V307) and the F1 gene of *Y. pestis* inserted therein -- (RCN-(F1+V307)). This construct expresses both the truncated V-antigen of *Y. pestis* and the F1 antigen of *Y. pestis*. The RCN-(F1+V307) construct may be used as an oral vaccine to protect and/or treat animals against plague.

[0016] The vaccines herein are used to protect and/or treat an animal against plague. The vaccines, when administered to an animal before its exposure to plague, induce protective immunity against plague. These vaccines may be administered orally in any form, including incorporation into a bait or animal feed.

[0017] The vaccines herein may further be used in a veterinary setting or other medical treatment environment, and orally administered and/or administered via injection to an animal susceptible to plague.

BRIEF DESCRIPTION OF THE DRAWINGS

[0018] In the drawings:

[0019] **Figure 1** is a diagram of vaccine constructs described herein. These include (1) RCN-IRES-tPA-YpF1 (designated herein as RCN-F1**)**, (2) RCN/Vfull, (3) RCN/Vt (also designated herein as RCN-V307 or RCN-$Vt_{(307)}$), and (4) RCN/F1/Vt (also designated herein as RCN-(F1+V307) or RCN-(F1+$Vt_{(307)}$), wherein both F1 and V307 are present in the same RCN vector).

[0020] **Figure 2** is a table that sets forth vaccine treatments and dosages administered to AJ mice via intramuscular inoculation and their response to challenge with *Yersinia pestis* (CO92; original strain obtained from US Army Institute of Infectious Diseases) at various dosages. Median survival time (MST) is the day that 50% of the animals succumbed to infection.

[0021] **Figure 3** is a photograph of a Western blot of RCN-V307, and RCN-Vfull infected vero cell monolayers.

[0022] **Figure 4** is a graph of the hazard ratio and 95% confidence intervals for rRCN vaccine treatments administered to mice i.m. either singly or in combination in relation to RCN-F1 at a dosage of 1×10^7. Letters in parenthesis indicate the dosages administered.

[0023] **Figure 5** is a graph of least squares means log anti-F1 titer for rRCN vaccine treatments administered to mice i.m. either singly or in combination. Letters indicate the dosages administered.

[0024] **Figure 6** is a graph of least squares mean log anti-V titer for rRCN vaccine treatments administered to mice i.m. either singly or in combination. Letters indicate the dosages administered.

[0025] **Figure 7** is a graph of the geometric mean serum IgG antibody titers against F1 and V antigens in black-tailed prairie dogs that were vaccinated

against plague by consuming RCN-F1 and RCN-V307 vaccine-laden baits or by parenteral injection of F1-V fusion protein. Mean pre-challenge titers of all vaccinates were higher than controls ($P < 0.05$). Mean titers of animals injected with F1-V fusion proteins were higher ($P < 0.001$) than those that consumed RCN vaccines in baits.

[0026] **Figure 8** is a Kaplan-Meier graph showing survival curves of black-tailed prairie dogs after *Y. pestis* challenge in relation to vaccine treatment. Survival of prairie dogs consuming RCN vaccine-laden baits was higher than the group that received injections of F1-V fusion protein ($P = 0.025$) and the control group ($P < 0.0001$).

[0027] **Figure 9** is a diagram showing the IRES-F1/Vt construct.

[0028] **Figure 10** is a sequence listing for the full amino acid and nucleotide sequences for *Y. pestis* LcrV (SEQ ID NOS: 1 and 2 respectively).

[0029] **Figure 11** is a sequence listing for the amino acid and nucleotide sequences for a novel shortened *Y. pestis* LcrV protein (SEQ ID NOS: 3 and 4 respectively).

[0030] **Figure 12** is a sequence listing for the nucleotide sequence of the IRES-FI/Vt fragment (SEQ ID NO: 5).

[0031] **Figure 13** is a sequence listing of is a sequence listing for the

amino acid and nucleotide sequences for a shortened *Y. pestis* LcrV protein with a frame shift mutation at the C-terminal (SEQ ID NOS: 6 and 7 respectively).

DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0032] The present invention relates to vaccines having improved efficacy for the treatment and/or protection of animals, and particularly of animals in the wild, against plague.

[0033] The vaccines of the present invention are recombinant raccoon poxvirus vaccines. They use recombinant raccoon poxviruses as vectors to deliver the F1 gene of *Y. pestis* (F1) and a novel truncated form of the LcrV (V) gene of *Y. pestis* to an animal susceptible to plague. The sequence listing of the full LcrV gene *Y. pestis* is provided herein as FIG. 10 (SEQ ID NOS: 1 and 2).

[0034] The sequence listing of the novel truncated form of the LcrV (V) gene of *Y. pestis* (designated herein as V307 or $Vt_{(307)}$) employed in the present invention is provided herein as FIG. 11 (SEQ ID NOS: 3 and 4).

[0035] Vaccines of the present invention may comprise (1) a recombinant RCN construct containing F1 therein, and a separate recombinant RCN construct containing V307 therein, wherein these recombinant constructs are both administered to an animal susceptible to plague (designated as (RCN-F1)+(RCN-V307)); or (2) a single recombinant RCN construct containing both F1 and V307 therein (designated as RCN-(F1+V307) or RCN(F1+$Vt_{(307)}$). The sequence listing of the combination F1/V307 fragment (also designated F1/$Vt_{(307)}$) inserted into RCN to make RCN-(F1+V307) is provided herein as FIG. 12 (SEQ ID NO: 5),

and entitled IRES-F1/Vt fragment.

[0036] The invention includes methods of preventing and/or treating an animal susceptible to plague, as well as a kit containing the vaccine constructs described herein, and instructions for use thereof.

[0037] Administration to an animal susceptible to plague of V307 in combination with F1, wherein recombinant raccoon poxvirus is used as a vector for delivery of these provides enhanced protection against plague.

[0038] It is to be noted that the term "a" or "an" entity refers to one or more of that entity; for example, an antigen refers to one or more antigens, or to at least one antigen. As such, the terms "a" (or "an"), "one or more" and "at least one" can be used interchangeably herein. It is also to be noted that the terms "comprising", "including", and "having" can be used interchangeably. Furthermore, an administration route "selected from the group consisting of" refers to one or more of the routes in that group, including combinations thereof.

[0039] As used herein, the terms immunize, immunized and immunization refer to the ability to elicit an immune response in an animal. An immune response can be humoral or cell-mediated, or a combination thereof. Methods to measure an immune response are known to those skilled in the art; examples of some of such methods are disclosed herein. In a preferred embodiment an immunized animal is protected from disease caused by the agent against which

the animal is being immunized. For example, in a preferred embodiment, immunization of an animal with a recombinant raccoon poxvirus expressing proteins from plague LcrV and F1 genes will protect that animal from plague. Such an animal preferably displays (i.e., has) a protective antibody liter. Particularly preferred are high plague antibody liters, as measured by Enzyme-linked immunosorbent assay (ELISA; see examples).

[0040] A recombinant raccoon poxvirus of the present invention can be formulated in an excipient that the animal to be treated can tolerate. As such, the present invention includes administration of a composition comprising a recombinant raccoon poxvirus, wherein the composition further comprises an excipient. Examples of such excipients include water, saline, Ringer's solution, dextrose solution, Hank's solution, and other aqueous solutions physiologically balanced salt solutions. Other useful formulations include suspensions containing viscosity enhancing agents, such as sodium carboxymethylcellulose, sorbitol, or dextran. Excipients can also include various amounts of additives, such as substances that enhance isotonicity and chemical stability. Examples of buffers include phosphate buffer, bicarbonate buffer and Tris buffer. Standard formulations can either be liquid injectable or solids which can be taken up in a suitable liquid as a suspension or solution for injection. Thus, in a non-liquid formulation, the excipient can comprise dextrose, human serum albumin, preservatives, etc., to which sterile water or saline can be added prior to administration. Oral methods of delivery can consist of solids as described in

further detail herein.

[0041] In one embodiment of the present invention, the recombinant raccoon poxvirus can also include an adjuvant and/or a carrier. One advantage of a recombinant raccoon poxvirus is that adjuvants and carriers are not required to produce an efficacious vaccine, and in some cases, the advantages of the poxviruses of the present invention would be precluded by the use of some adjuvants. However, it should be noted that use of adjuvants or carriers is not precluded by the present invention. Adjuvants are typically substances that generally enhance the immune response of an animal to a specific antigen. Suitable adjuvants include, but are not limited to, other bacterial cell wall components; aluminum-based salts, calcium-based salts; silica; polynucleotides, toxoids; serum proteins; other viral coat proteins; other bacterial-derived preparations; block copolymer adjuvants, and saponins and their derivatives. Carriers are typically compounds that increase the half-life of a therapeutic composition in the treated animal. Suitable carriers include, but are not limited to, polymeric controlled release formulations, biodegradable implants, liposomes, bacteria, viruses, oils, esters and glycols.

[0042] The term "sequence homology" or “homology” means the proportion of base matches between two nucleic acid sequences or the proportion amino acid matches between two amino acid sequences. When

sequence homology is expressed as a percentage, e.g., 50%, the percentage denotes the proportion of matches over the length of sequence that is compared to some other sequence. Gaps (in either of the two sequences) are permitted to maximize matching; gap lengths of 15 bases or less are usually used, 6 bases or less are preferred with 2 bases or less more preferred.

[0043] Two amino acid sequences are homologous if there is a partial or complete identity between their sequences. For example, 85% homology means that 85% of the amino acids are identical when the two sequences are aligned for maximum matching. Gaps (in either of the two sequences being matched) are allowed in maximizing matching; gap lengths of 5 or less are preferred with 2 or less being more preferred. Alternatively and preferably, two protein sequences (or 60 polypeptide sequences derived from them of at least 30 amino acids in length) are homologous, as this term is used herein, if they have an alignment score of at more than 5 (in standard deviation units) using the program ALIGN with the mutation data matrix and a gap penalty of 6 or greater. See Dayhoff, M. O., in Atlas of Protein Sequence and Structure, 1972, volume 5, National Biomedical Research Foundation, pp. 101-110, and Supplement 2 to this volume, pp. 1-10. The two sequences or parts thereof are more preferably homologous if their amino acids are greater than or equal to 50% identical when optimally aligned using the ALIGN program.

[0044] Recombinant raccoon poxviruses have been used as delivery systems for heterologous (i.e., foreign) nucleic acid molecules – see USP, 5,266,313 (Esposito et al.), USP 6,106,841 (Osorio et al.), Osorio et al. [16], and Esposito et al. [43]. These references set forth descriptions of suitable raccoon poxvirus vectors (RCN) that may be employed in the present invention (see, for example, USP '313, at column 4, lines 11+). In addition, these references describe that specific heterologous nucleic acid molecules encoding antigens have been inserted into the thimidine kinase (TK) gene of RCN by homologous recombination. Such an insertion inactivates the TK gene, markedly reducing raccoon poxvirus virulence. The RCN vectors, as well as the description of TK gene insertion methodology, described in these references may be employed in making the novel RCN-V307 construct and RCN(F1+V307) construct of the present invention. The teachings set forth in USP 5,266,313, USP 6,106,841, Osorio et al. [16], and Esposito et al. [43] are incorporated by reference herein in their entirety.

[0045] A recombinant raccoon poxvirus of the present invention includes not only a recombinant raccoon poxvirus genome but also an envelope and core in which the genome is packaged. The viral envelope and core are preferably a raccoon poxvirus envelope and a raccoon poxvirus core, encoded at least in part by raccoon poxvirus genes, thereby imparting to the recombinant raccoon poxvirus the host range of a natural raccoon poxvirus isolate. It is to be noted,

however, that the present invention also includes recombinant raccoon poxviruses having envelopes and/or cores that have been modified to, for example, alter (e.g., broaden, narrow, or completely change) the host range of the recombinant raccoon poxvirus genome. Such modifications can be accomplished by one skilled in the art by, for example, modifying raccoon poxvirus envelope and/or core genes and/or replacing such genes with those of another virus. Altered genes can be located on the raccoon poxvirus genome itself and/or in the genome of the cell in which the recombinant virus is produced.

[0046] A recombinant raccoon poxvirus genome of the present invention is a raccoon poxvirus genome in which nucleotides have been deleted, inserted, substituted or inverted using recombinant techniques known to those skilled in the art such that the recombinant raccoon poxvirus genome is no longer the same as a natural genome. A recombinant raccoon poxvirus genome of the present invention includes a (i.e., one or more) heterologous nucleic acid molecule. As used herein, a heterologous nucleic acid molecule is a nucleic acid molecule isolated from a source other than raccoon poxvirus. In a preferred embodiment, a heterologous nucleic acid molecule of the present invention encodes a protein, such as a heterologous antigen, that is, a non-raccoon poxvirus antigen.

[0047] A heterologous nucleic acid molecule of the present invention is

operatively linked to a transcription control region, meaning that the heterologous nucleic acid molecule is expressed as an RNA and/or protein by the recombinant viral genome upon infection into a cell. The transcription control region can be endogenous to the genome or the heterologous nucleic acid molecule can be operatively linked to an exogenous transcription control region to form a recombinant molecule. Such a recombinant molecule can be introduced into the genome by methods known to those skilled in the art, for example by homologous recombination.

[0048] A suitable transcription control sequence is any sequence that allows for transcription of a heterologous nucleic acid molecule of the present invention. Examples include, but are not limited to, poxvirus promoters such as pII, p7.5, and synthetic promoters, as well as other viral promoters such as CMV promoters.

[0049] A suitable location for a heterologous nucleic acid molecule or recombinant molecule of the present invention is in an essential region, a non-essential region, or an intergenic region of the raccoon poxvirus genome. Examples include, but are not limited to, a thymidine kinase gene, a hemaglutinin gene, a serpin gene, a cytokine receptor gene, and an interferon receptor gene.

[0050] One embodiment of the present invention is a recombinant raccoon poxvirus that also comprises a nucleic acid molecule encoding an

immunomodulator. Suitable immunomodulators include compounds that enhance certain immune responses as well as compounds that suppress certain immune responses. Compounds that enhance the immune response include compounds that preferentially enhance humoral immunity as well as compounds that preferentially enhance cell-mediated immunity. Suitable compounds can be selected depending on the desired outcome. Suitable immunomodulators include, but are not limited to, cytokines, chemokines, superantigens, and other immunomodulators as well as compounds that induce the production of cytokines, chemokines and other immunomodulators. Examples of such compounds include, but are not limited to, granulocyte macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), macrophage colony stimulating factor (M-CSF), colony stimulating factor (CSF), erythropoietin (EPO), interleukin 2 (IL-2), interleukin-3 (IL-3), interleukin 4 (IL- 4), interleukin 5 (IL-5), interleukin 6 (IL-6), interleukin 7 (IL-7), interleukin 8 (IL-8), interleukin 10 (IL-10), interleukin 12 (IL-12), interferon gamma, interferon gamma inducing factor I (IGIF), transforming growth factor beta (TGF-β) RANTES (regulated upon activation, normal T-cell expressed and presumably secreted), macrophage inflam-matory proteins (e.g., MIP-1 alpha and MIP-1 beta), and Leishmania elongation initialing factor (LEIF).

[0051] A suitable composition for administering to animals in accordance with the present invention can include one or more recombinant raccoon

poxviruses, each including one or more heterologous nucleic acid molecules, as disclosed herein. As such, a composition of the present invention can be multivalent, that is, either delivering one or more antigens derived from a single source or antigens from multiple sources, such as those disclosed herein.

[0052] As used herein, the term "vaccine" means a composition which, when administered to an animal, stimulates an immune response against an antigen. A vaccine may comprise a protein, recombinant protein, DNA or RNA which upon introduction into a host, is able to provoke the immune response including but not limited to the production of antibodies, cytokines and other cellular responses. A vaccine may be used for the prevention of or prophylaxis against a disease, to include the passive or active immunization with antibodies or vaccines of the invention such that the disease or infection does not occur. By "active immunity to infection", we mean the ability of an organism to produce specific responses such as production of cytokines, lymphokines, antibodies or other substances, or cellular capacity to inhibit or retard infection in response to a contact with an antigen. By "passive immunity to infection" we mean the transfer to a host of the specific antibodies or other substances or cells capable of inhibiting or retarding infection.

[0053] Heterologous nucleic acid molecules, recombinant molecules, recombinant raccoon poxvirus genomes, recombinant raccoon poxviruses and compositions of the present invention can be produced by methods known lo

those skilled in the art.

[0054] Applicants used the Herman strain raccoon pox virus [8] discussed in Esposito et al. Applicants obtained it from the CDC. The raccoon pox virus used is the same as that having ATCC designation VR-838™, classification: Poxviridae, Orthopoxvirus; Strain: Herman; Original Source: Isolated by Y.F. Herman from respiratory tract of raccoon with no clinical symptoms, Maryland, USA, 1964; depositor: J.H. Nakana).

[0055] The RCN-F1 construct is well known in the art. A detailed description of an RCN-F1 construct of the type that may be employed herein is set forth in Mencher et al. [13], Osorio, et al. [16], and Rocke et al. [31] all of which are incorporated herein in their entirety. These references teach that experiments were conducted wherein RCN-F1 incorporated into baits were offered to black-tailed prairie dogs, and that immunized prairie dogs had higher survival rates (56% and 38%, in [13] and [31] respectively) than unimmunized control animals (12% and 11%, respectively).

[0056] In order to improve vaccine efficacy and potentially provide protection against F1 negative strains of *Y. pestis*, applicants constructed and optimized a novel RCN-vectored vaccine construct containing therein a novel truncated form of the LcrV (V) gene – referred to herein as V307 or $Vt_{(307)}$. Because the LcrV (or V) antigen has been associated by others with the

suppression of gamma interferon and tumor necrosis factor alpha *in vivo* [17, 18], applicants constructed this novel, truncated form (V307) of the LcrV gene, removing the purportedly immunosuppressive sequences. The nucleic acid sequence, and amino acid sequence corresponding to V307 is set forth herein as FIG 11 (SEQ ID NOS: 3 and 4). The method set forth in Overheim et al. [19], which is hereby incorporated by reference in its entirety, was used to remove the purportedly immunosuppressive sequence from the LcrV gene (FIG. 10 and SEQ ID NOS: 1 and 2) and hence, construct the novel V307 gene (FIG 11 and SEQ ID NOS: 3 and 4). Although Overheim et al. identifies numerous truncated forms of the LcrV gene and how to make them, it does not teach the specific V307 gene of the present invention. The novel RCN-vectored vaccine construct containing the novel V307 therein is designated herein as RCN-V307 or RCN-$Vt_{(307)}$.

[0057] Methods employed herein in making the RCN-vectored constructs, and the truncated form of the V gene (V307) can be broadly described as follows:

Construction of a recombinant raccoon poxvirus (RCNV) shuttle vector

[0058] Raccoon poxvirus vector created by modifying the vaccinia virus thymidine kinase (*tk*) gene-containing pKB3 plasmid are known in the art (Esposito et al., 1988 [43]**).** The known constructs include an additional unique polylinker restriction sites, and vaccinia *tk* sequences replaced by RCNV *tk* sequences. The resulting pTK vector was designed such that foreign DNA

cloned into the polylinker region is flanked by RCNV *tk* sequences and can recombine into the *tk* gene of a wild-type RCNV.

Construction of pTK/Vfull and pTK/Vt(307) shuttle vector

[0059] The plasmid pCD1 was used as a template for PCR amplification of the full length LcrV sequence of *Y. pestis* (FIG. 10 and SEQ ID NOS: 1 and 2). The PCR amplified product was cloned into a pre-existing plasmid pTK-IRES-tPA-YpF1 [16] such that LcrV sequence replaced YpF1 sequence. The resulting plasmid pTK/Vfull displayed the full length LcrV sequence, flanked by the RCNV *tk* sequences, and preceded by the secretory signal of tissue plasminogen activator (tPA) and the internal ribosomal entry site (IRES) of encephalomyocarditis virus. The operon is driven by the vaccinia virus promoter P11 (late class). The combination of IRES and tPA before a sequence has been shown to strongly increase its expression [16].

[0060] To obtain pTK/$Vt_{(307)}$, a stop codon was engineered into the full length LcrV sequence at the position 918. The truncated sequence was then inserted as above in the plasmid pTK-IRES-tPA-YpF1 to obtain pTK/$Vt_{(307)}$. As a result of this truncation, 19 C-terminal amino acids will be missing in the LcrV protein which is shortened to 307 amino acids. The lack of C-terminal amino acids reduces anti-inflammatory properties observed with the full length LcrV without interfering with its immunogenicity, leading to a better immune activation

[19].

Construction of pTK/F1/Vt(307) shuttle vector

[0061] Both F1 and LcrV from *Y. pestis* have been shown to induce a protective immunity against a challenge with plague. Moreover, F1-V fusion protein is now considered as the standard for vaccination against plague in animals and humans. The goal of the pTK/F1/$Vt_{(307)}$ construct is to combine both F1 and the novel truncated LcrV in a RCN virus, conferring a better protection.

[0062] The F1 gene was isolated together with IRES and tPA from the pre-existing plasmid pTK-IRES-tPA-YpF1 by PCR. The truncated LcrV gene $Vt_{(307)}$ was isolated together with tPA from the plasmid pTK/$Vt_{(307)}$ also by PCR.

[0063] Two synthetic super early/late promoters (sE/L) were synthesized back-to-back and cloned into the pTK plasmid, resulting in a pTK-sE/L(-/+) plasmid comprising two sE/L promoters in opposite directions flanked by RCNV *tk* sequences.

[0064] The isolated IRES-tPA-F1 sequence was cloned in pTK-sE/L(-/+) immediately after the sE/L promoter oriented in the positive direction and the isolated tPA-$Vt_{(307)}$ sequence was cloned immediately after the sE/L promoter oriented in the negative direction. The resulting pTK/F1/$Vt_{(307)}$ shuttle vector then comprises both IRES-tPA-F1 and tPA-$Vt_{(307)}$ sequences back-to-back, driven by

sE/L promoters (see FIG. 9). The removal of the IRES before the tPA-$Vt_{(307)}$ sequence is necessary due to interferences that occur during the RNA transduction process when two IRES are present in a same transcript.

Construction of recombinant RCN virus constructs

[0065] All the plasmids were sequenced and checked for mutations before attempting RCNV recombination. RCN-Vfull, RCN-$Vt_{(307)}$, and RCN-(F1+$Vt_{(307)}$) virus constructs were produced by homologous recombination as described elsewhere (Mackett et al., 1982) using techniques well known in the art.

[0066] Briefly, flasks of BSC-1 African green monkey kidney cells were infected with wild-type raccoon poxvirus and were then transfected with one of the plasmid shuttles, pTK-Vfull, pTK-$Vt_{(307)}$ or pTK-F1-$Vt_{(307)}$ using LIPOFECTAMIN (Gibco, Grand Island, NY), but it is understood that other suitable transfection reactions may be used in this process. The resulting recombinant viruses were plaque purified three times in RAT-2 thymidine kinase mutant rat embryo cells in the presence of bromodeoxyuridine (BrdU) to select for *tk* recombinants.

[0067] Vero cells were then infected with the selected recombinant RCNV clone and incubated at 37°C, 5% CO_2 until 100% cytopathic effect was obtained. Cells were detached and the culture was centrifuged at 660g for 5 minutes. The

cell pellet was resuspended in cold 10mM Tris buffer and homogenized in a dounce homogenizer on ice, then sonicated 3x15 sec at 6W. The lysate was then centrifuged for 80 min at 33,000g on a 36% sucrose gradient. The pelleted virus was re-suspended in 1mM Tris, pH=9 and stored in aliquots at -70°C until use.

[0068] The resulting recombinant RCN viruses (also referred to herein as recombinant RCN constructs), are identified herein as RCN-Vfull, RCN-V307 or RCN-$Vt_{(307)}$, and RCN-(F1+V307) or RCN-(F1+$Vt_{(307)}$). RCN-F1 was previously described.

[0069] The recombinant RCN constructs described herein are combined with a suitable excipient (carrier suitable for delivery) prior to delivery to animals susceptible to plague. Suitable excipients include those well known in the art that would be suitable for oral administration (i.e., in the form of baits, pills, elixirs (liquid), etc.), and those suitable for injection, such as phosphate buffered saline. Selection of a suitable excipient is well within the skill of the art.

[0070] Animal studies were conducted to assess the effectiveness of vaccines containing these constructs against plague challenge.

Vaccination of Mice via Injection

[0071] Mice were vaccinated via injection using both constructs, RCN-F1

and RCN-V307. Survival of these vaccinated mice upon plague challenge was significantly enhanced over mice that had been vaccinated with either construct alone (see Example 1)

[0072] Although a dual injection vaccine (injection of RCN-F1 and separate injection of RCN-V307) is not a desired means with which to vaccinate animals in the wild, it is certainly a suitable means for veterinary, and physician office use. Moreover, mouse studies using the combination vaccine construct, RCN(F1+V307) in a single injection has shown equal protection to the dual injection experiments conducted (Rocke, unpublished data).

Oral Vaccination of Black-Tailed Prairie Dogs (Cynomys ludovicianus)

[0073] Applicants conducted studies that demonstrated that consumption of baits containing both RCN-F1 and RCN-V307, by prairie dogs resulted in near complete protection to subsequent plague challenge. Their survival was found to be greater than that of prairie dogs injected subcutaneously (SC) with F1-V fusion protein, a vaccine demonstrated to induce immunity to plague in mice and other mammals (Powell et al. [32]), including black-footed ferrets (Rocke et al. [33]). See Example 2, below. Comparison was made between the effectiveness of baits containing RCN-F1 and RCN-V307 vs. SC injection using the F1-V fusion protein because the fusion protein is considered by most plague researchers to

be the "gold standard" for plague vaccines. The F1-V fusion protein is presently in clinical trials.

[0074] The following examples are provided for the purposes of illustration and are not intended to limit the scope of the present invention.

EXAMPLE 1

Vaccination of Mice with RCN-V307 in combination with RCN-F1

Materials and Methods Used for Vaccination of Mice via Injection

[0075] Two RCN-vectored vaccines containing different forms of the low calcium response V gene (LcrV) were designed in an attempt to improve vaccine efficacy and to potentially provide protection against F1 negative strains of *Y. pestis*. The different forms of LcrV used to design the RCN-vectored vaccines are designated herein as Vfull (FIG. 10 and SEQ ID NOS: 1 and 2) and V307 (the novel, truncated V307 gene – FIG. 11 and SEQ ID NOS: 3 and 4). Vfull and V307 were then each inserted into separate RCN vectors (resulting in RCN-V307 and RCN-Vfull). They were then each individually tested in mice in combination with RCN-F1 via simultaneous injections – ((RCN-Vfull + RCN-F1) and (RCN-V307 + RCN-F1)).

[0076] Serological analyses and animal challenge studies revealed that

vaccination of mice with RCN-F1 in combination with the truncated V construct (RCN-V307) provided the greatest protection when compared with RCN-F1 in combination with RCN-Vfull or RCN-F1 alone.

[0077] **Cells and Viruses.** Rat embryonic fibroblasts [Rat-2 (ATCC # CRL-1764)] and African green monkey kidney epithelial cells [BSC-1 (ATCC # CCL-26) and Vero (ATCC # CCL-18)] were maintained at 37ºC and 5% CO_2 in Medium 199 supplemented with 0.01 g/L L-glutamine and 5% fetal bovine serum (FBS) and were used for culturing virus. Raccoon poxvirus (RCN) Herman strain [8], having ATCC designation VR-838, was mixed 1:1 with trypsin-versene solution (0.05% trypsin; 0.02% EDTA in Earle's Balanced Salt Solution) and incubated for 15 min at 37℃ to release infectious particles from aggregates that may have formed upon storage prior to inoculation into cells.

[0078] **Construction of pTK transfer vectors.** Construction of RCN-F1 was described previously by Osorio et al. [16] the teachings of which are incorporated by reference herein in their entirety.

[0079] For RCN-V constructs, various versions of the *Y. pestis lcrV* gene (Vfull and V307) were cloned into the polylinker region of the pTK shuttle vector [16] so that the introduced DNA and upstream *cis*-acting elements (the p11 late promoter of vaccinia virus and the EMCV-IRES) were flanked by RCN thymidine kinase gene (*tk*) sequences. The introduced genes (lacking their native translation initiation codons) were inserted in-frame behind the tPA secretory

signal sequence (aa 2-23). Two versions of *lcrV* were each PCR-amplified and separately cloned into the pTK vector to construct (1) pTK-tPA-Vfull (RCN-Vfull), expressing the full-length (326 aa) antigen (PCR primers: forward 5' CATATGATTAGAGCCTACGAA 3' (SEQ ID NO: 8); reverse 5' GGATCCTCATTTACCAGACGT 3'(SEQ ID NO: 9)) and (2) pTK-tPA-Vt_{307} (RCN-V307), expressing a 307-aa C-terminally truncated V antigen (PCR primers: forward 5' GGCGCCGGCATTAGAGCCTACGAACAA 3' (SEQ ID NO: 10); reverse 5' GCGGATCCTCAACGGTTCAGT GCTTC 3' (SEQ ID NO: 11)).

[0080] **Generation of recombinant RCN constructs.** The pTK shuttle vectors described above were used to deliver each version (full-length or truncated) of the *Y. pestis lcrV* gene into the RCN genome through *tk*-driven homologous recombination. The process of generating *tk*-disrupted RCN recombinants (rRCN) has previously been described in detail [16].

[0081] Briefly, BSC-1 cells at 80% confluence were infected at a multiplicity of infection (MOI) of 0.06 with wild type RCN **(**Herman strain, ATCC designation VR-838**)**. Infected cells were then transfected in serum-free Opti-MEM (Invitrogen, Carlsbad, CA) with a mixture containing 4 µg of plasmid DNA [16] and 10 µL of LIPOFECTAMINE 2000 (Invitrogen, Carlsbad, CA) per well of a 6-well plate, according to the instructions provided by the manufacturer. The cell medium was replaced 5 h later. Three days post-infection/transfection, cells were freeze-thawed three times and the lysate was re-plated on Rat-2 cells in

M199 medium containing 5% FCS and 50 µg/ml 5-bromo-2-deoxyuridine (BrdU). Following a 2 h incubation, the medium was removed and the infected monolayer overlaid with 1X MEM containing 5% FCS and 1.2% low melt agarose. In the presence of BrdU, only *tk*$^-$ rRCN give rise to plaque forming units (pfu). Individual BrdU-resistant plaques were picked approximately 14 days post-inoculation (PI) and passaged twice through Rat-2 cells grown in BrdU-containing medium. Purified viral clones were then amplified for large scale purification in Vero cells as described previously [16].

[0082] ***In vitro* expression of rRCN constructs.** The *in vitro* expression of *Y. pestis* LcrV antigens by our rRCN constructs was determined by Western blot as described previously [16], using polyclonal mouse anti-LcrV antiserum and goat anti-mouse secondary antibody conjugated to alkaline phosphatase obtained from KPL, Inc. (Gaithersburg, MD).

[0083] **Animal Studies.** Four animal studies were conducted with the rRCN/*Y. pestis* vaccine constructs. The first study was designed to determine the most effective RCN-V vaccine construct. Groups of 12 mice each received the vaccine formulations singly or combined as listed in the table of FIG 2. All rRCN constructs were inoculated intramuscularly (IM) in the thigh with a volume of 0.2 ml. Animals that received two rRCN constructs were inoculated with each virus in separate thighs. RCN-F1 was inoculated at a dosage level of 10^7 pfu (the level shown to be protective in previous studies), whereas the RCN-V

constructs were inoculated at a dosage level of 10^8 pfu. A group of 11 mice received the empty vector virus (RCN-TK-) and was used as a negative control. All animals were boosted (same formulations, dosage, and route) on day 28 post-initial inoculation. Four weeks after the boost, all animals were challenged with the CO92 isolate of *Y. pestis* [provided by the United States Army Medical Research Institute of Infectious Diseases (USAMRIID)]. Each group of 12 mice was subdivided into groups of animals and each received either 70,000 cfu (3,500 LD50), 700,000 cfu (35,000 LD50), or 7,000,000 cfu (350,000 LD50) of *Y. pestis*. Stock aliquots of the bacteria were prepared and quantified as previously described [16], and diluted in sterile saline. A volume of 0.2 ml of each dilution was administered to each mouse by i.d. injection in the abdominal region. Plate counts of the challenge inoculum confirmed the dose administered, and concurrent mouse tests confirmed its virulence. The mice were monitored for 24 days for signs of illness or death. Animals with obvious clinical signs (labored breathing, severe lethargy) were humanely euthanized as were all survivors at the end of the 24-day post-infection period.

[0084] The second and third experiments were designed to determine the most effective vaccine dosages. Groups of 6-8 week old AJ mice (8-12 mice per group) were vaccinated IM with the vaccine combinations listed in Table 1 of FIG 2. One group of mice in each experiment received RCN-TK- and served as the negative control. The animals were boosted with the same formulations and

dosages on day 28 PI. Four weeks after the boost, the animals were challenged with *Y. pestis* via i.d. injection and monitored for morbidity and mortality for 21 days as described above.

[0085] A fourth experiment was conducted to confirm results of the previous experiments. Groups of 6-8 week old AJ mice (8 mice per group) were immunized and boosted as described above with single (RCN-F1, RCN-Vfull, RCN-V307) or combined ((RCN-F1+ RCN-Vfull), (RCN-F1 + RCN-V307), (RCN-F1 + RCN-TK-)) vaccines. A control group of 4 mice received RCN-TK- only. All constructs were inoculated at a dosage level of 5×10^7 pfu, the level that gave the best protection in experiment 3. Plague-induced mortality was verified in selected mice in each experiment by isolation of *Y. pestis* specific DNA sequences from bacterial culture via PCR. Frozen carcasses were thawed and necropsied, and liver samples were plated on blood agar plates (Becton-Dickinson, Franklin Lakes, NJ) at 28°C for up to 72 hr. Suspect colonies were cultured in brain heart infusion broth (Difco, Lawrence, Kansas). The DNA was subsequently extracted from the culture broth and stored at -20°C. For PCR, primers specific for the *Y. pestis* F1 gene [7] were used to amplify DNA fragments that were fractionated and directly visualized using standard techniques.

[0086] **Serology.** Blood samples (50 μl) were collected from the medial saphenous vein of each mouse at the times of initial vaccination, boost, and

challenge; blood samples were also obtained from all survivors at study termination. Serum was collected and stored at -20°C.

[0087] Antibody titers to *Y. pestis* F1 and V were determined using ELISA with antigens supplied by USAMRIID as described previously [16]. Serum samples were serially diluted 4-fold from 1:160 to 1:163,840 and test samples were run in duplicate. Titers of 1:160 were considered negative. The highest dilution that was positive (exceeded the mean of four negative control samples by three standard deviations) was considered the endpoint and its reciprocal value recorded as the titer.

[0088] **Statistical Analyses.** All analyses were performed with SAS statistical software (SAS Institute Inc., Cary NC). The effects of treatments on survival rates were examined with the Cox proportional hazards model, which takes into account time to death as well as survival rates. We used the stratified version of the Cox model, with experiment as the stratifying variable. This model assumes that the relative effects of the treatments remain the same between experiments, but the model permits the baseline survivorship to vary freely. Antibody titers were transformed by calculating the $\log_{10}$ of the titer in order to normalize the data and reduce skew. The effect of treatments on the log antibody titers were determined using analysis of variance with additive treatment and experiment effects. To adjust for experiment effects, the SAS least squares means (population marginal means) for the treatments and pair-wise significance

tests for each of them were computed using the SAS PDIFF option. For data display, we present the least squares means along with +/- two standard errors computed from the treatment groups pooled across experiments. We also analyzed each experiment individually for verification of the combined analysis; because the results were consistent, we report only those for the combined analysis.

Results

[0089] ***In vitro*** **expression of V antigen by RCN viruses.** Two RCN-vectored vaccines were constructed containing either the full length *lcrV* (RCN-Vfull) gene or a truncated form (RCN-V307). That particular truncation was chosen because it was the location of a convenient restriction site (*Xho* I), and previous studies by Overheim, et al., [19], which is incorporate by reference herein in its entirety, showed a similar truncation (at amino acid 301) reduced the immunosuppressive properties of the LcrV protein without compromising protection against plague challenge in mice. The *in vitro* expression of RCN-Vfull and RCN-V307 vaccines was examined by western blot analyses at 24 and 48 hours PI (FIG 3). As expected from our previous studies on RCN-F1 [16], the use of the EMCV-IRES and the tPA secretory signal resulted in strong expression levels of the V307 and Vfull antigens in the cell pellet and supernatant of Vero-infected cells, both at 24 and 48 hours PI.

[0090] **Animal Studies.** To evaluate the efficacy and immunogenicity of our RCN-V constructs, we conducted a series of sequential experiments and analyzed the data with statistical procedures that allowed us to compare the combined results. The proportion of animals that survived plague challenge in each treatment group and the median survival time (MST) for those groups with ≤ 50% survival are included in Table 1 of FIG 2. In every case, all the control animals that received the empty vector (TK-) died from plague challenge within 3-4 days PI. Interestingly, nearly all of the animals that received either RCN-Vfull or RCN-V307 alone did not survive challenge either. In contrast animals that received RCN-V constructs in combination with RCN-F1 had better survival rates. Using a Cox proportional hazards model, stratified by experiment, we first compared survival of all treatment groups against survival of groups that received RCN-F1 alone at a dosage of 1 x 10^7 pfu. All vaccinated groups with a hazard ratio (HR) < 1.0 relative to RCN-F1 (1 x 10^7 pfu) are included in FIG 4. There was no difference in the hazard ratio (HR = 0.27; P=0.42) between RCN-F1 administered at 1 x 10^7 pfu or 5 x 10^7 pfu, nor did the addition of RCN-TK- at either dosage significantly alter protection (HR = 0.46; P=0.27 and HR= 0.20; P= 0.33). The biggest improvement over RCN-F1 alone was obtained when this construct was administered in combination with RCN-V307 at dosages of 5 x 10^7 pfu (HR = 0.06, P =0.01). With this treatment, 89% of mice were protected following challenge with 700,000 cfu of *Y. pestis*. The next best treatment was 1 x 10^7 pfu RCN-F1 and 1 x 10^8 pfu RCN-V307 (HR = 0.25; P=0.01). The

combination of RCN-F1 and RCN-Vfull at either of the dosages tested did not significantly improve survival (P >0.10) of plague-challenge mice relative to RCN-F1 alone.

[0091] We also performed a Cox proportional hazards analysis that included anti-F1 and anti-V titers in addition to all of the treatment groups. The hazard ratios were 0.50 (P=0.006) and 0.59 (P=0.006) respectively, indicating that increased survival was associated with both increased anti-F1 and anti-V antibodies. By running the model without treatment effects and with just anti-F1 and anti-V antibodies, we constructed a likelihood ratio statistic for a treatment effect of G^2=14.1 (df=14), which is not significant (P>0.5). This result suggests that the treatment effect is primarily modulated through antibody levels. It is worth noting that the hazard ratio for each antibody was adjusted for the presence of the other. The fact that both anti-F1 and anti-V antibodies remain significant when both are present in the model means that they both contribute independently to the survival of the challenge subjects.

[0092] Antibody titers were compared between treatment groups using analysis of variance with additive experiment and treatment effects. Antibody titers to F1 were significantly elevated (P < 0.0001) in all treatment groups that received RCN-F1 alone or in combination with another RCN construct compared to controls (TK-) and vaccinates that did not receive the RCN-F1 construct (data not shown). Population marginal means for anti-F1 titers of all treatment groups

that received RCN-F1 alone or in combination with another RCN construct are shown in FIG 5. No difference in mean anti-F1 antibody titer was noted between RCN-F1 administered alone at a dosage of 1 x 10^7 or 5 x 10^7 pfu and any of the other groups that received an additional construct (RCN-TK-, RCN-V307, or RCN-Vfull) as long as the construct was administered at an equivalent dosage. In those groups that received RCN-F1 at a dosage of 1 x 10^7 pfu and a larger dosage of the second construct (5 x 10^7 or 1 x 10^8 pfu), anti-F1 titers were significantly lower ($P < 0.005$) than that of RCN-F1 alone. This appears to have resulted from the additional virus and not the V protein as this was observed in groups that received the empty vector (RCN-TK^-) as well as RCN-V307 or RCN-Vfull in combination with RCN-F1.

[0093] Anti-V antibody titers were significantly elevated ($P < 0.0001$) in all treatment groups that received RCN-Vfull or RCN-V307 alone or in combination with RCN-F1 compared to controls (TK-) and vaccinated groups that received RCN-F1 or RCN-F1+RCN-TK^- (data not shown). Population marginal means for those groups with elevated anti-V titers are shown in FIG 6. No difference in anti-V titer was noted between the group that received RCN-Vfull alone and those that received RCN-Vfull in combination with RCN-F1 ($P > 0.05$). Likewise, the anti-V titers of groups that received RCN-V307 at a dosage of 5 x 10^7 pfu alone or in combination with an equivalent dosage of RCN-F1 were not significantly different ($P > 0.05$). However, groups that received RCN-V307 at a

dosage of 1 x 10^7 pfu or in combination with RCN-F1 at 1 x 10^7 pfu had a lower mean anti-V titer than the RCN-Vfull group ($P < 0.001$).

[0094] Discussion of results of vaccination of mice via injection.

We had previously reported that mice [16] and prairie dogs [13] immunized with an rRCN-vectored vaccine expressing *Y. pestis* F1 antigen (RCN-F1) were protected against plague challenge. At a dosage of 1 x 10^7 pfu, the RCN-F1 vaccine provided full protection in vaccinated mice upon challenge with 100,000 cfu of *Y. pestis* and partial protection (56%) of orally immunized prairie dogs challenged with 130,000 cfu.

[0095] Here we disclose a new vaccine construct expressing the truncated form of the V gene (RCN-V307). What has been demonstrated herein is that the new vaccine construct (RCN-V307) significantly improved the survival of mice ($P = 0.01$) when administered in combination with RCN-F1. The majority (89%) of mice vaccinated with a combination of RCN-F1 and RCN-V307 at a dosage of 5 x 10^7 for each construct survived a plague challenge dose of 700,000 cfu. In contrast, increasing the dosage of RCN-F1 alone to 5 x 10^7 pfu did not significantly improve survival of plague-challenged mice ($P=0.42$).

[0096] Interestingly, administration of RCN-Vfull in combination with RCN-F1 was not as effective as administration of RCN-V307 in combination with RCN-

F1. As a major virulence factor of *Y. pestis,* full length LcrV antigen has been shown to trigger the release of interleukin 10 (IL-10) by host immune cells, a cytokine that suppresses innate immune functions [18, 20]. The elevated IL-10 suppresses the release of pro-inflammatory cytokines, such as tumor necrosis factor alpha and gamma interferon, altering the defense mechanisms required to combat the pathogenesis of plague [18, 20]. This immunosuppressive property of full-length LcrV reduces its desirability as a vaccine candidate, but recently Overheim, *et al.* [19] reported the construction of several LcrV variants and demonstrated their use as recombinant vaccines against plague. In their studies, a truncated LcrV antigen lacking amino acid residues 301 to 326 (rV11) elicited immune responses that protected mice against a lethal challenge with *Y. pestis*. Compared to full-length LcrV, rV11 (as well as other constructs) displayed a reduced ability to release IL-10 from mouse and human macrophages, enabling an increased response by the innate immune system. The RCN-V307 construct developed in this study contains a similar deletion of 19 aa at the C-terminus of the LcrV gene (308-326). The increased protective efficacy of the RCN-V307 construct compared to RCN-Vfull is believed to have resulted from removal of the immunosuppressive region located within the truncated sequence.

[0097] Our data analysis suggests that the effect of vaccination was primarily mediated by induction of antibody to F1 and V antigens. Although anti-F1 had more influence than anti-V when both antibodies were present, both

appeared to contribute independently to increased survival of challenged mice. The F1 antigen is a capsule-like protein expressed by *Y. pestis* at 37°C, after the bacteria begins multiplying in eukaryotic tissues, but not at 28°C [21], the approximate body temperature of fleas and the temperature we used to grow our *Y. pestis* challenge inoculum. Early in flea-transmitted plague infection (and presumably in our challenge system), the bacteria is highly sensitive to phagocytosis and is taken up by tissue macrophages and perhaps also epithelial cells [22], where they gain resistance to phagocytosis through the type III secretion system and proteins encoded by the virulence plasmid common to all pathogenic *Yersinia* species (Yops). Once the thermally-induced F1 antigen is expressed, it renders the pathogen even more able to resist phagocytocis by preventing binding between *Y. pestis* and phagocytic cells [23]. This resistance to uptake by phagocytes and neutrophils allows the pathogen to rapidly multiply, leading to a lethal systemic infection. Therefore, antibody to F1 in vaccinated mice may reduce the ability of *Y. pestis* to evade phagocytosis upon i.d. inoculation.

[0098] Antibody to V antigen in vaccinated mice that also had anti-F1 antibody clearly improved their response to i.d. challenge in our study, perhaps by blocking the immunomodulatory activity of *Y. pestis* LcrV [18], and/or by blocking the delivery of Yops in infected macrophage-like cells [24]. However, nearly all (23/24) of the animals vaccinated with single RCN-V constructs died

upon *Y. pestis* challenge, even at our lowest challenge dose of 3,500 LD50s (70,000 cfu), suggesting anti-V antibody alone was insufficient to protect the animals. Other studies have demonstrated the suitability of the LcrV antigen as a vaccine against $F1^+$ and $F1^-$ *Y. pestis* when delivered as a single subunit protein [25, 26] or DNA vaccine [27] at challenge doses as high as 10^7 cfu [28]. In these studies, the subunit vaccines were all delivered in the presence of Alhydrogel, an aluminum adjuvant known to significantly boost antibody response [29], and the time between the boost and challenge was typically 4-8 weeks or even longer, whereas in our study we challenged animals 2 weeks post-boost. It has been shown by several investigators that anti-V antibody develops much more slowly than anti-F1 antibody [25, 30], and it is possible that given more time, mice vaccinated with our single RCN-V constructs might improve their response to plague challenge. However, when administered in combination with RCN-F1, the RCN-V307 vaccine construct significantly increased protection against plague in mice. The best survival and highest anti-F1 and anti-V antibody titers in vaccinated mice were derived from a combination of 5×10^7 pfu for each virus, a feasible dosage from a production standpoint.

[0099] Although a dual injection vaccine is not a desired or suitable final product for vaccinating animals in the field such as free-ranging prairie dogs, such a vaccine is useful for the treatment of domestic animals. We disclose that a combination vaccine vectored by RCN that contains both F1 and V antigens is

a better alternative than single vaccines containing F1 or V alone.

[00100] This example demonstrates that the combination of RCN-F1 and RCN-V307 vaccines would go farthest towards meeting the goal of developing a safe vaccine that provides rapid treatment/protection against plague exposure for ultimate use in free-ranging animals, such as prairie dogs, via oral immunization.

EXAMPLE 2

Oral Vaccination of Prairie Dogs with RCN-based Vaccines

Materials and Methods for Oral Vaccination of Black-Tailed Prairie Dogs (*Cynomys ludovicianus)* –

[00101] Experimental Animals. Adult black-tailed prairie dogs (males and females) were captured from wild colonies near Wall, SD, USA (43.992N, 102.241W) and then transported to the U.S. Geological Survey National Wildlife Health Center (NWHC, Madison, WI, USA). At the time of capture, plague was not considered endemic in this region. The animals were dusted with carbaryl prior to shipment, and, upon arrival at NWHC, they were inspected for external parasites (none were found), injected with an ANTHELMINTHIC (200 µg/kg of Ivermectin, Merck & Co., Inc, West Point, PA), then treated with 200 µl of ADVANTAGE FLEA CONTROL (Imidacloprid; Bayer HealthCare, Shawnee Mission, KS), via external application to the skin on the back of the neck. Electronic microchip identification units (Avid Identification Systems, Inc., Folsom, LA) were inserted SC into each animal between the scapula. Prairie dogs were group-housed in isolation rooms with approximately 180 ft^2 of floor space. BETA CHIPS (Northwestern Productions Corporation, Warrensburg, NY) covered the floor; and for shelter, custom-made stainless steel nest boxes with connecting lengths of PVC pipe were provided. An alfalfa-based pelleted food

(approximately 50 g per animal per day) and fresh vegetables (broccoli, carrot, green beans, and sweet potato chunks) were provided once daily. Water was available *ad libitum*.

[00102] Administration of oral RCN-vectored vaccines. Two raccoon pox-vectored recombinant plague vaccine constructs were produced. The first, RCN-IRES-tPA-YpF1 (designated as RCN-F1 herein), was produced as previously described in Osorio et al. [16], and stored at –70 °C in 2 ml aliquots until bait production. The second construct, RCN-IRES-tPA-YpV307 (designated as RCN-V307) was prepared using the V gene truncated at amino acid position 307.

[00103] Briefly, each vaccine construct was created by replacing the thymidine kinase (TK) gene of RCN with the gene for *Y. pestis* F1 or V307 and associated regulatory elements (a poxvirus promoter, an internal ribosome entry site, and the secretory leader from tissue plasminogen activator). Virus stocks were thawed and diluted to 5 X 10^7 $TCID_{50}$/ml in Hank's medium (Gibco BRL, Carlsbad, CA) supplemented with 5% glycerin (Sigma, St. Louis, MO) immediately before adding to sweet potato gelatin baits. Preparation of baits and validation of vaccine titer was described in Mencher et al. **[13]**, incorporated by reference herein in its entirety. Briefly, 10g shredded organic yams (sweet potatoes**)** were mixed with 10 ml of Hank's medium with glycerin and 6.2% gelatin (w/v), and after the vaccine was gently mixed in, the baits were

refrigerated to harden. Placebo baits were prepared similarly but contained RCN vector with the TK gene deleted (RCN-TK$^-$) but no inserted genes.

[00104] Prairie dogs were prepared for bait administration by withholding fresh vegetables for 48 hr and food pellets for 12-18 hr. All animals were then identified by microchips and individually placed in pet carriers with a small food dish containing either one vaccine-laden bait or one placebo bait, according to the experimental group. After 2-4 hr, all animals were released, and bait consumption was recorded for each individual. This process was performed again the next day to ensure bait consumption. Baits containing both RCN-F1 and RCN-V307 were offered to prairie dogs (n= 16) at 30 day intervals (days 1-2 and 29-30). Upon back titration of prepared baits and stocks, it was determined that the titer of the RCN-F1 construct included in the baits was too low (~10^5 pfu) to induce an effective immune response (based on previous experience, Rocke, unpubl. data), so a new lot was prepared and baits containing only RCN-F1 were offered to prairie dogs on days 122 and 143. For the final bait administration on day 172, both RCN-F1 and RCN-V307 constructs were mixed together in one bait and offered to the prairie dogs. In all, prairie dogs consumed baits with approximately 10^7 pfu of each vaccine construct 3 times. Placebo baits were given to a separate group of prairie dogs (n=8) at each interval.

[00105] **Administration of F1-V fusion vaccine.** To compare the performance of our orally delivered vaccine with a well-studied injectable protein

vaccine based on the same plague antigens (F1 and V), we vaccinated a separate group of prairie dogs (n=12) with F1-V fusion protein provided by the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID). This F1-V fusion protein and our methods of preparing the vaccine-adjuvant mixture for SC injection were described previously (Powell et al. 2005 [32]; Rocke et al. 2004 [34]). Briefly, the antigen was diluted in Modified Dulbecco's medium (Sigma, St. Louis, MO), mixed 1:1 with 0.2% Alhydrogel (United Vaccines, Middleton, WI), and rocked gently overnight at 4°C. Prairie dogs were vaccinated with 40 µg of the diluted F1-V fusion protein mixed with adjuvant (0.5 ml volume) via SC inoculation. Control animals (n=7) received a placebo of 0.5 ml of the adjuvant (without protein antigen) suspended in Dulbecco's medium. Each group received a booster injection of protein or diluent (same dose and volume) 30 days later.

[00106] ***Y. pestis*** **challenge.** Three weeks after consumption of the final vaccine-laden bait and ten weeks after the injectable vaccine administration, animals were challenged with the CO92 wild type isolate of *Y. pestis* (provided by USAMRIID). Stock aliquots of the bacteria, prepared and quantified as previously described (Osorio et al.[16]) were diluted 1000-fold in sterile saline. A volume of 0.2 ml of this solution was administered to each prairie dog by SC injection in the right hip region. Plate counts of the challenge inoculum verified that a dose of approximately 70,000 cfu (3,500 mouse intradermal LD_{50}) was

given to each prairie dog, and concurrent mouse tests confirmed its virulence. (The LD_{50} of this inoculum for prairie dogs is unknown.) Prairie dogs were monitored for 28 days for signs of illness or death. Animals with obvious clinical signs (labored breathing, disinclination to move) were humanely euthanized as were all survivors at the end of the 28-day period. Carcasses were frozen for future necropsy.

[00107] Upon necropsy, tissue samples from lung, liver, spleen, and skin or any visible abscess at the site of inoculation were cultured in brain heart infusion broth (Difco, Lawrence, Kansas) and on blood agar plates (Becton-Dickinson, Franklin Lakes, NJ) at 28°C for up to 72 hr. Plague-induced mortality was verified by isolating *Y. pestis* specific DNA sequences from bacterial colonies using PCR and primers specific for the *Y. pestis* F1 gene [7]. DNA fragments were fractionated and directly visualized using standard techniques.

[00108] **Serology.** Blood samples (≤ 300 µl) were collected from the medial saphenous vein of each prairie dog 1-2 days before the priming vaccination and 1-2 days prior to challenge; blood samples were also obtained from survivors post-challenge. Serum was collected and stored at -20°C until analyses.

[00109] Antibody titers to *Y. pestis* F1 antigen were determined using ELISA with F1 antigen and V antigens supplied by USAMRIID, as described

previously (Rocke et al. 2008b [31]). Briefly, serum samples were serially diluted fourfold from 1:160 to 1:40,960; test samples were run in duplicate. Each plate also contained four replicates of a negative control serum sample and two replicates of a positive control serum sample. A horseradish-peroxidase labeled anti-prairie dog IgG custom-prepared by Bethyl Laboratories (Montgomery, TX) was diluted 1:100 and used as the secondary antibody. Titers < 1:160 were considered negative and recorded as 1:40. The highest dilution that was positive (exceeded the mean of four negative control samples by three standard deviations) was considered the endpoint and its reciprocal value recorded as the titer.

[00110] Statistical analyses. All statistical analyses were performed using SAS software (SAS 2005, SAS Institute Inc., Cary, NC). The Kaplan-Meier survival analysis was used to calculate survival curves, followed by log-rank tests to determine significance. Because Kaplan Meier analysis showed that survival was not significantly different between the two control groups (log rank chi square; P = 0.37), they were pooled into one group for further analyses. Antibody titers were transformed by calculating the $\log_{10}$ of the titer to symmetize the data and improve the accuracy of confidence interval coverage. For comparing antibody titers between groups, we used a robust linear models approach, which involves first transforming the data to ranks and then applying the appropriate linear models tests: a t-test for two groups or a one-way analysis of variance for more than two groups. This approach is equivalent to the

nonparametric Wilcoxon and Kruskal-Wallis tests, respectively (Conover and Iman 1981 [36]). For comparing antibody titers pre- and post-vaccination or pre- and post-challenge, a matched-pairs analysis was used.

[00111] **Results**. The preferred embodiments of this invention are compositions and methods of vaccine production that produce antibody response and survival to challenge of prairie dogs orally vaccinated against plague with RCN-based vaccines in comparison to controls and animals that received an injectable vaccine. Prairie dogs consumed ≥ 75% of the RCN vaccine-laden bait offered to them. No adverse reactions were observed in any of the animals that consumed these or that received the injectable F1-V vaccine.

[00112] Prairie dogs that consumed RCN vaccine-laden baits had significantly higher ($P < 0.05$) mean IgG titers to F1 and V antigens pre-challenge compared to their pre-vaccination levels and the pre-challenge titers of control animals (FIG 7), although the rise was only about 10-20 fold. In comparison, animals that received F1-V vaccine injections had a significantly higher rise in titer (70-100 fold) compared to their pre-vaccination levels and significantly higher levels ($P < 0.0001$) than both oral vaccinates and controls. Interestingly, in oral vaccinates that survived challenge, IgG titers to F1 and V antigens increased significantly (df 14, $P < 0.001$) from a mean pre-challenge titer of 1:738 and 1:843 respectively to a mean post-challenge titer of 1:16,708 and 1:7648. In contrast, mean post-challenge titers of animals that received F1-V fusion vaccine

injections did not rise significantly (df 6, P >0.05) over their peak pre-challenge values. Anti-F1 antibody was detected in the one surviving control animal at a very high titer (1:163,480), but no anti-V antibody was detected.

[00113] Upon challenge with *Y. pestis,* 15/16 prairie dogs that consumed vaccine-laden baits survived compared to 7 /12 prairie dogs that received F1-V fusion vaccine injections and 1/15 controls. *Yersinia pestis* was cultured from tissues of those that died, confirming that plague was the cause of death. Kaplan Meier analysis showed that a significant difference in survival (FIG 8) was evident between the three treatment groups ($P < 0.0001$), and that vaccinates, regardless of treatment, survived better ($P < 0.0001$) than the controls. Survival of prairie dogs consuming RCN vaccine-laden baits was higher than the group that received injections of F1-V fusion protein ($P = 0.025$). For animals that succumbed to plague, whether vaccinated or not, no overt difference ($P > 0.27$) in time to death was evident between these three groups.

[00114] Discussion of results of oral vaccination of prairie dogs

We disclose an oral vaccine to protect prairie dogs against plague with proven immunogenicity and protection induced from their consumption of baits containing recombinant RCN vaccine vectors expressing *Y. pestis* antigens (within scope of the present invention) in comparison to injection of the well-characterized F1-V fusion subunit vaccine. The F1-V fusion vaccine is

considered the "gold standard" for the treatment and/or prevention against plague.

[00115] Surprisingly, a higher level of protection against plague was achieved in prairie dogs consuming recombinant RCN vaccine-laden baits than with parenteral vaccination of F1-V fusion protein, despite the fact that the fusion protein vaccine stimulated much higher serum IgG titers. Others have shown that F1-V fusion protein fully protects mice against *Y. pestis* challenge doses up to 9 x 10^6 LD_{50} (Powell et al. 2005 **[32]**, Heath et al. 1998 **[7]**), and single-dose vaccinations even protected mice against aerosol challenge by wild type and non-encapsulated (F1 -) *Y. pestis* (Powell et al. 2005 **[32]**). However, in prairie dogs vaccinated with two injections of F1-V fusion protein in this study, only 56% of the animals survived challenge with approximately 3,500 mouse LD_{50} of *Y. pestis*. This corroborates an earlier study conducted similarly in which 5/8 prairie dogs vaccinated with F1-V fusion vaccine survived challenge with the same dose of plague administered three weeks after receiving the booster injection (Rocke, unpubl. data). In contrast, in prairie dogs orally vaccinated with recombinant RCN vaccines, nearly all (94%) survived *Y. pestis* challenge, even though their mean IgG titers to F1 and V antigens (< 1:1000) were more than 10-fold lower than animals vaccinated with F1-V fusion protein. This suggests a profound difference in the role of humoral immunity in evading plague infection between mice and prairie dogs.

[00116] Typically, the gastrointestinal tract has well developed lymphoid tissue, enabling the stimulation of both systemic and mucosal immunity, so it is likely that other aspects of the immune system were stimulated in the orally vaccinated prairie dogs. For example, other investigators have shown that an oral plague vaccine using *Salmonella*-vectored F1 and V antigens resulted in elevations in both serum IgG and secretory IgA in laboratory mice (Yang et al. 2007 [42]), resulting in near complete protection against a *Y. pestis* challenge dose of 1000 LD_{50}. Unfortunately, in prairie dogs, we were only able to measure serum IgG, as anti-prairie dog IgA is not currently available, and techniques for measuring cellular immunity are likewise limited for outbred animals such as these.

[00117] We dislcose that a second, non-F1 antigen is important for inducing immune protection in prairie dogs. In two previous studies (Mencher et al. 2004, *supra* **[13]**; Rocke et al. 2008b, *supra* **[31]**), prairie dogs were orally vaccinated with baits containing only RCN-F1, and even after consuming bait three times, only about 50% survived plague challenge. Here, prairie dogs were provided baits with RCN-V307 which expresses a truncated form of the V gene, in addition to RCN-F1, and nearly all survived plague challenge. The significantly improved prairie dog survival was due to addition of RCN-V307.

[00118] The nucleic acid molecules of the present invention inserted into the RCN vector – F1, V307, and (F1+V307) – may be readily prepared by

methods known in the art, for example, by directly synthesizing the nucleic acid sequence using methods and equipment known in the art such as automated oligonucleotide synthesizers, PCR technology, recombinant DNA techniques, and the like.

[00119] The vaccines of the present invention may be incorporated/placed into baits that are able to maintain vaccine stability. Those baits that are suitable for field use could be used to broadcast the vaccine-laden baits from planes or other vehicles. One having ordinary skill in the art with knowledge of the present invention will be able to identify baits suitable for use herein.

[00120] Although the vaccine within the scope of the present invention is primarily described with reference to being suitable for oral administration, and in particular for delivery to animals found in the wild in the form of a bait, the route of vaccine administration/delivery is not intended to be so limited. Note that administration of the RCN-vectored vaccine via injection was found to significantly improve survival of plague-challenged mice. Applicants submit that the RCN-vectored vaccine of the present invention may be administered orally or by injection. The vaccine may be incorporated into any carrier suitable for delivery of said vaccine to an animal – i.e., to include carriers suitable for injection or oral administration (capsule, pill, liquid, etc.). Selection of a suitable carrier is well within the skill of the art. In addition to being useful to prevent the spread of plague in the wild, the invention also has significant veterinary

application, and may be administered in an office setting to animals susceptible to plague.

[00121] Although RCN was used as the vector in these studies, other poxviruses could act as suitable vectors for insertion and delivery of F1, V307 and F1-V307. Moreover, although the showings herein relate to use of a specifically identified RCN vector, one having ordinary skill in the art will recognize that, and be able to select, other RCN vectors, if available, that may be suitably employed herein as well.

[00122] V307 and $Vt_{(307)}$ are used interchangeably herein.

[00123] While particular embodiments of the present invention have been shown and described, it will be obvious to those skilled in the art that changes and modifications may be made without departing from this invention. Therefore, it is intended that the claims herein are to include all such obvious changes and modifications as fall within the true spirit and scope of this invention.

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We claim:

1. An isolated polypeptide having the amino acid sequence of SEQ ID NO: 3, or a conservatively substituted variant thereof exhibiting *Y. pestis* antigenic behavior.

2. The polypeptide of claim 1 wherein the polypeptide has at least about 50% homology with SEQ ID NO: 3.

3. The polypeptide of claim 1 wherein the polypeptide has at least about 60% homology with SEQ ID NO: 3.

4. The polypeptide of claim 1 wherein the polypeptide has at least about 70% homology with SEQ ID NO: 3.

5. The polypeptide of claim 1 wherein the polypeptide has at least about 80% homology with SEQ ID NO: 3.

6. The polypeptide of claim 1 wherein the polypeptide has at least about 90% homology with SEQ ID NO: 3.

7. An isolated oligonucleotide having the nucleotide sequence of SEQ ID NO: 4, or a conservatively substituted variant thereof exhibiting *Y. pestis* antigenic behavior.

8. The oligonucleotide of claim 7 wherein the oligonucleotide has at least about 50% homology with SEQ ID NO: 4.

9. The oligonucleotide of claim 7 wherein the oligonucleotide has at least about 60% homology with SEQ ID NO: 4.

10. The oligonucleotide of claim 7 wherein the oligonucleotide has at least about 70% homology with SEQ ID NO: 4.

11. The oligonucleotide of claim 7 wherein the oligonucleotide has at least about 80% homology with SEQ ID NO: 4.

12. The oligonucleotide of claim 7 wherein the oligonucleotide has at least about 90% homology with SEQ ID NO: 4.

13. A recombinant raccoon poxvirus comprising a recombinant poxvirus genome having the heterologous nucleic acid molecule of SEQ ID NO:

4, or a conservatively substituted variant thereof exhibiting *Y. pestis* antigenic behavior.

14. The recombinant raccoon poxvirus of claim 13 wherein the heterologous nucleic acid molecule has at least 50% homology with SEQ ID NO: 4.

15. The recombinant raccoon poxvirus of claim 13 wherein the heterologous nucleic acid molecule has at least 60% homology with SEQ ID NO: 4.

16. The recombinant raccoon poxvirus of claim 13 wherein the heterologous nucleic acid molecule has at least 70% homology with SEQ ID NO: 4.

17. The recombinant raccoon poxvirus of claim 13 wherein the heterologous nucleic acid molecule has at least 80% homology with SEQ ID NO: 4.

18. The recombinant raccoon poxvirus of claim 13 wherein the heterologous nucleic acid molecule has at least 90% homology with SEQ ID NO: 4.

19. A recombinant raccoon poxvirus comprising a recombinant poxvirus genome having at least one heterologous nucleic acid molecule integral to SEQ ID NO: 5, or a conservatively substituted variant thereof exhibiting *Y. pestis* antigenic behavior.

20. The recombinant raccoon poxvirus of claim 19 wherein the recombinant poxvirus has at least 50% homology with SEQ ID NO: 5.

21. The recombinant raccoon poxvirus of claim 19 wherein the recombinant poxvirus has at least 60% homology with SEQ ID NO: 5.

22. The recombinant raccoon poxvirus of claim 19 wherein the recombinant poxvirus has at least 70% homology with SEQ ID NO: 5.

23. The recombinant raccoon poxvirus of claim 19 wherein the recombinant poxvirus has at least 80% homology with SEQ ID NO: 5.

24. The recombinant raccoon poxvirus of claim 19 wherein the recombinant poxvirus has at least 90% homology with SEQ ID NO: 5.

25. The recombinant raccoon poxvirus of claim 19 wherein the heterologous nucleic acid molecule is SEQ ID NO: 4.

26. The recombinant raccoon poxvirus of claim 19 wherein the heterologous nucleic acid molecule encodes the fraction 1 (F1) capsular antigen of Y. pestis.

27. The recombinant raccoon poxvirus of claim 19 further comprising the heterologous nucleic acid molecules: SEQ ID NO: 4 and the fraction 1 (F1) capsular antigen of *Y. pestis*.

28. A vaccine comprising any one of claims 1-27.

29. The vaccine of claim 28 further comprising an excipient suitable for delivery of said vaccine to an animal.

30. The vaccine of claim 29 wherein said excipient is suitable for oral administration.

31. The vaccine of claim 30 wherein said excipient is amenable to voluntary consumption by an animal.

32. The vaccine of claim 28 further comprising an adjuvant.

33. A method for vaccinating animals comprising administering to said animals an effective amount of the vaccine of claim 28.

34. A method for vaccinating animals comprising administering to said animals an effective amount of any one of the vaccines of claims 29-32.

35. The method of claim 33, wherein said animal is selected from a group consisting of: a black-tailed prairie dog (*Cynomys ludovicianus*) or a black-footed ferret.

36. The method of claim 34, wherein said animal is selected from a group consisting of: a black-tailed prairie dog (*Cynomys ludovicianus*) or a black-footed ferret.

37. A kit for vaccinating animals against plague, wherein said kit comprises:

the vaccine of claim 28; and,

instructions for use thereof; and,

container means.

38. A kit for vaccinating animals against plague, wherein said kit comprises:

any one of the vaccines of claim 29-32; and,

instructions for use thereof; and,

container means.

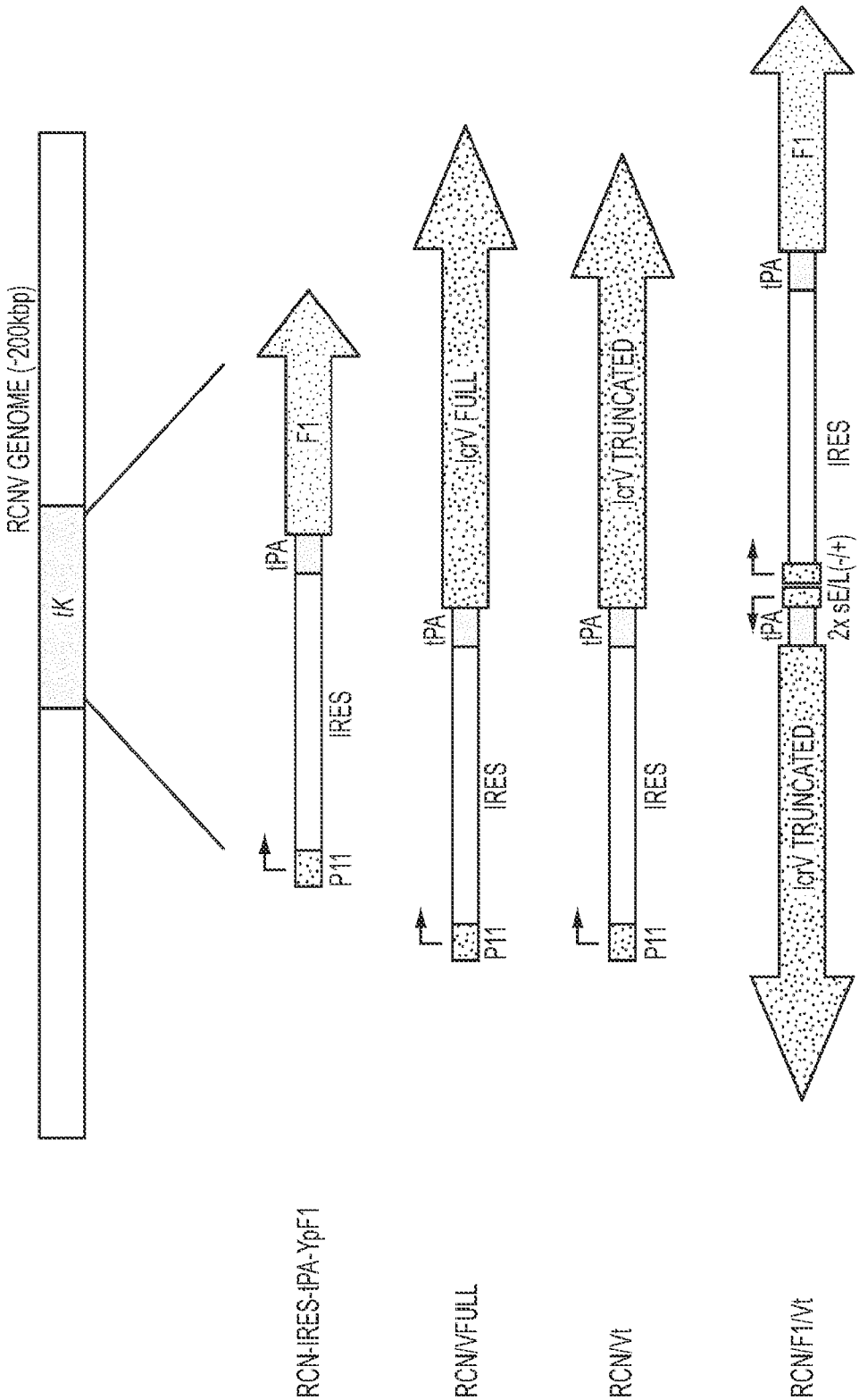
RCNV GENOME (~200kbp)
IK
tPA
F1
IRES
P11
RCN-IRES-tPA-YpF1
IgV FULL
RCNVFULL
IgV TRUNCATED
RCNVt
2x sEIL(-/+)
RCNF1Vt

FIG. 1

EXPT.#	n	VACCINE	DOSAGES (pfu)	% SURVIVAL TO *Y. PESTIS* CHALLENGE (MST)		
				70,000 cfu	700,000 cfu	7,000,000 cfu
1	12	RCN-TK-	$1X10^7$	0 (3)	0 (2)	0 (2)
1	12	RCN-F1	$1X10^7$	0 (8)	25 (12)	0 (2)
1	12	RCN-F1+ RCN V307	$1X10^7$, $1X10^8$	100	75	25 (2)
1	12	RCN-F1+ RCN VFULL	$1X10^7$, $1X10^8$	50 (11)	50 (13)	25 (3)
2	8	RCN-TK-	$1X10^7$	0 (2)	ND	0 (2)
2	8	RCN-F1	$1X10^7$	100	ND	0 (3)
2	8	RCN-V307	$1X10^7$	0 (2)	ND	0 (2)
2	8	RCN-F1+ RCN V307	$1X10^7$, $1X10^7$	75	ND	25 (4)
3	8	RCN-TK-	$1X10^7$, $1X10^7$	ND	0 (2)	ND
3	8	RCN-F1+TK	$1X10^7$, $1X10^8$	ND	25 (4)	ND
3	8	RCN-F1+RCN V307	$1X10^7$, $1X10^8$	ND	50 (8)	ND
3	8	RCN-F1+RCN V307	$1X10^7$, $5X10^7$	ND	63	ND
3	8	RCN-F1+RCN V307	$5X10^7$, $5X10^7$	ND	89	ND
4	4	RCN-TK-	$5X10^7$	ND	0 (4)	ND
4	8	RCN-F1	$5X10^7$	ND	50 (14)	ND
4	8	RCN-V307	$5X10^7$	ND	0 (3)	ND
4	8	RCN-VFULL	$5X10^7$	ND	13 (3)	ND
4	8	RCN-F1+TK-	$5X10^7$, $5X10^7$	ND	63	ND
4	8	RCN-F1+RCN V307	$5X10^7$, $5X10^7$	ND	89	ND
4	8	RCN-F1+RCN VFULL	$5X10^7$, $5X10^7$	ND	75	ND

FIG. 2

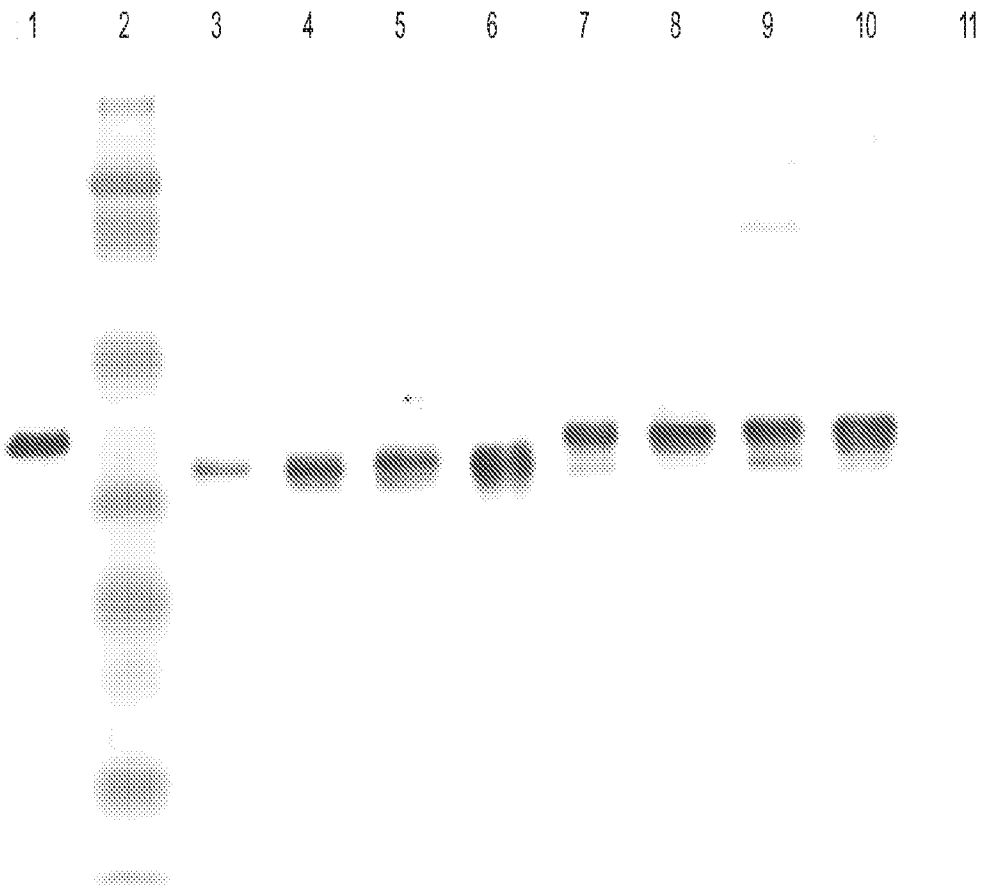

FIG. 3

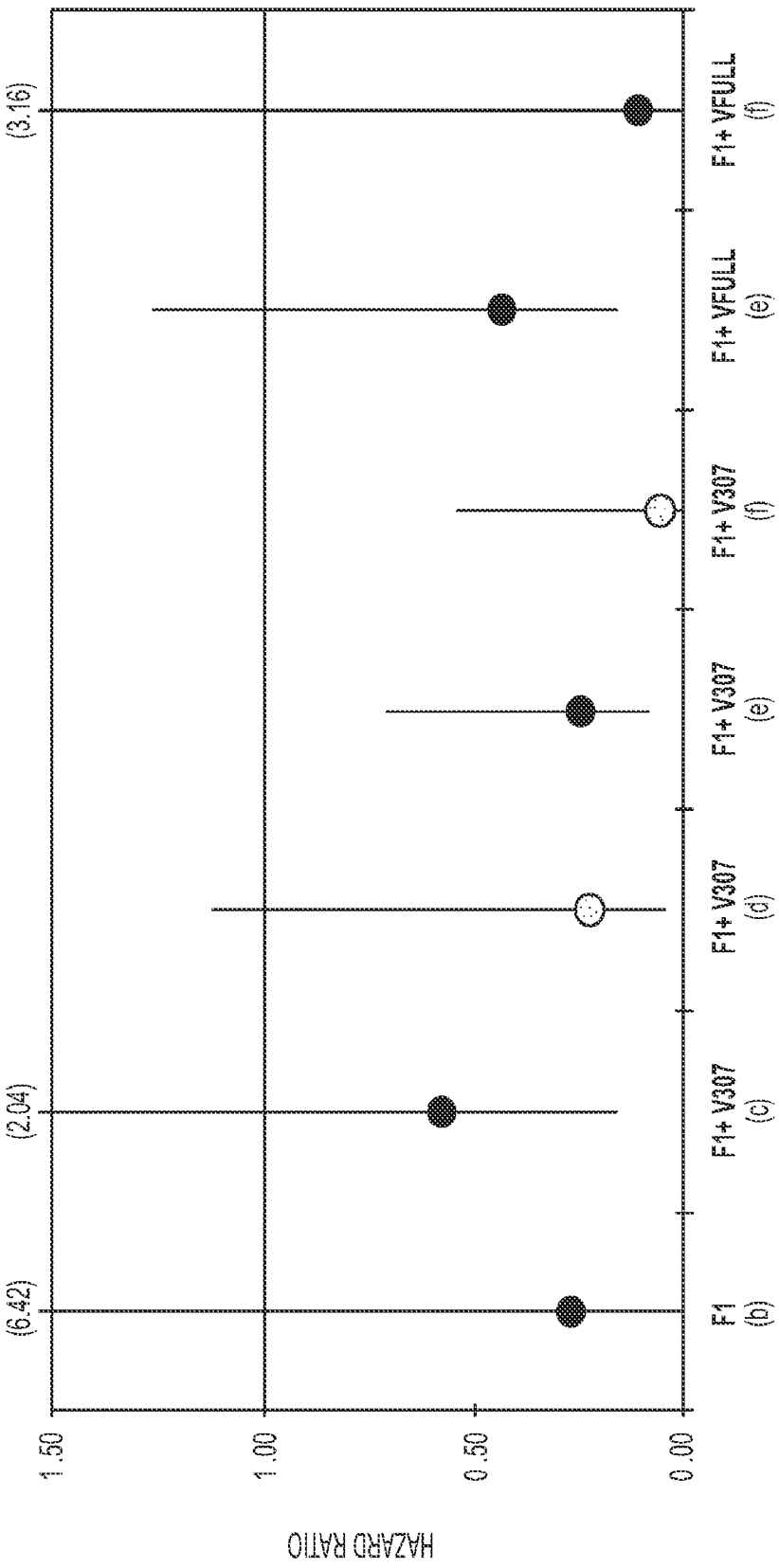
HAZARD RATIO
1.50
1.00
0.50
0.00
(6.42)
(2.04)
(3.16)
F1 (b)
F1+ V307 (c)
F1+ V307 (d)
F1+ V307 (e)
F1+ V307 (f)
F1+ VFULL (e)
F1+ VFULL (f)
VACCINE TREATMENT
(b) 5 x 10^7
(c) 1 x 10^7, 1 x 10^7
(d) 1 x 10^7, 5 x 10^7
(e) 1 x 10^7, 1 x 10^8
(f) 5 x 10^7, 5 x 10^7

FIG. 4

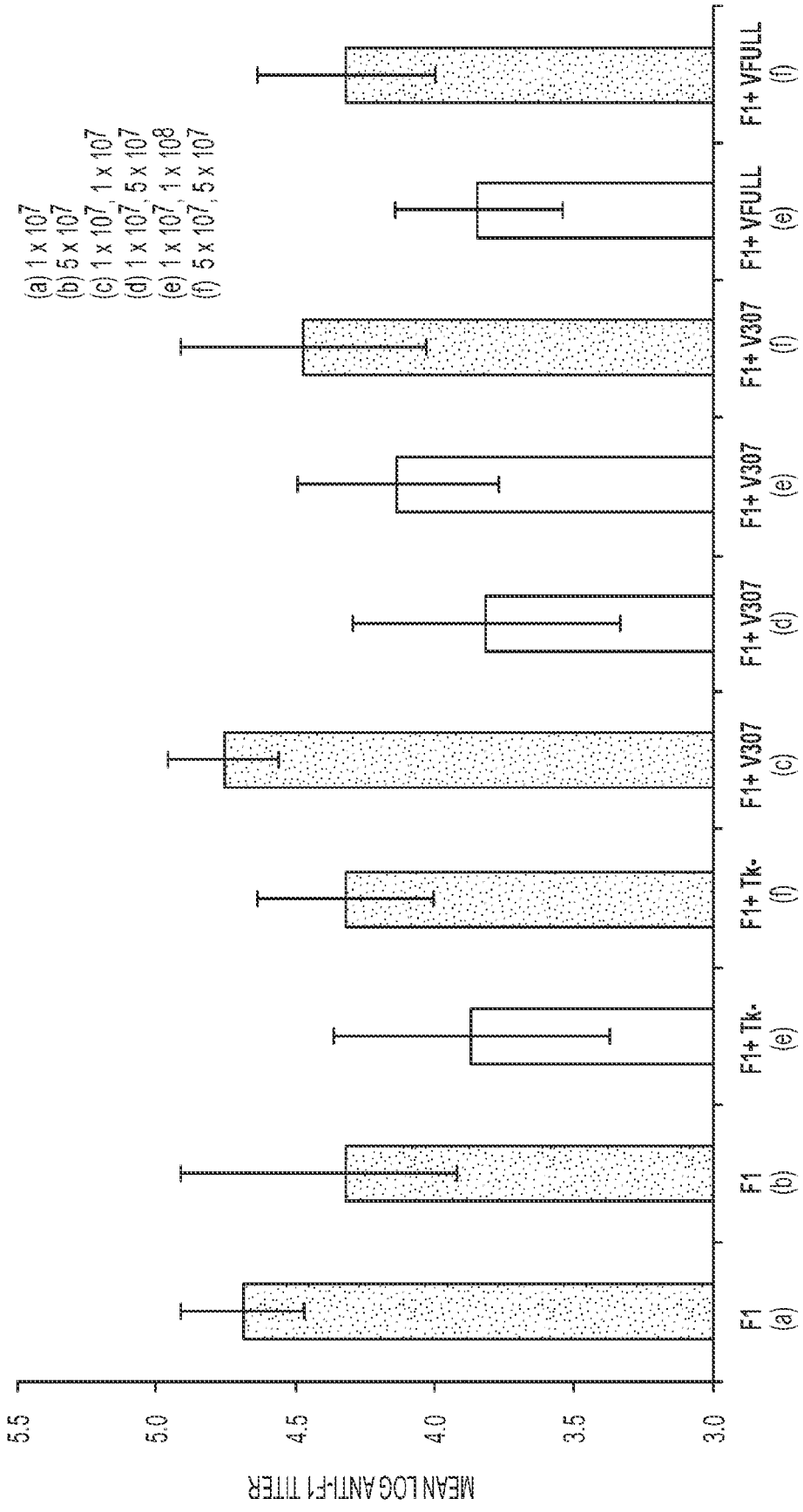
(a) 1 x 10^7
(b) 5 x 10^7
(c) 1 x 10^7, 1 x 10^7
(d) 1 x 10^7, 5 x 10^7
(e) 1 x 10^7, 1 x 10^8
(f) 5 x 10^7, 5 x 10^7
MEAN LOG ANTI-F1 TITER
5.5
5.0
4.5
4.0
3.5
3.0
F1 (a)
F1 (b)
F1+ Tk- (e)
F1+ Tk- (f)
F1+ V307 (c)
F1+ V307 (d)
F1+ V307 (e)
F1+ V307 (f)
F1+ VFULL (e)
F1+ VFULL (f)
VACCINE TREATMENT

FIG. 5

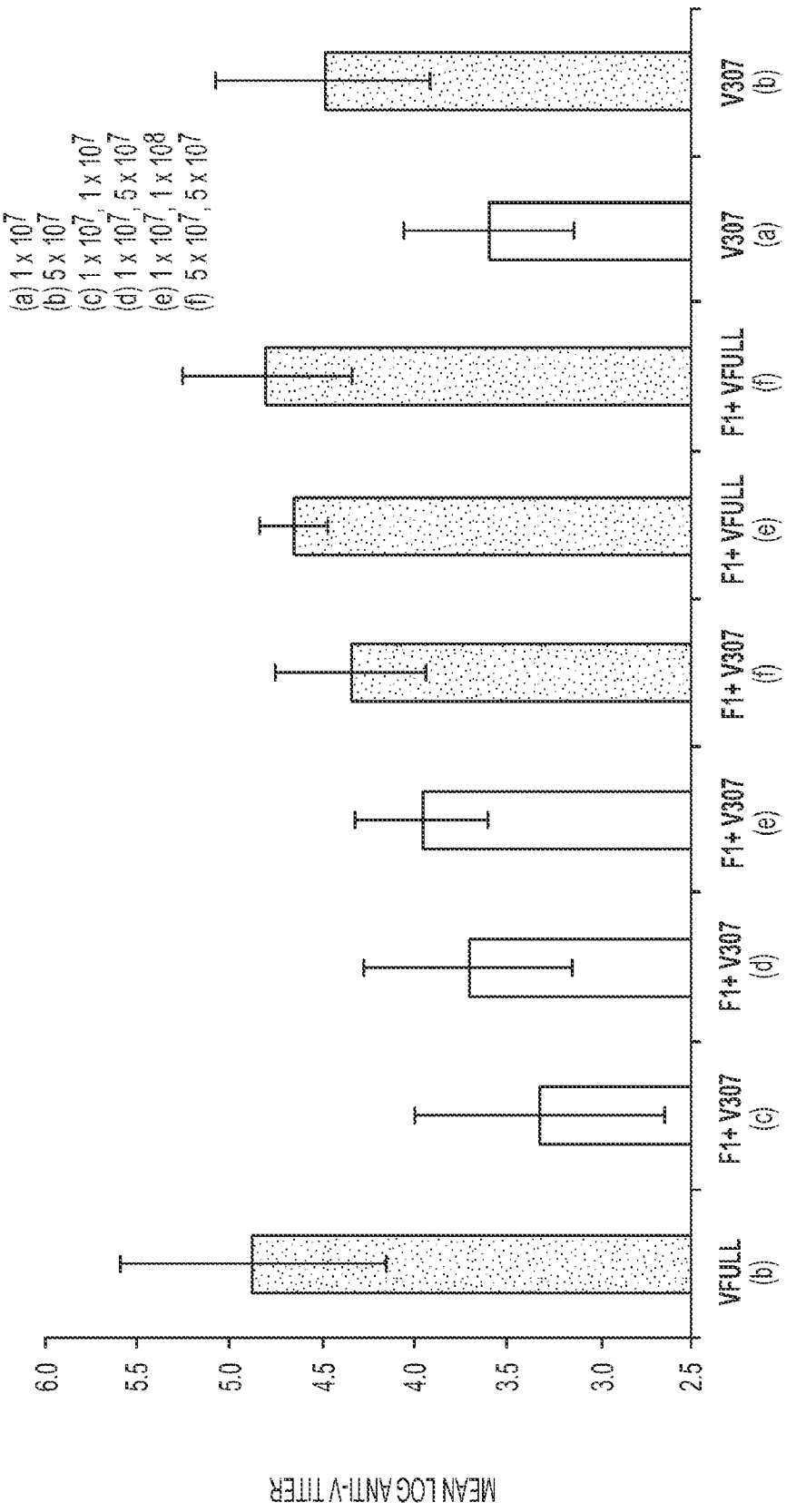
MEAN LOG ANTI-V TITER
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
VACCINE TREATMENT
VFULL (b)
F1+ V307 (c)
F1+ V307 (d)
F1+ V307 (e)
F1+ V307 (f)
F1+ VFULL (e)
F1+ VFULL (f)
V307 (a)
V307 (b)
(a) 1 x 10^7
(b) 5 x 10^7
(c) 1 x 10^7, 1 x 10^7
(d) 1 x 10^7, 5 x 10^7
(e) 1 x 10^7, 1 x 10^8
(f) 5 x 10^7, 5 x 10^7

FIG. 6

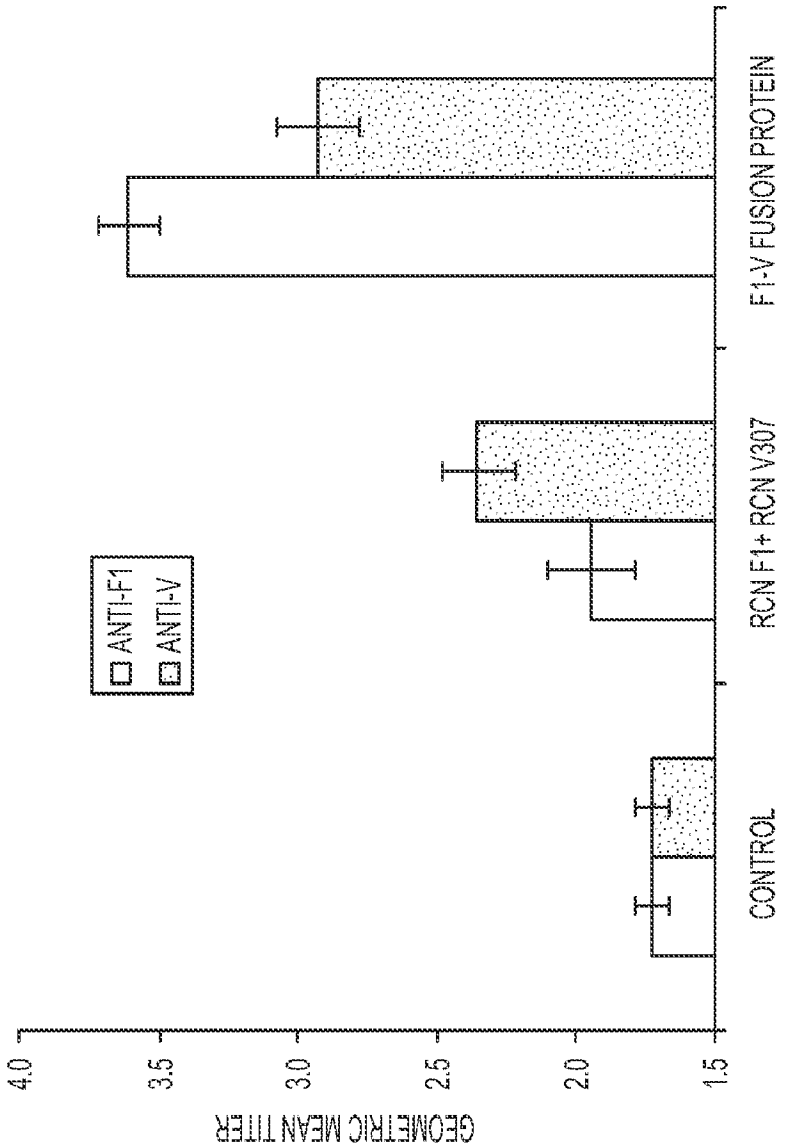
GEOMETRIC MEAN TITER
4.0
3.5
3.0
2.5
2.0
1.5
ANTI-F1
ANTI-V
CONTROL
RCN F1+ RCN V307
F1-V FUSION PROTEIN
TREATMENT

FIG. 7

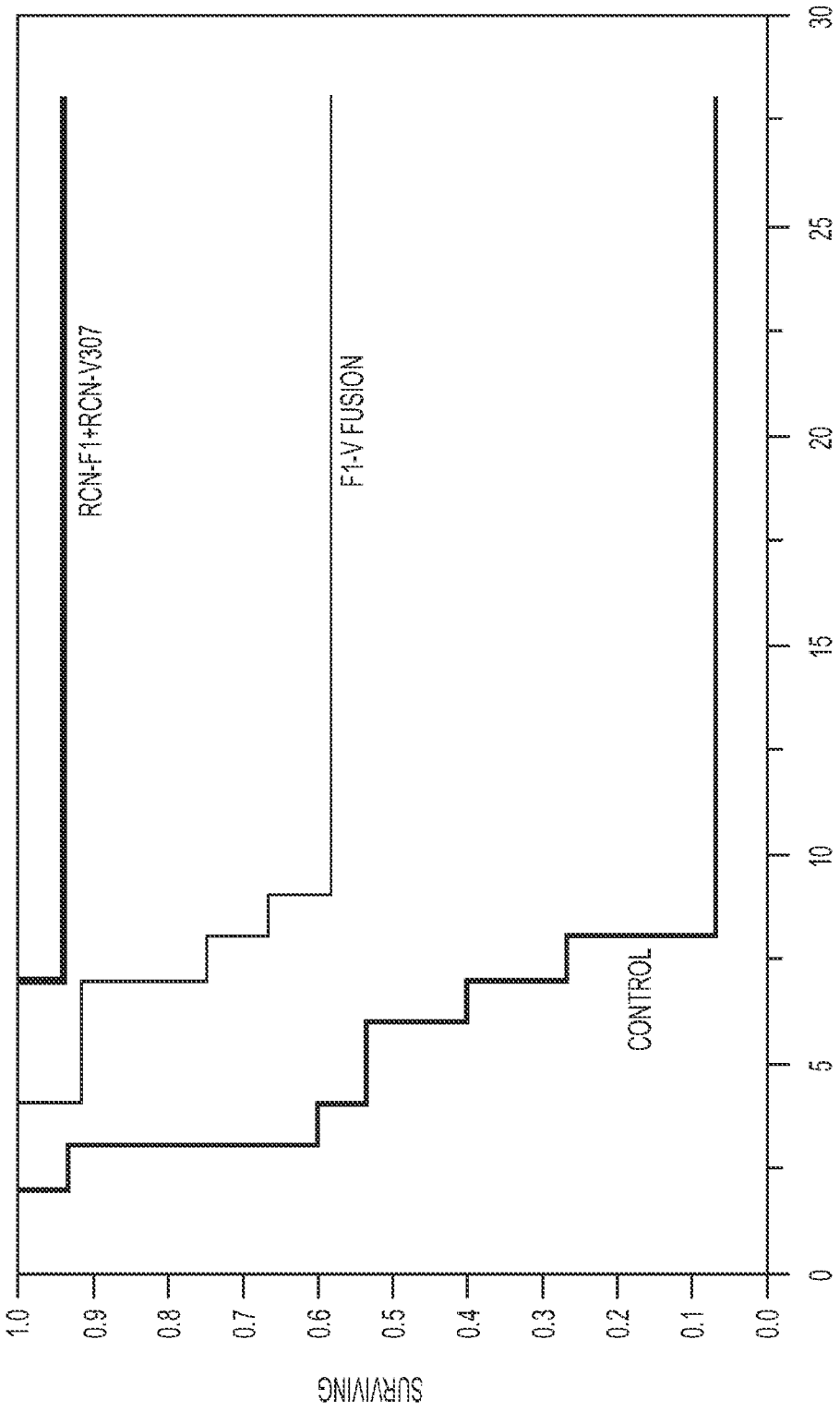
RON-F1+RON-V307
F1-V FUSION
CONTROL
SURVIVING
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
5
10
15
20
25
30
DAY TO DEATH

FIG. 8

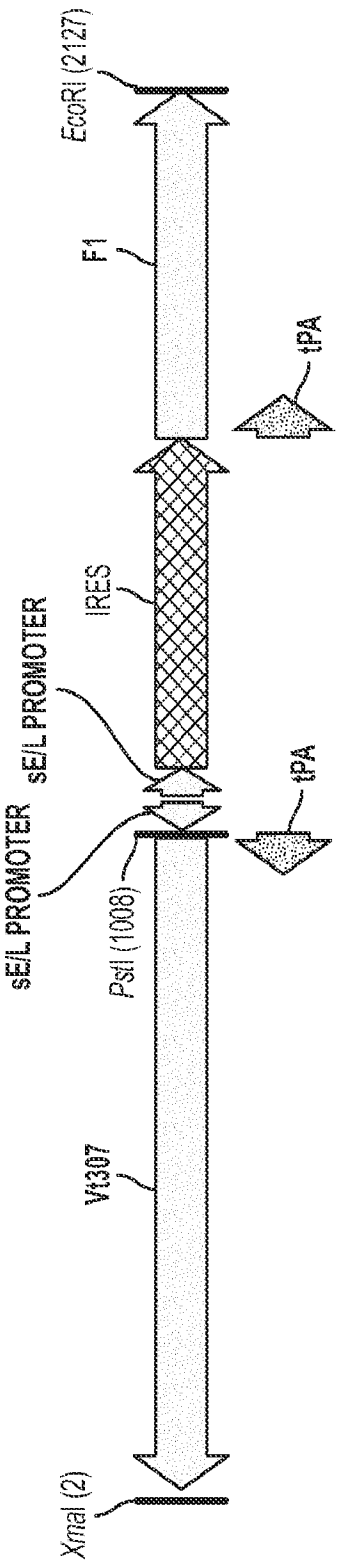
EcoRI (2127)
F1
tPA
IRES
sE/L PROMOTER
sE/L PROMOTER
tPA
PstI (1008)
Vt307
XmaI (2)
IRES-F1/Vt FRAGMENT
2131 bp

FIG. 9

Y. PESTIS LcrV (VFULL)

```
M  I  R  A  Y  E  Q  N  P  Q  H  F  I  E  D  L  E  K  V  R  V  E  Q  L  T  G
atgattagagcctacgaacaaaacccacaacattttattgaggatctagaaaaagttagggtggaacaacttactggt
tactaatctcggatgcttgttttgggtgttgtaaaataactcctagatctttttcaatcccaccttgttgaatgacca

H  G  S  S  V  L  E  E  L  V  Q  L  V  K  D  K  N  I  D  I  S  I  K  Y  D  P
catggttcttcagttttagaagaattggttcagttagtcaaagataaaaatatagatatttccattaaatatgatccc
gtaccaagaagtcaaaatcttcttaaccaagtcaatcagtttctatttttatatctataaaggtaatttatactaggg

R  K  D  S  E  V  F  A  N  R  V  I  T  D  D  I  E  L  L  K  K  I  L  A  Y  F
agaaaagattcggaggtttttgccaatagagtaattactgatgatatcgaattgctcaagaaaatcctagcttatttt
tcttttctaagcctccaaaaacggttatctcattaatgactactatagcttaacgagttcttttaggatcgaataaaa

L  P  E  D  A  I  L  K  G  G  H  Y  D  N  Q  L  Q  N  G  I  K  R  V  K  E  F
ctacccgaggatgccattcttaaaggcggtcattatgacaaccaactgcaaaatggcatcaagcgagtaaaagagttc
gatgggctcctacggtaagaatttccgccagtaatactgttggttgacgttttaccgtagttcgctcatttttctcaag

L  E  S  S  P  N  T  Q  W  E  L  R  A  F  M  A  V  M  H  F  S  L  T  A  D  R
cttgaatcatcgccgaatacacaatgggaattgcgggcgttcatggcagtaatgcatttctctttaaccgccgatcgt
gaacttagtagcggcttatgtgttacccttaacgcccgcaagtaccgtcattacgtaaagagaaattggcggctagca

I  D  D  D  I  L  K  V  I  V  D  S  M  N  H  H  G  D  A  R  S  K  L  R  E  E
atcgatgatgatattttgaaagtgattgttgattcaatgaatcatcatggtgatgcccgtagcaagttgcgtgaagaa
atgctactactataaaactttcactaacaactaagttacttagtagtaccactacgggcatcgttcaacgcacttctt

L  A  E  L  T  A  E  L  K  I  Y  S  V  I  Q  A  E  I  N  K  H  L  S  S  S  G
ttagctgagcttaccgccgaattaaagatttattcagttattcaagccgaaattaataagcatctgtctagtagtggc
aatcgactcgaatggcggcttaatttctaaataagtcaataagttcggctttaattattcgtagacagatcatcaccg

T  I  N  I  H  D  K  S  I  N  L  M  D  K  N  L  Y  G  Y  T  D  E  E  I  F  K
accataaatatccatgataaatccattaatctcatggataaaaatttatatggttatacagatgaagagatttttaaa
tggtatttataggtactatttaggtaattagagtacctatttttaaatataccaatatgtctacttctctaaaaattt

A  S  A  E  Y  K  I  L  E  K  M  P  Q  T  T  I  Q  V  D  G  S  E  K  K  I  V
gccagcgcagagtacaaaattctcgagaaaatgcctcaaaccaccattcaggtggatgggagcgagaaaaaaatagtc
cggtcgcgtctcatgttttaagagctcttttacggagtttggtggtaagtccacctaccctcgctctttttttatcag

S  I  K  D  F  L  G  S  E  N  K  R  T  G  A  L  G  N  L  K  N  S  Y  S  Y  N
tcgataaaggactttcttggaagtgagaataaaagaaccggggcgttgggtaatctgaaaaactcatactcttataat
agctatttcctgaaagaaccttcactcttatttcttggccccgcaacccattagacttttgagtatgagaatatta

K  D  N  N  E  L  S  H  F  A  T  T  C  S  D  K  S  R  P  L  N  D  L  V  S  Q
aaagataataatgaattatctcactttgccaccacctgctcggataagtccaggccgctcaacgacttggttagccaa
tttctattattacttaatagagtgaaacggtggtggacgagcctattcaggtccggcgagttgctgaaccaatcggtt

K  T  T  Q  L  S  D  I  T  S  R  F  N  S  A  I  E  A  L  N  R  F  I  Q  K  Y
aaaacaactcagctgtctgatattacatcacgttttaattcagctattgaagcactgaaccgtttcattcagaaatat
ttttgttgagtcgacagagtagaatgtagtgcaaaattaagtcgataacttcgtgacttggcaaagtaagtctttata

D  S  V  M  Q  R  L  L  D  D  T  S  G  K  *
gattcagtgatgcaacgtctgctagatgacacgtctggtaaatga
ctaagtcactacgttgcagacgatctactgtgcagaccatttact
```

FIG. 10

Y. *PESTIS* Vt(307)

```
M  I  R  A  Y  E  Q  N  P  Q  H  F  I  E  D  L  E  K  V  R  V  E  Q  L  T  G
atgattagagcctacgaacaaaacccacaacattttattgaggatctagaaaaagttagggtggaacaacttactggt
tactaatctcggatgcttgttttgggtgttgtaaaataactcctagatctttttcaatcccaccttgttgaatgacca

H  G  S  S  V  L  E  E  L  V  Q  L  V  K  D  K  N  I  D  I  S  I  K  Y  D  P
catggttcttcagttttagaagaattggttcagttagtcaaagataaaaatatagatatttccattaaatatgatccc
gtaccaagaagtcaaaatcttcttaaccaagtcaatcagtttctatttttatatctataaaggtaatttatactaggg

R  K  D  S  E  V  F  A  N  R  V  I  T  D  D  I  E  L  L  K  K  I  L  A  Y  F
agaaaagattcggaggtttttgccaatagagtaattactgatgatatcgaattgctcaagaaaatcctagcttatttt
tcttttctaagcctccaaaaacggttatctcattaatgactactatagcttaacgagttcttttaggatcgaataaaa

L  P  E  D  A  I  L  K  G  G  H  Y  D  N  Q  L  Q  N  G  I  K  R  V  K  E  F
ctacccgaggatgccattcttaaaggcggtcattatgacaaccaactgcaaaatggcatcaagcgagtaaaagagttc
gatgggctcctacggtaagaatttccgccagtaatactgttggttgacgttttaccgtagttcgctcattttctcaag

L  E  S  S  P  N  T  Q  W  E  L  R  A  F  M  A  V  M  H  F  S  L  T  A  D  R
cttgaatcatcgccgaatacacaatgggaattgcgggcgttcatggcagtaatgcatttctctttaaccgccgatcgt
gaacttagtagcggcttatgtgttacccttaacgcccgcaagtaccgtcattacgtaaagagaaattggcggctagca

I  D  D  D  I  L  K  V  I  V  D  S  N  N  H  H  G  D  A  R  S  K  L  R  E  E
atcgatgatgatattttgaaagtgattgttgattcaatgaatcatcatggtgatgcccgtagcaagttgcgtgaagaa
atgctactactataaaactttcactaacaactaagttacttagtagtaccactacgggcatcgttcaacgcacttctt

L  A  E  L  T  A  E  L  K  I  Y  S  V  I  Q  A  E  I  N  K  H  L  S  S  S  G
ttagctgagcttaccgccgaattaaagatttattcagttattcaagccgaaattaataagcatctgtctagtagtggc
aatcgactcgaatggcggcttaatttctaaataagtcaataagttcggctttaattattcgtagacagatcatcaccg

T  I  N  I  H  D  K  S  I  N  L  M  D  K  N  L  Y  G  Y  T  D  E  E  I  F  K
accataaatatccatgataaatccattaatctcatggataaaaatttatatggttatacagatgaagagatttttaaa
tggtatttataggtactatttaggtaattagagtacctatttttaaatataccaatatgtctacttctctaaaaattt

A  S  A  E  Y  K  I  L  E  K  M  P  Q  T  T  I  Q  V  D  G  S  E  K  K  I  V
gccagcgcagagtacaaaattctcgagaaaatgcctcaaaccaccattcaggtggatgggagcgagaaaaaaatagtc
cggtcgcgtctcatgttttaagagctcttttacggagtttggtggtaagtccacctaccctcgctctttttttatcag

S  I  K  D  F  L  G  S  E  N  K  R  T  G  A  L  G  N  L  K  N  S  Y  S  Y  N
tcgataaaggactttcttggaagtgagaataaaagaaccgggcgttgggtaatctgaaaaactcatactcttataat
agctatttcctgaaagaaccttcactcttatttcttggccccgcaacccattagacttttgagtatgagaatatta

K  D  N  N  E  L  S  H  F  A  T  T  C  S  D  K  S  R  P  L  N  D  L  V  S  Q
aaagataataatgaattatctcactttgccaccacctgctcggataagtccaggccgctcaacgacttggttagccaa
tttctattattacttaatagagtgaaacggtggtggacgagcctattcaggtccggcgagttgctgaaccaatcggtt

                                                                    307
K  T  T  Q  L  S  D  I  T  S  R  F  N  S  A  I  E  A  L  N  R  *
aaaacaactcagctgtctgatattacatcacgttttaattcagctattgaagcactgaacgttga
ttttgttgagtcgacagagtagaatgtagtgcaaaattaagtcgataacttcgtgacttggcaact
```

FIG. 11

IRES-F1/Vt FRAGMENT

```
     XmaI
   1 CCCGGGCGCG CCTCAACGGT TCAGTGCTTC AATAGCTGAA TTAAAACGTG ATGTAATATC AGACAGCTGA GTTGTTTTTT GGCTAACCAA GTCGTTGAGC
     GGGCCCGCGC GGAGTTGCCA AGTCACGAAG TTATCGACTT AATTTTGCAC TACATTATAG TCTGTCGACT CAACAAAAAA CCGATTGGTT CAGCAACTCG
 101 GGCCTGGACT TATCCGAGCA GGTGGTGGCA AAGTGAGATA ATTCATTATT ATCTTTATTA TAAGAGTATG AGTTTTTCAG ATTACCCAAC GCCCCGGTTC
     CCGGACCTGA ATAGGCTCGT CCACCACCGT TTCACTCTAT TAAGTAATAA TAGAAATAAT ATTCTCATAC TCAAAAAGTC TAATGGGTTG CGGGGCCAAG
 201 TTTTATTCTC ACTTCCAAGA AAGTCCTTTA TCGAGACTAT TTTTTTCTCG CTCCCATCCA CCTGAATGGT GGTTTGAGGC ATTTTCTCGA GAATTTTGTA
     AAAATAAGAG TGAAGGTTCT TTCAGGAAAT AGCTCTGATA AAAAAAGAGC GAGGGTAGGT GGACTTACCA CCAAACTCCG TAAAAGAGCT CTTAAAACAT
 301 CTCTGCGCTG GCTTTAAAAA TCTCTTCATC TGTATAACCA TATAAATTTT TATCCATGAG ATTAATGGAT TTATCATGGA TATTTATGGT GCCACTACTA
     GAGACGCGAC CGAAATTTTT AGAGAAGTAG ACATATTGGT ATATTTAAAA ATAGGTACTC TAATTACCTA AATAGTACCT ATAAATACCA CGGTGATGAT
 401 GACAGATGCT TATTAATTTC GGCTTGAATA ACTGAATAAA TCTTTAATTC GGCGGTAAGC TCAGCTAATT CTTCACGCAA CTTGCTACGG GCATCACCAT
     CTGTCTACGA ATAATTAAAG CCGAACTTAT TGACTTATTT AGAAATTAAG CCGCCATTCG AGTCGATTAA GAAGTGCGTT GAACGATGCC CGTAGTGGTA
 501 GATGATTCAT TGAATCAACA ATCACTTTCA AAATATCATC ATCGATACGA TCGGCGGTTA AAGAGAAATG CATTACTGCC ATGAACGCCC GCAATTCCCA
     CTACTAAGTA ACTTAGTTGT TAGTGAAAGT TTTATAGTAG TAGCTATGCT AGCCGCCAAT TTCTCTTTAC GTAATGACGG TACTTGCGGG CGTTAAGGGT
 601 TTGTGTATTC GGCGATGATT CAAGGAACTC TTTTACTCGC TTGATGCCAT TTTGCAGTTG GTTGTCATAA TGACCGCCTT TAAGAATGGC ATCCTCGGGT
     AACACATAAG CCGCTACTAA GTTCCTTGAG AAAATGAGCG AACTACGGTA AAACGTCAAC CAACAGTATT ACTGGCGGAA ATTCTTACCG TAGGAGCCCA
 701 AGAAAATAAG CTAGGATTTT CTTGAGCAAT TCGATATCAT CAGTAATTAC TCTATTGGCA AAAACCTCCG AATCTTTTCT GGCATCATAT TTAATGGAAA
     TCTTTTATTC GATCCTAAAA GAACTCGTTA AGCTATAGTA GTCATTAATG AGATAACCGT TTTTGGAGGC TTAGAAAAGA CCCTAGTATA AATTACCTTT
 801 TATCTATATT TTTATCTTTG ACTAACTGAA CCAATTCTTC TAAAACTGAA GAACCATGAC CAGTAAGTTG TTCCACCCTA ACTTTTTCTA GATCCTCAAT
     ATAGATATAA AAATAGAAAC TGATTGACTT GGTTAAGAAG ATTTTGACTT CTTGGTACTG GTCATTCAAC AAGGTGGGAT TGAAAAAGAT CTAGGAGTTA
 901 AAAATGTTGT GGGTTTTGTT CGTAGGCTCT AATGCCGGCA GAAACGAAGA CTGCTCCACA CAGCAGCAGC ACACAGCAGA GCCCTCTCTT CATTGCATCC
     TTTTACAACA CCCAAAACAA GCATCCGAGA TTACGGCCGT CTTTGCTTCT GACGAGGTGT GTCGTCGTCG TGTGTCGTCT CGGGAGAGAA GTAACGTAGG
       PstI
1001 ATCTGCAGTA TTTATATTCC AAAAAAAAAA AATAAAATTT CAATTTTTCC TAGTAAAAAT TGAAATTTTA TTTTTTTTTT TTGGAATATA AATAGTCGAC
     TAGACGTCAT AAATATAAGG TTTTTTTTTT TTATTTTAAA GTTAAAAAGG ATCATTTTTA ACTTTAAAAT AAAAAAAAAA AACCTTATAT TTATCAGCTG
1101 GTTATTTTCC ACCATATTGC CGTCTTTTGG CAATGTGAGG GCCCGGAAAC CTGGCCCTGT CTTCTTGACG AGCATTCCTA GGGGTCTTTC CCCTCTCGCC
     CAATAAAAGG TGGTATAACG GCAGAAAACC GTTACACTCC CGGGCCTTTG GACCGGGACA GAAGAACTGC TCGTAAGGAT CCCCAGAAAG GGGAGAGCGG
1201 AAAGGAATGC AAGGTCTGTT GAATGTCGTG AAGGAAGCAG TTCCTCTGGA AGCTTCTTGA AGACAAACAA CGTCTGTAGC GACCCTTTGC AGGCAGCGGA
     TTTCCTTACG TTCCAGACAA CTTACAGCAC TTCCTTCGTC AAGGAGACCT TCGAAGAACT TCTGTTTGTT GCAGACATCG CTGGGAAACG TCCGTCGCCT
1301 ACCCCCCACC TGGCGACAGG TGCCTCTGCG GCCAAAAGCC ACGTGTATAA GATACACCTG CAAAGGCGGC ACAACCCCAG TGCCACGTTG TGAGTTGGAT
     TGGGGGGTGG ACCGCTGTCC ACGGAGACGC CGGTTTTCGG TGCACATATT CTATGTGGAC GTTTCCGCCG TGTTGGGGTC ACGGTGCAAC ACTCAACCTA
1401 AGTTGTGGAA AGAGTCAAAT GGCTCTCCTC AAGCGTATTC AACAAGGGGC TGAAGGATGC CCAGAAGGTA CCCCATTGTA TGGGATCTGA TCTGGGGCCT
     TCAACACCTT TCTCAGTTTA CCGAGAGGAG TTCGCATAAG TTGTTCCCCG ACTTCCTACG GGTCTTCCAT GGGGTAACAT ACCCTAGACT AGACCCCGGA
1501 CGGTGCACAT GCTTTACATG TGTTTAGTCG AGGTTAAAAA AACGTCTAGG CCCCCCGAAC CACGGGGACG TGGTTTTCCT TTGAAAAACA CGATGATAAT
     GCCACGTGTA CGAAATGTAC ACAAATCAGC TCCAATTTTT TTGCAGATCC GGGGGGCTTG GTGCCCCTGC ACCAAAAGGA AACTTTTTGT GCTACTATTA
1601 ATGGATGCAA TGAAGAGAGG GCTCTGCTGT GTGCTGCTGC TGTGTGGAGC AGTCTTCGTT TCTGCCGGCG CAGATTTAAC TGCAAGCACC ACTGCAACGG
     TACCTACGTT ACTTCTCTCC CGAGACGACA CACGACGACG ACACACCTCG TCAGAAGCAA AGACGGCCGC GTCTAAATTG ACGTTCGTGG TGACGTTGCC
1701 CAACTCTTGT TGAACCAGCC CGCATCACTC TTACATATAA GGAAGGCGCT CCAATTACAA TTATGGACAA TGGAAACATC GATACAGAAT TACTTGTTGG
     GTTGAGAACA ACTTGGTCGG GCGTAGTGAG AATGTATATT CCTTCCGCGA GGTTAATGTT AATACCTGTT ACCTTTGTAG CTATGTCTTA ATGAACAACC
1801 TACGCTTACT CTTGGCGGCT ATAAAACAGG AACCACTAGC ACATCTGTTA ACTTTACAGA TGCCGCGGGT GATCCCATGT ACTTAACATT TACTTCTCAG
     ATGCGAATGA GAACCGCCGA TATTTTGTCC TTGGTGATCG TGTAGACAAT TGAAATGTCT ACGGCGCCCA CTAGGGTACA TGAATTGTAA ATGAAGAGTC
1901 GATGGAAATA ACCACCAATT CACTACAAAA GTGATTGGCA AGGATTCTAG AGATTTTGAT ATCTCTCCTA AGGTAAACGG TGAGAACCTT GTGGGGGATG
     CTACCTTTAT TGGTGGTTAA GTGATGTTTT CACTAACCGT TCCTAAGATC TCTAAAACTA TAGAGAGGAT TCCATTTGCC ACTCTTGGAA CACCCCCTAC
2001 ACGTCGTCTT GGCTACGGGC AGCCAGGATT TCTTTGTTCG CTCAATTGGT TCCAAAGGCG GTAAACTTGC AGCAGGTAAA TACACTGATG CTGTAACCGT
     TGCAGCAGAA CCGATGCCCG TCGGTCCTAA AGAAACAAGC GAGTTAACCA AGGTTTCCGC CATTTGAACG TCGTCCATTT ATGTGACTAC GACATTGGCA
                                 EcoRI
2101 AACCGTATCT AACCAAGGAT CCTAAGAATT C
     TTGGCATAGA TTGGTTCCTA GGATTCTTAA G
```

FIG. 12

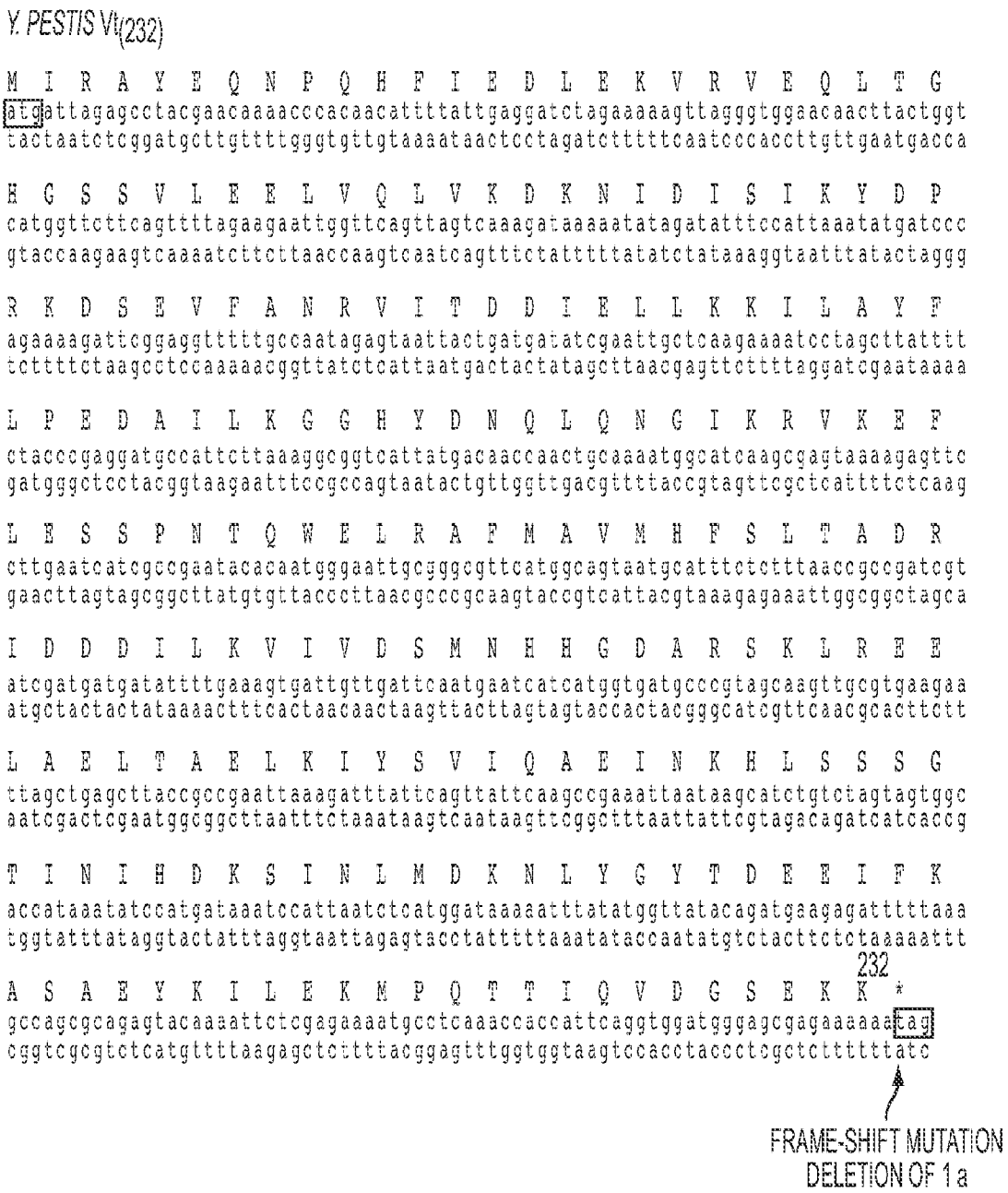
Y. PESTIS Vt(232)
M I R A Y E Q N P Q H F I E D L E K V R V E Q L T G
atgattagagcctacgaacaaaacccacaacattttattgaggatctagaaaaagttagggtggaacaacttactggt
tactaatctcggatgcttgttttgggtgttgtaaaataactcctagatctttttcaatcccaccttgttgaatgacca
H G S S V L E E L V Q L V K D K N I D I S I K Y D P
catggttcttcagttttagaagaattggttcagttagtcaaagataaaaatatagatatttccattaaatatgatccc
gtaccaagaagtcaaaatcttcttaaccaagtcaatcagtttctatttttatatctataaaggtaatttatactaggg
R K D S E V F A N R V I T D D I E L L K K I L A Y F
agaaaagattcggaggtttttgccaatagagtaattactgatgatatcgaattgctcaagaaaatcctagcttatttt
tcttttctaagcctccaaaaacggttatctcattaatgactactatagcttaacgagttcttttaggatcgaataaaa
L P E D A I L K G G H Y D N Q L Q N G I K R V K E F
ctacccgaggatgccattcttaaaggcggtcattatgacaaccaactgcaaaatggcatcaagcgagtaaaagagttc
gatgggctcctacggtaagaatttccgccagtaatactgttggttgacgttttaccgtagttcgctcattttctcaag
L E S S P N T Q W E L R A F M A V M H F S L T A D R
cttgaatcatcgccgaatacacaatgggaattgcgggcgttcatggcagtaatgcatttctctttaaccgccgatcgt
gaacttagtagcggcttatgtgttacccttaacgcccgcaagtaccgtcattacgtaaagagaaattggcggctagca
I D D D I L K V I V D S M N H H G D A R S K L R E E
atcgatgatgatattttgaaagtgattgttgattcaatgaatcatcatggtgatgcccgtagcaagttgcgtgaagaa
atgctactactataaaactttcactaacaactaagttacttagtagtaccactacgggcatcgttcaacgcacttctt
L A E L T A E L K I Y S V I Q A E I N K H L S S S G
ttagctgagcttaccgccgaattaaagatttattcagttattcaagccgaaattaataagcatctgtctagtagtggc
aatcgactcgaatggcggcttaatttctaaataagtcaataagttcggctttaattattcgtagacagatcatcaccg
T I N I H D K S I N L M D K N L Y G Y T D E E I F K
accataaatatccatgataaatccattaatctcatggataaaaatttatatggttatacagatgaagagatttttaaa
tggtatttataggtactatttaggtaattagagtacctatttttaaatataccaatatgtctacttctctaaaaattt
232
A S A E Y K I L E K M P Q T T I Q V D G S E K K *
gccagcgcagagtacaaaattctcgagaaaatgcctcaaaccaccattcaggtggatgggagcgagaaaaaatag
cggtcgcgtctcatgttttaagagctctttacggagtttggtggtaagtccacctaccctcgctctttttatc
FRAME-SHIFT MUTATION
DELETION OF 1 a

FIG. 13

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 10/55229

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 9/68 (2011.01)
USPC - 424/93.48
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)
USPC: 424/93.48
IPC(8): A61K 9/68 (2011.01)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 424/184.1

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Dialog Classic (EPO, WIPO, US Patents, Biotech, Medline); PubWEST (PGPB,USPT, etc.); Google Patents; Free Patents Online: vaccine, Y. pestis, antigen, excipient, adjuvant, orally, polypeptide, container

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2009/009759 A2 (YUSIBOV et al.) 15 January 2009 (15.01.2009) para [0012], [0040], [0063], [0199]-[0200], [0204], [0208], [0213], [0219], [0223], [0225], [0234], [0240], [0245], [0250], [0253], [0258], [0269], [0283]; SEQ ID NO: 47; claim 2	1-6, 28-33 and 37
A	WO 2006/060728 A2 (SCHNEEWIND et al.) 08 June 2006 (08.06.2006)	1-6, 28-30 and 32-33

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

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

Date of the actual completion of the international search	Date of mailing of the international search report
09 February 2011 (09.02.2011)	09 MAR 2011
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. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 10/55229

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.: 34-36 and 38
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.

Group I: claims 1-6, 28-33, 37, drawn to an isolated polypeptide having the amino acid sequence of SEQ 10 NO: 3, or a conservatively substituted variant thereof exhibiting Y. pestis antigenic behavior.

Group II, claims 7-18, 28-33, 37, drawn to an isolated oligonucleotide having the nucleotide sequence of SEQ ID NO: 4, or a conservatively substituted variant thereof exhibiting Y. pestis antigenic behavior, and a recombinant raccoon poxvirus comprising said SEQ ID NO:4.

**********See Supplemental Box to continue **********

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.:
1-6, 28-33 and 37

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
Information on patent family members

International application No.
PCT/US 10/55229

********** Supplemental Box **********

Continuation from Box III. LOU:

Group III, claims 19-33, 37, drawn to a recombinant raccoon poxvirus comprising a recombinant poxvirus genome having at least one heterologous nucleic acid molecule integral to SEQ IDs NO: 5.

The inventions listed as Groups I-III do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The inventions of Groups I do not include the inventive concept of a recombinant raccoon poxvirus, as required by Groups II-III. In addition, US 2006/0246084 A1 to BRUBAKER, et al. discloses, in the context of Yersinia polypeptide vaccines, antibodies and immunomodulatory proteinS (title), the claimed polypeptide having the amino acid seqeunce of SEQ ID NO:3 (amino acids 1-307 of SEQ ID NO 5, length 326 amino acids).

The inventions of Groups II-III share the technical feature of a recombinant raccoon poxvirus. However, this shared technical feature does not represent a contribution over prior art as being anticipated by a paper titled "Protection of black-tailed prairie dogs (Cynomys ludovicianus) against plague after voluntary consumption of baits containing recombinant raccoon poxvirus vaccine" by Mencher, et al. (Infect Immun. 2004, 72(9):5502-5) that discloses "[a] recombinant raccoon poxvirus, expressing the F1 antigen of Y. pestis...". (Abstract). As said recombinant raccoon poxvirus was known at the time of the invention, this cannot be considered a special technical feature that would otherwise unify the groups.

Another technical feature of the inventions listed as Group II-III is the specific nucleic acid sequence recited therein. As no significant structural similarities can readily be ascertained among the amino acid sequences, the inventions do not share a special technical feature. Without a shared special technical feature, the inventions lack unity with one another.

Groups I-III therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.