

RECOMBINANT EQUINE HERPESVIRUS-1 VACCINE CONTAINING MUTATED GLYCOPROTEIN C AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

5 [0001] This application claims benefit of US provisional application Serial No. 61/613,151 filed March 20, 2012.

FIELD OF THE INVENTION

[0002] The present invention relates to compositions or vaccines for combating equine
10 herpesvirus infections in animals. Specifically, the present invention provides compositions or vaccines that contain a recombinant EHV-1 that elicit an immune response in animals against equine herpesvirus, including compositions comprising said recombinant EHV-1, methods of vaccination against equine herpesvirus, and kits for use with such methods and compositions.

15 BACKGROUND OF THE INVENTION

[0003] Equine Herpesvirus type 1 (EHV-1) is one of the most important and prevalent pathogens of equine populations worldwide (Ma et al., J. of General Virology 91, 1817-1822, 2010). Together with its close relatives varicella-zoster virus, bovine Herpesvirus type 1, pseudorabies virus and EHV-4, EHV-1 forms the genus *Varicellovirus* in the subfamily *Alphaherpesvirinae* of
20 the family *Herpesviridae* of the order *Herpesvirales* (Davison et al., The order *Herpesvirales*. Arch Virol 154, 171-177, 2009). Diseases caused by EHV-1 range from mild rhinopneumonitis and abortion in pregnant mares to neurological disease that is frequently lethal in affected horses (Allen et al., Prog Vet Microbiol Immunol 2, 78-144, 1986; Carroll et al., Aust Vet J 62, 345-346, 1985; Crabb et al., Adv Virus Res 45, 153-190, 1995). The pathogenesis of EHV infection
25 is very complex. Natural infection occurs through inhalation or ingestion of the infectious virus. Within a few days of the virus can be found in leucocytes, where it is protected from recognition and attacks by the immune system. The virus disseminates via cell-associated viremia to secondary sites of replication (Allen et al., Proceedings 8th Equine Infectious Disease Conference, Dubai 23-26, pp 129-146, 1998).

30 [0004] EHV-1 harbors a 150kb double-stranded DNA genome that is highly conserved among strains. A neuropathogenic strain Ab4 (GenBank accession No. AY665713) and a nonneuropathogenic strain V592 (GenBank accession No. AY464052) were extensively

characterized and showed a nucleotide variation rate of approximately 0.1% (Nugent et al., J. Virol 80, 4047-4060, 2006). It was found that only a minority of EHV-1 strains are capable of inducing neurological disorders, although all strains can cause respiratory disease and abortion (Mumford et al., J. Reprod Fertil Suppl 35, 509-518, 1987; Ostlund, Vet Clin North Am Equine Pract 9, 283-294, 1993; Wilson, Vet Clin North Am Equine Pract 13, 53-72, 1997). Recently, epidemiological as well as reverse-genetic studies have shown that a single-nucleotide polymorphism at position 2254 (G/A2254) of open reading frame 30 (ORF30), encoding viral DNA polymerase (Pol), will lead to a variation at the amino acid position 752 (D/N752), which is associated with the virus's neuropathogenic potential (Goodman et al., J Biol Chem 281, 18193-18200, 2007; Van de Walle et al., J. Infect Dis 200, 20-25, 2009; Smith et al., Vet. Microbiol., 141, 5-11, 2010). It has been shown that residue 752 in the essential Pol of EHV-1 is not required for virus growth and that the N752 mutation confers a drug-sensitive phenotype to the virus (Ma et al., 2010).

[0005] Glycoprotein C (gC) of EHV-1 was shown to play important role in the early steps of infection and in release of virions (Osterrieder, Virus Research 2, 165, 1999). Glycoprotein C of EHV-1 is non-essential for virus growth. It mediates primary attachment, and is required for efficient virus replication in primary equine cells (Osterrieder, 1999).

[0006] Conventional killed vaccines provide only partial clinical and virological protection against respiratory infections, but do not prevent cell-associated viremia. Considering the susceptibility of animals, including humans, to herpesvirus, a means of preventing herpesvirus infection and protecting animals is essential. Accordingly, there is a need for an effective vaccine against herpesvirus.

[0007] Citation or identification of any document in this application is not an admission that such document is available as prior art to the present invention.

SUMMARY OF THE INVENTION

[0008] The invention provides a composition or vaccine that contains a recombinant Equine Herpesvirus -1 (EHV-1). In particular, the present invention provides a recombinant EHV-1 that contains a mutated Glycoprotein C (gC) gene that is non-functional. The gC gene of the recombinant EHV-1 may be deleted. The gC gene may encode a mutated gC protein wherein the N-terminal region of the gC protein is deleted. The recombinant EHV-1 may further comprise a

DNA polymerase (Pol) gene encoding a Pol having an asparagine (N) at the amino acid position 752. The recombinant EHV-1 may comprise a DNA polymerase (Pol) gene encoding a Pol having an asparagine (N) at the amino acid position 752. The EHV-1 may be EHV-1 RacL strain.

5 [0009] The invention provides methods for inducing an immunogenic or protective response against EHV-1, as well as methods for preventing EHV-1 or disease state(s) caused by EHV-1, comprising administering the composition or vaccine of the present invention. The invention also provides methods of vaccinating an animal comprising at least one administration of the composition or recombinant EHV-1.

10 [0010] These and other embodiments are disclosed or are obvious from and encompassed by, the following Detailed Description.

BRIEF DESCRIPTION OF DRAWINGS

[0011] The following detailed description, given by way of example, and which is not intended
15 to limit the invention to specific embodiments described, may be understood in conjunction with the accompanying figures, incorporated herein by reference, in which:

[0012] Figure 1 is the table showing the corresponding SEQ ID NO assigned to polynucleotide and protein sequences.

[0013] Figures 2A-2C depict the cloning scheme. Figure 2D shows the amino acid mutation in
20 EHV-1 polymerase.

[0014] Figures 3A-3B show the indirect immunofluorescence assay (IFA) of RK13-Pol and RK13.

[0015] Figures 4A-4B depict the growth of L11- Δ Pol EYFP in RK13-Pol v. L11- Δ Pol EYFP in RK13.

25 [0016] Figures 5A-5F depict the IFA of RacL11, L11_D752N Δ gC and L11_D752N Δ gC rev using anti-EHV-1 gC MAb.

[0017] Figure 6 depicts the *in vitro* growth of RacL11, L11_D752N, L11_D752N Δ gC and L11_D752N Δ gC rev measured by attachment assay.

[0018] Figure 7 depicts the *in vitro* growth of RacL11, L11_D752N, L11_D752N Δ gC and
30 L11_D752N Δ gC rev determined by comparing plaque sizes.

[0019] Figure 8 depicts the growth kinetics of RacL11, L11_D752N, L11_D752NΔgC and L11_D752NΔgC rev by extracellular and intracellular titers.

[0020] Figures 9A and 9B depict the mean virus titers in lungs infected with RacL11, L11_D752N, L11_D752NΔgC, L11_D752N ΔgC rev 2 and PBS (control) 2 and 4 days post infection (p.i.), 2 and 4 days post challenge (p.c).

[0021] Figure 10 depicts the histopathological changes of the lungs of mice infected with RacL11, L11_D752N, L11_D752NΔgC, L11_D752N ΔgC rev on day 2 p.i.

[0022] Figures 11A-11B depict the serology against EHV-1 using the complement fixation (CF) and virus neutralization (VN) tests.

10 [0023] Figure 12 depicts the duration of viraemia in vaccinated horses.

[0024] Figure 13 depicts the virus shedding in nasal swabs from the vaccinated horses.

[0025] Figures 14A-14C show the polynucleotide and protein sequence alignments of EHV-1 DNA polymerase.

[0026] Figures 15A-15B show the polynucleotide and protein sequence alignments of EHV-1 glycoprotein C.

15 [0027] Figure 16 shows the DNA and protein sequences.

DETAILED DESCRIPTION

[0028] It is noted that in this disclosure and particularly in the claims, terms such as “comprises”, “comprised”, “comprising” and the like can have the meaning attributed to it in U.S. Patent law; e.g., they can mean “includes”, “included”, “including”, and the like; and that terms such as “consisting essentially of” and “consists essentially of” have the meaning ascribed to them in U.S. Patent law, e.g., they allow for elements not explicitly recited, but exclude elements that are found in the prior art or that affect a basic or novel characteristic of the invention.

[0029] Unless otherwise noted, technical terms are used according to conventional usage. Definitions of common terms in molecular biology may be found in Benjamin Lewin, Genes V. published by Oxford University Press, 1994 (ISBN 0-19-854287-9); Kendrew et al. (eds.), The Encyclopedia of Molecular Biology, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Robert A. Meyers (ed.), Molecular Biology and Biotechnology: a Comprehensive Desk Reference, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8).

[0030] The singular terms “a”, “an”, and “the” include plural referents unless context clearly indicates otherwise. Similarly, the word “or” is intended to include “and” unless the context clearly indicates otherwise. The word “or” means any one member of a particular list and also includes any combination of members of that list.

5 [0031] The term “EHV-1 N strain” as used herein refers to any EHV-1 strain which has an asparagine (N) at the amino acid position 752 of its DNA polymerase. The EHV-1 N strain may be wild-type strain that comprises a DNA polymerase comprising an asparagine (N) at the amino acid position 752. The EHV-1 N strain may be mutated or recombinant EHV-1 strain wherein the DNA polymerase is engineered to have an asparagine (N) at the amino acid position 752.

10 [0032] The term “EHV-1 D strain” as used herein refers to any EHV-1 strain which has an Aspartic acid (D) at the amino acid position 752 of its DNA polymerase. The EHV-1 D strain may be a wild-type strain that comprises a DNA polymerase comprising an aspartic acid (D) at the amino acid position 752. The EHV-1 D strain may be a mutated or recombinant EHV-1 strain wherein the DNA polymerase is engineered to have an aspartic acid (D) at the amino acid
15 position 752.

[0033] By “animal” is intended mammals, human, birds, and the like. The animal may be selected from the group consisting of equine (e.g., horse, zebra, donkey), canine (e.g., dogs, wolves, foxes, coyotes, jackals), feline (e.g., lions, tigers, domestic cats, wild cats, other big cats, and other feline including cheetahs and lynx), ovine (e.g., sheep), bovine (e.g., cattle, cow,
20 buffalo), swine (pig), avian (e.g., chicken, duck, goose, turkey, quail, pheasant, parrot, finches, hawk, crow, ostrich, emu and cassowary), primate (e.g., prosimian, tarsier, monkey, gibbon, ape), and fish. The term “animal” also includes an individual animal in all stages of development, including embryonic and fetal stages.

[0034] The terms “polypeptide” and “protein” are used interchangeably herein to refer to a
25 polymer of consecutive amino acid residues.

[0035] The term “nucleic acid”, “nucleotide”, and “polynucleotide” refers to RNA or DNA and derivatives thereof, such as those containing modified backbones. It should be appreciated that the invention provides polynucleotides comprising sequences complementary to those described herein. Polynucleotides according to the invention can be prepared in different ways (e.g. by
30 chemical synthesis, by gene cloning etc.) and can take various forms (e.g. linear or branched, single or double stranded, or a hybrid thereof, primers, probes etc.).

[0036] The term “gene” is used broadly to refer to any segment of polynucleotide associated with a biological function. Thus, genes or polynucleotides include introns and exons as in genomic sequence, or just the coding sequences as in cDNAs, such as an open reading frame (ORF), starting from the start codon (methionine codon) and ending with a termination signal (stop codon). Genes and polynucleotides can also include regions that regulate their expression, such as transcription initiation, translation and transcription termination. Thus, also included are promoters and ribosome binding regions (in general these regulatory elements lie approximately between 60 and 250 nucleotides upstream of the start codon of the coding sequence or gene; Doree S M *et al.*; Pandher K *et al.*; Chung J Y *et al.*), transcription terminators (in general the terminator is located within approximately 50 nucleotides downstream of the stop codon of the coding sequence or gene; Ward C K *et al.*). Gene or polynucleotide also refers to a nucleic acid fragment that expresses mRNA or functional RNA, or encodes a specific protein, and which includes regulatory sequences.

[0037] As used herein, the term “antigen” or “immunogen” means a substance that induces a specific immune response in a host animal. The antigen may comprise a whole organism, killed, attenuated or live; a subunit or portion of an organism; a recombinant vector containing an insert expressing an epitope, polypeptide, peptide, protein, or fragment thereof with immunogenic properties; a piece or fragment of nucleic acid capable of inducing an immune response upon presentation to a host animal; a protein, a polypeptide, a peptide, an epitope, a hapten, or any combination thereof. Alternately, the immunogen or antigen may comprise a toxin or antitoxin.

[0038] By definition, an epitope is an antigenic determinant that is immunologically active in the sense that once administered to the host, it is able to evoke an immune response of the humoral (B cells) and/or cellular type (T cells). These are particular chemical groups or peptide sequences on a molecule that are antigenic. An antibody specifically binds a particular antigenic epitope on a polypeptide. Specific, non-limiting examples of an epitope include a tetra- to pentapeptide sequence in a polypeptide, a tri- to penta-glycoside sequence in a polysaccharide. In the animal most antigens will present several or even many antigenic determinants simultaneously. Such a polypeptide may also be qualified as an immunogenic polypeptide and the epitope may be identified as described further.

[0039] An “isolated” biological component (such as a nucleic acid or protein or organelle) refers to a component that has been substantially separated or purified away from other biological

components in the cell of the organism in which the component naturally occurs, for instance, other chromosomal and extra-chromosomal DNA and RNA, proteins, and organelles. Nucleic acids and proteins that have been “isolated” include nucleic acids and proteins purified by standard purification methods. The term also embraces nucleic acids and proteins prepared by recombinant technology as well as chemical synthesis.

[0040] The term “purified” as used herein does not require absolute purity; rather, it is intended as a relative term. Thus, for example, a purified polypeptide preparation is one in which the polypeptide is more enriched than the polypeptide is in its natural environment. A polypeptide preparation is substantially purified such that the polypeptide represents several embodiments at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 98%, of the total polypeptide content of the preparation. The same applies to polynucleotides. The polypeptides disclosed herein can be purified by any of the means known in the art.

[0041] The present invention provides a composition or vaccine comprising a recombinant viral vector Equine Herpesvirus-1 (EHV-1). In one aspect, the present invention provides a recombinant EHV-1 that comprises a mutated Glycoprotein C (gC) gene. In another aspect, the present invention provides a recombinant EHV-1 that comprises a DNA polymerase having an asparagine (N) at the amino acid position 752. In yet another aspect, the present invention provides a recombinant EHV-1 wherein the EHV-1 is an EHV-1 N strain. The recombinant EHV-1 N strain may comprise a mutated gC gene. In yet another aspect, the recombinant EHV-1 comprises a mutated gC gene and a DNA polymerase having an asparagine (N) at the amino acid position 752. The composition or vaccine of the present invention may further comprise a pharmaceutically or veterinary acceptable vehicle, diluent, adjuvant, or excipient.

[0042] The term “composition” comprises any vaccine or immunological composition, once it has been injected to a host, including canines, felines, equine and humans, that induces an immune response in the host, and/or protects the host from leukemia, and/or which may prevent implantation of the parasite, and/or which may prevent disease progression in infected subjects, and/or which may limit the diffusion of runaway parasites to internal organs. This may be accomplished upon vaccination according to the present invention through the induction of cytokine secretion, notably IFN-gamma secretion (as example of a method of measurement of IFN-gamma secretion, the Quantikine® immunoassay from R&D Systems Inc. (catalog number# CAIF00) could be used (Djoba Siawaya JF *et al.*)).

[0043] The present invention provides a recombinant EHV-1 comprising a DNA polymerase having an asparagine (N) at the amino acid position 752. Homologs of polypeptides of EHV-1 DNA polymerase are intended to be within the scope of the present invention. As used herein, the term "homologs" includes orthologs, analogs and paralogs. The term "anologs" refers to two polynucleotides or polypeptides that have the same or similar function, but that have evolved separately in unrelated organisms. The term "orthologs" refers to two polynucleotides or polypeptides from different species, but that have evolved from a common ancestral gene by speciation. Normally, orthologs encode polypeptides having the same or similar functions. The term "paralogs" refers to two polynucleotides or polypeptides that are related by duplication within a genome. Paralogs usually have different functions, but these functions may be related. Analogs, orthologs, and paralogs of a wild-type polypeptide can differ from the wild-type polypeptide by post-translational modifications, by amino acid sequence differences, or by both. In particular, homologs of the invention will generally exhibit at least 80-85%, 85-90%, 90-95%, or 95%, 96%, 97%, 98%, 99% sequence identity, with all or part of the wild-type polypeptide or polynucleotide sequences, and will exhibit a similar function.

[0044] In one aspect of the present invention, the recombinant EHV-1 comprises an EHV-1 DNA polymerase comprising an asparagine (N) at the amino acid position 752 or equivalent position of a polypeptide having at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8% or 99.9% sequence identity to SEQ ID NO: 2, 4, 6, 37, 13, 14, or 15. In another aspect, the present invention provides fragments and variants of the EHV-1 DNA polymerases, which may readily be prepared by one of skill in the art using well-known molecular biology techniques. Variants are homologous polypeptides having an amino acid sequence at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8% or 99.9% identical to the amino acid sequence as set forth in SEQ ID NO: 2, 4, 6, 37, 13, 14, or 15. Variants include allelic variants. The term "allelic variant" refers to a polynucleotide or a polypeptide containing polymorphisms that lead to changes in the amino acid sequences of a protein and that exist within a natural population (e.g., a virus species or variety). Such natural allelic variations can typically result in 1- 5% variance in a polynucleotide or a polypeptide. Allelic variants can be identified by sequencing the nucleic acid sequence of interest in a number of different species, which can be readily carried out by using hybridization probes to identify the same genetic locus

in those species. Any and all such nucleic acid variations and resulting amino acid polymorphisms or variations that are the result of natural allelic variation and that do not alter the functional activity of gene of interest, are intended to be within the scope of the invention.

[0045] As used herein, the term "derivative" or "variant" refers to a polypeptide, or a nucleic acid encoding a polypeptide, that has one or more conservative amino acid variations or other minor modifications such that (1) the corresponding polypeptide has substantially equivalent function when compared to the wild type polypeptide or (2) an antibody raised against the polypeptide is immunoreactive with the wild-type polypeptide. These variants or derivatives include polypeptides having minor modifications of the EHV-1 DNA polymerase primary amino acid sequences that may result in peptides which have substantially equivalent activity as compared to the unmodified counterpart polypeptide. Such modifications may be deliberate, as by site-directed mutagenesis, or may be spontaneous. The term "variant" further contemplates deletions, additions and substitutions to the sequence, so long as the polypeptide functions to produce an immunological response as defined herein. The modifications may be any amino acid change at amino acid positions other than position 752 of SEQ ID NO: 2, 4, 6, 37, 13, 14, or 15.

[0046] The term "conservative variation" denotes the replacement of an amino acid residue by another biologically similar residue, or the replacement of a nucleotide in a nucleic acid sequence such that the encoded amino acid residue does not change or is another biologically similar residue. In this regard, particularly preferred substitutions will generally be conservative in nature, i.e., those substitutions that take place within a family of amino acids. For example, amino acids are generally divided into four families: (1) acidic--aspartate and glutamate; (2) basic--lysine, arginine, histidine; (3) non-polar--alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar--glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine. Phenylalanine, tryptophan, and tyrosine are sometimes classified as aromatic amino acids. Examples of conservative variations include the substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another hydrophobic residue, or the substitution of one polar residue for another polar residue, such as the substitution of arginine for lysine, glutamic acid for aspartic acid, or glutamine for asparagine, and the like; or a similar conservative replacement of an amino acid with a structurally related amino acid that will not have a major effect on the biological activity. Proteins having substantially the same amino acid sequence as the reference molecule but

possessing minor amino acid substitutions that do not substantially affect the immunogenicity of the protein are, therefore, within the definition of the reference polypeptide. All of the polypeptides produced by these modifications are included herein. The term "conservative variation" also includes the use of a substituted amino acid in place of an unsubstituted parent amino acid provided that antibodies raised to the substituted polypeptide also immunoreact with the unsubstituted polypeptide.

[0047] Procedures to determine fragments of polypeptide and epitope such as, generating overlapping peptide libraries (Hemmer B. *et al.*), Pepscan (Geysen H. M. *et al.*, 1984; Geysen H. M. *et al.*, 1985; Van der Zee R. *et al.*; Geysen H. M.) and algorithms (De Groot A. *et al.*; Hoop T. *et al.*; Parker K. *et al.*), can be used in the practice of the invention, without undue experimentation. Generally, antibodies specifically bind a particular antigenic epitope. Specific, non-limiting examples of epitopes include a tetra- to penta- peptide sequence in a polypeptide, a tri- to penta glycoside sequence in a polysaccharide. In animals most antigens will present several or even many antigenic determinants simultaneously. Preferably wherein the epitope is a protein fragment of a larger molecule it will have substantially the same immunological activity as the total protein.

[0048] In one aspect, the present invention provides a polynucleotide encoding an EHV-1 DNA polymerase comprising an asparagine (N) at the amino acid position 752 or an equivalent position of a polypeptide having at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8% or 99.9% sequence identity to SEQ ID NO: 2, 4, 6, 37, 13, 14, or 15, or a conservative variant, an allelic variant, a homolog or an immunogenic fragment comprising at least eight or at least ten consecutive amino acids of one of these polypeptides, or a combination of these polypeptides.

[0049] In another aspect, the present invention provides a polynucleotide having a nucleotide sequence as set forth in SEQ ID NO: 1, 3, 5, or 36, or a variant thereof. In yet another aspect, the present invention provides a polynucleotide having at least 70%, 75%, 80%, 85%, 90%, 95%, 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8% or 99.9% sequence identity to SEQ ID NO: 1, 3, 5 or 36, or a variant thereof.

[0050] The polynucleotides of the disclosure include sequences that are degenerate as a result of the genetic code, e.g., optimized codon usage for a specific host. As used herein, "optimized" refers to a polynucleotide that is genetically engineered to increase its expression in a given

species. To provide optimized polynucleotides coding for an EHV-1 DNA polymerase, the DNA sequence of the EHV-1 DNA polymerase gene can be modified to 1) comprise codons preferred by highly expressed genes in a particular species; 2) comprise an A+T or G+C content in nucleotide base composition to that substantially found in said species; 3) form an initiation sequence of said species; or 4) eliminate sequences that cause destabilization, inappropriate polyadenylation, degradation and termination of RNA, or that form secondary structure hairpins or RNA splice sites. Increased expression of EHV-1 DNA polymerase in said species can be achieved by utilizing the distribution frequency of codon usage in eukaryotes and prokaryotes, or in a particular species. The term “frequency of preferred codon usage” refers to the preference exhibited by a specific host cell in usage of nucleotide codons to specify a given amino acid. There are 20 natural amino acids, most of which are specified by more than one codon. Therefore, all degenerate nucleotide sequences are included in the disclosure as long as the amino acid sequence of the EHV-1 DNA polymerase encoded by the nucleotide sequence is functionally unchanged.

15 **[0051]** In one aspect, the present invention provides a recombinant EHV-1 that contains a mutated Glycoprotein C (gC) gene. The term “mutated gC gene” refers to the gC gene of EHV-1 that is altered or engineered which results in a non-functional gC protein upon expression. The alteration or engineering of the gC gene includes mutation or deletion of a segment of the gC gene which is essential for the expression of a functional gC protein. The deletion of the gC gene may be a deletion of the polynucleotides encoding the amino-terminal region of the gC protein. The deleted amino-terminal regions of the gC protein may be any length, for example, a region of 1-10 amino acids, or 11-20 amino acids, or 21-30 amino acids, or 31-40 amino acids, or 41-60 amino acids, or 61-90 amino acids, or 91-120 amino acids, or 121-160 amino acids. The term “mutated gC gene” also includes deletion of the entire gC gene of EHV-1 wherein gC protein is not expressed.

25 **[0052]** In one aspect, the present invention provides a recombinant EHV-1 wherein the Glycoprotein C (gC) gene in the native (wild-type) EHV-1 genome encoding the gC protein is deleted. The term “Glycoprotein C (gC) gene” includes any gene or polynucleotide that encodes the Glycoprotein C (gC) of EHV-1, and homologs, fragments or variants thereof. The gC gene may encode a gC protein having at least 75%, 80%, 85%, 90%, 95%, 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8% or 99.9% sequence identity to

SEQ ID NO: 8, 10, or 12, or a variant thereof. The gC gene having at least 75%, 80%, 85%, 90%, 95%, 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8% or 99.9% sequence identity to SEQ ID NO:7, 9, or 11 is also encompassed in the present invention. In another aspect, the present invention provides a recombinant EHV-1 wherein the Glycoprotein C (gC) gene in the native (wild-type) EHV-1 genome encoding the gC protein is altered or engineered resulting in a mutated gC protein. In yet another aspect, the engineered gC gene encodes a mutated gC protein wherein the N-terminal region of the gC protein is deleted.

[0053] The sequence identity between two amino acid sequences may be established by the NCBI (National Center for Biotechnology Information) pairwise blast and the blosum62 matrix, using the standard parameters (see, e.g., the BLAST or BLASTX algorithm available on the "National Center for Biotechnology Information" (NCBI, Bethesda, Md., USA) server), as well as in Altschul *et al.*; and thus, this document speaks of using the algorithm or the BLAST or BLASTX and BLOSUM62 matrix by the term "blasts".

[0054] Alternatively or additionally, the term "identity", for instance, with respect to a nucleotide or amino acid sequence, may indicate a quantitative measure of homology between two sequences. The percent sequence homology may be calculated as: $(N_{ref} - N_{dif}) * 100 / N_{ref}$, wherein N_{dif} is the total number of non-identical residues in the two sequences when aligned and wherein N_{ref} is the number of residues in one of the sequences. Hence, the DNA sequence AGTCAGTC will have a sequence identity of 75% with the sequence AATCAATC ($N_{ref} = 8$; $N_{dif} = 2$).

[0055] Alternatively or additionally, "identity" with respect to sequences can refer to the number of positions with identical nucleotides or amino acids divided by the number of nucleotides or amino acids in the shorter of the two sequences wherein alignment of the two sequences can be determined in accordance with the Wilbur and Lipman algorithm (Wilbur and Lipman), for instance, using a window size of 20 nucleotides, a word length of 4 nucleotides, and a gap penalty of 4, and computer-assisted analysis and interpretation of the sequence data including alignment can be conveniently performed using commercially available programs (e.g., Intelligenetics™ Suite, Intelligenetics Inc. CA). When RNA sequences are said to be similar, or have a degree of sequence identity or homology with DNA sequences, thymidine (T) in the DNA sequence is considered equal to uracil (U) in the RNA sequence. Thus, RNA sequences are

within the scope of the invention and can be derived from DNA sequences, by thymidine (T) in the DNA sequence being considered equal to uracil (U) in RNA sequences.

[0056] The sequence identity or sequence similarity of two amino acid sequences, or the sequence identity between two nucleotide sequences can be determined using Vector NTI

5 software package (Invitrogen, 1600 Faraday Ave., Carlsbad, CA).

[0057] Recombinant vectors disclosed herein may include a polynucleotide encoding a polypeptide, a variant thereof or a fragment thereof. Recombinant vectors may include plasmids and viral vectors and may be used for *in vitro* or *in vivo* expression. Recombinant vectors may include further a signal peptide. Signal peptides are short peptide chain (3-60 amino acids long) that direct the post-translational transport of a protein (which are synthesized in the cytosol) to certain organelles such as the nucleus, mitochondrial matrix, endoplasmic reticulum, chloroplast, apoplast and peroxisome. Typically, the naturally occurring EHV-1 proteins may be translated as precursors, having an N-terminal signal peptide sequence and a “mature” protein domain. The signal peptide may be cleaved EHV-1 protein or a peptide signal from a secreted protein e.g. the signal peptide from the tissue plasminogen activator protein (tPA), in particular the human tPA (S. Frieznier Degen *et al.*; R. Rickles *et al.*; D. Berg. *et al.*), or the signal peptide from the Insulin-like growth factor 1 (IGF1), in particular the equine IGF1 (K. Otte *et al.*), the canine IGF1 (P. Delafontaine *et al.*), the feline IGF1 (WO03/022886), the bovine IGF1 (S. Lien *et al.*), the porcine IGF1 (M. Muller *et al.*), the chicken IGF1 (Y. Kajimoto *et al.*), the turkey IGF1 (GenBank accession number AF074980). The signal peptide from IGF1 may be natural or optimized which may be achieved by removing cryptic splice sites and/or by adapting the codon usage. Upon translation, the unprocessed polypeptide may be cleaved at a cleavage site to lead to the mature polypeptide. The cleavage site may be predicted using the method of Von Heijne (1986).

25 [0058] A plasmid may include a DNA transcription unit, for instance a nucleic acid sequence that permits it to replicate in a host cell, such as an origin of replication (prokaryotic or eukaryotic). A plasmid may also include one or more selectable marker genes and other genetic elements known in the art. Circular and linear forms of plasmids are encompassed in the present disclosure.

[0059] In a further aspect, the present invention relates to an *in vivo* expression vector comprising a polynucleotide sequence, which contains and expresses *in vivo* in a host the EHV-1 antigen, polypeptides and/or variants or fragments thereof.

[0060] The *in vivo* expression vector may include any transcription unit containing a
5 polynucleotide or a gene of interest and those essential elements for its *in vivo* expression. These expression vectors may be plasmids or recombinant viral vectors. For *in vivo* expression, the promoter may be of viral or cellular origin. In one embodiment, the promoter may be the cytomegalovirus (CMV) early promoter (CMV-IE promoter), the SV40 virus early or late promoter or the Rous Sarcoma virus LTR promoter, a promoter of a cytoskeleton gene, such as
10 the desmin promoter (Kwissa M. *et al.*), or the actin promoter (Miyazaki J. *et al.*). When several genes are present in the same plasmid, they may be provided in the same transcription unit or in different units.

[0061] As used herein, the term “plasmid” may include any DNA transcription unit comprising a polynucleotide according to the invention and the elements necessary for its *in vivo* expression in
15 a cell or cells of the desired host or target; and, in this regard, it is noted that a supercoiled or non-supercoiled, circular plasmid, as well as a linear form, are intended to be within the scope of the invention. The plasmids may also comprise other transcription-regulating elements such as, for example, stabilizing sequences of the intron type. In several embodiments, the plasmids may include the first intron of CMV-IE (WO 89/01036), the intron II of the rabbit beta-globin gene
20 (van Ooyen *et al.*), the signal sequence of the protein encoded by the tissue plasminogen activator (tPA; Montgomery *et al.*), and/or a polyadenylation signal (polyA), in particular the polyA of the bovine growth hormone (bGH) gene (US 5,122,458) or the polyA of the rabbit beta-globin gene or of SV40 virus.

[0062] The pharmaceutically acceptable vehicles or diluents or excipients or adjuvants of use are
25 conventional. *Remington's Pharmaceutical Sciences*, by E. W. Martin, Mack Publishing Co., Easton, PA, 15th Edition (1975), describes compositions and formulations suitable for pharmaceutical delivery of the polypeptides, plasmids, viral vectors herein disclosed. In general, the nature of the vehicle or excipient will depend on the particular mode of administration being employed. For instance, parenteral formulations usually comprise injectable fluids that include
30 pharmaceutically and physiologically acceptable fluids such as water, physiological saline, balanced salt solutions, aqueous dextrose, glycerol or the like as a vehicle. For solid

compositions (for example, freeze-dried pastille, powder, pill, tablet, or capsule forms), conventional non-toxic solid vehicles or diluents or excipients or adjuvants can include, for example, pharmaceutical grades of mannitol, lactose, starch, or magnesium stearate. In addition to biologically neutral vehicles or diluents or excipients or adjuvants, immunogenic compositions to be administered can contain minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, preservatives, and pH buffering agents and the like, for example sodium acetate or sorbitan monolaurate.

[0063] The compositions or vaccines according to the instant invention may include recombinant vectors encoding any polypeptide or antigen according to the present invention as described above.

[0064] Multiple insertions may be done in the same vector using different insertion sites or using the same insertion site. When the same insertion site is used, each polynucleotide insert, which may be any polynucleotide of the present invention aforementioned, may be inserted under the control of the same and/or different promoters. The insertion can be done tail-to-tail, head-to-head, tail-to-head, or head-to-tail. IRES elements (Internal Ribosome Entry Site, see EP 0803573) can also be used to separate and to express multiple inserts operably linked to the same and/or different promoters.

[0065] More generally, the present invention encompasses *in vivo* expression vectors including any plasmid (EP-A2-1001025; Chaudhuri P.) containing and expressing *in vivo* in a host the polynucleotide or gene of EHV-1 polypeptide, variant or fragment as described above and elements necessary for its *in vivo* expression.

[0066] In a specific, non-limiting example, the pVR1020 or pVR1012 plasmid (VICAL Inc.; Luke C. *et al.*; Hartikka J. *et al.*), pVR2001-TOPA (or pVR2001-TOPO) (Oliveira F. *et al.*) or pAB110 (US 6,852,705) can be utilized as a vector for the insertion of a polynucleotide sequence. The pVR1020 plasmid is derived from pVR1012 and contains the human tPA signal sequence. The pVR1020 is a plasmid backbone available from Vical, Inc., (San Diego, CA) which has been previously used, see, e.g., US Patent Nos. 6,451,769 and 7,078,507. As described in Oliveira *et al.*, plasmid pVR2001-TOPO (or pVR2001-TOPA) is pVR1020 modified by the addition of topoisomerases flanking the cloning site and containing coding for and expressing a signal secretory peptide, for example, tissue plasminogen activator signal peptide (tPA), that increases the likelihood of producing a secreted protein (Oliveira F. *et al.*).

[0067] Each plasmid may comprise or contain or consist essentially of, the polynucleotide according to the present invention, operably linked to a promoter or under the control of a promoter or dependent upon a promoter, wherein the promoter may be advantageously adjacent to the polynucleotide for which expression is desired. In general, it is advantageous to employ a strong promoter that is functional in eukaryotic cells. One example of a useful promoter may be the immediate early cytomegalovirus promoter (CMV-IE) of human or murine origin, or it may optionally have another origin such as from rat or guinea pig. The CMV-IE promoter may comprise the actual promoter part, which may or may not be associated with the enhancer part. Reference can be made to EP 260 148, EP 323 597, US 5,168,062, 5,385,839, and 4,968,615, as well as to WO 87/03905. The CMV-IE promoter may advantageously be a human CMV-IE (Boshart M. *et al.*) or murine CMV-IE. In more general terms, the promoter may have either a viral or a cellular origin. A strong viral promoter other than CMV-IE that may be usefully employed in the practice of the invention is the early/late promoter of the SV40 virus or the LTR promoter of the Rous sarcoma virus. A strong cellular promoter that may be usefully employed in the practice of the invention is the promoter of a gene of the cytoskeleton, such as the desmin promoter (Kwissa M. *et al.*), or the actin promoter (Miyazaki J. *et al.*). Functional sub fragments of these promoters, i.e., portions of these promoters that maintain adequate promoter activity, are included within the present invention, e.g. truncated CMV-IE promoters according to WO 98/00166 or US 6,156,567 and may be used in the practice of the invention. A promoter useful in the practice of the invention consequently may include derivatives and/or sub fragments of a full-length promoter that maintain adequate promoter activity and hence function as a promoter, and which may advantageously have promoter activity that is substantially similar to that of the actual or full-length promoter from which the derivative or sub fragment is derived, e.g., akin to the activity of the truncated CMV-IE promoters of US 6,156,567 in comparison to the activity of full-length CMV-IE promoters. Thus, a CMV-IE promoter in the practice of the invention may comprise or consist essentially of or consist of the promoter portion of the full-length promoter and/or the enhancer portion of the full-length promoter, as well as derivatives and/or sub fragments thereof.

[0068] Advantageously, the plasmids comprise or consist essentially of other expression control elements. It is especially advantageous to incorporate stabilizing sequence(s), e.g., intron sequence(s), for example, the first intron of the hCMV-IE (WO 89/01036), the intron II of the

rabbit β -globin gene (van Ooyen *et al.*). As to the polyadenylation signal (polyA) for the plasmids and viral vectors other than poxviruses, use can be made of the poly(A) signal of the bovine growth hormone (bGH) gene (*see* US 5,122,458), or the poly(A) signal of the rabbit β -globin gene or the poly(A) signal of the SV40 virus.

5 [0069] More generally, the present invention encompasses *in vivo* expression vectors including any recombinant viral vector containing a polynucleotide or gene encoding one or more EHV-1 polypeptide, variants or fragments as described above, including any elements necessary for its *in vivo* expression.

[0070] The recombinant viral vector may be a Herpesvirus, such as an equine Herpesvirus-1 (EHV-1) as described above. The EHV-1 vector may be derived from the RacH strain, the RacL strain, the Ab4 strain, the V592 strain, the Kentucky D strain (TACC No. VR-700), the 438/77 strain (ATCC No. VR-2229), the AB69 strain (ATCC No. VR-2581), the EHV-1 NY03 strain, or a combination of EHV-1 RacH or RacL strains. In one embodiment the polynucleotide to be expressed is inserted under the control of a promoter functional in eukaryotic cells,
10 advantageously a CMV-IE promoter (murine or human). A poly(A) sequence and terminator sequence can be inserted downstream the polynucleotide to be expressed, e.g. bovine growth hormone or a rabbit β -globin gene polyadenylation signal.

[0071] In one embodiment, the viral vector could be Newcastle Disease Virus (US2012/052089). In another embodiment, the viral vector could be selected from, for example, the poxviruses,
20 especially avipox viruses, such as fowlpox viruses or canarypox viruses. In one embodiment, the fowlpox virus is a TROVAC (*see* WO 96/40241). In another embodiment, the canarypox vector is an ALVAC. The use of these recombinant viral vectors and the insertion of polynucleotides or genes of interest are fully described in US 5,174,993; US 5,505,941 and US 5,766,599 for fowlpox, and in US 5,756,103 for canarypox. More than one insertion site inside the viral
25 genome could be used for the insertion of multiple genes of interest.

[0072] For recombinant vectors based on a poxvirus vector, a vaccinia virus or an attenuated vaccinia virus, (for instance, MVA, a modified Ankara strain obtained after more than 570 passages of the Ankara vaccine strain on chicken embryo fibroblasts; *see* Stickl & Hochstein-Mintzel; Sutter *et al.*; available as ATCC VR-1508; or NYVAC, *see* US 5,494,807, and U.S.
30 Patent No. 5,494,807 which discuss the construction of NYVAC, as well as variations of NYVAC with additional ORFs deleted from the Copenhagen strain vaccinia virus genome, as

well as the insertion of heterologous coding nucleic acid molecules into sites of this recombinant, and also, the use of matched promoters; see also WO 96/40241), an avipox virus or an attenuated avipox virus (e.g., canarypox, fowlpox, dovepox, pigeonpox, quailpox, ALVAC or TROVAC; see, e.g., U.S. Patent Nos. 5,505,941, 5,494,807) can be used. Attenuated canarypox viruses are described in US 5,756,103 (ALVAC) and WO 01/05934. Reference is also made to US5,766,599 which pertains to the attenuated fowlpox strain TROVAC. Reference is made to the canarypox available from the ATCC under access number VR-111. Numerous fowlpox virus vaccination strains are also available, e.g. the DIFTOSEC CT strain marketed by MERIAL and the NOBILIS VARIOLE vaccine marketed by INTERVET. For information on the method used to generate recombinants thereof and how to administer recombinants thereof, the skilled artisan can refer documents cited herein and to WO 90/12882, e.g., as to vaccinia virus, mention is made of U.S. Patents Nos. 4,769,330, 4,722,848, 4,603,112, 5,110,587, 5,494,807, and 5,762,938 *inter alia*; as to fowlpox, mention is made of U.S. Patents Nos. 5,174,993, 5,505,941 and 5,766,599 *inter alia*; as to canarypox, mention is made of U.S. Patent No. 5,756,103 *inter alia*. When the expression vector is a vaccinia virus, insertion site or sites for the polynucleotide or polynucleotides to be expressed are advantageously at the thymidine kinase (TK) gene or insertion site, the hemagglutinin (HA) gene or insertion site, the region encoding the inclusion body of the A type (ATI). In the case of canarypox, advantageously the insertion site or sites are ORF(s) C3, C5 and/or C6. In the case of fowlpox, advantageously the insertion site or sites are ORFs F7 and/or F8. The insertion site or sites for MVA virus are advantageously as in various publications, including Carroll M. W. *et al.*; Stittelaar K. J. *et al.*; Sutter G. *et al.*; and, in this regard it is also noted that the complete MVA genome is described in Antoine G., Virology, which enables the skilled artisan to use other insertion sites or other promoters. Advantageously, the polynucleotide to be expressed is inserted under the control of a specific poxvirus promoter, e.g., the vaccinia promoter 7.5 kDa (Cochran *et al.*), the vaccinia promoter I3L (Riviere *et al.*), the vaccinia promoter HA (Shida), the cowpox promoter ATI (Funahashi *et al.*), the vaccinia promoter H6 (Taylor J. *et al.*; Guo P. *et al.* J.; Perkus M. *et al.*).

[0073] Any of the polynucleotides disclosed here may be expressed *in vitro* by DNA transfer or expression vectors into a suitable host cell. The host cell may be prokaryotic or eukaryotic. The term "host cell" also includes any progeny of the subject host cell. Methods of stable transfer, meaning that the foreign polynucleotide is continuously maintained in the host cell, are known in

the art. Host cells may include bacteria (for example, *Escherichia coli*), yeast, insect cells, and vertebrate cells. Methods of expressing DNA sequences in eukaryotic cells are well known in the art. As a method for *in vitro* expression, recombinant Baculovirus vectors (for example, Autographa California Nuclear Polyhedrosis Virus (AcNPV)) may be used with the nucleic acids disclosed herein. For example, polyhedrin promoters may be utilized with insect cells (for example, *Spodoptera frugiperda* cells, like Sf9 cells available at the ATCC under the Accession number CRL 1711, or Sf21 cells) (see for example, Smith *et al.*; Pennock *et al.*; Vialard *et al.*; Verne A.; O'Reilly *et al.*; Kidd I. M. & Emery V.C.; EP 0370573; EP 0265785; US 4,745,051). For expression, the BaculoGold Starter Package (Cat # 21001K) from Pharmingen (Becton Dickinson) may be used. As a method for *in vitro* expression, recombinant *E. coli* may be used with a vector. For example, when cloning in bacterial systems, inducible promoters such as arabinose promoter, pL of bacteriophage lambda, plac, ptrp, ptac (ptrp-lac hybrid promoter), and the like may be used. Transformation of a host cell with recombinant DNA may be carried out by conventional techniques are well known to those skilled in the art. Where the host is prokaryotic, such as *E. coli*, competent cells which are capable of DNA uptake can be prepared from cells harvested after exponential growth phase and subsequently treated by the CaCl₂ method using procedures well known in the art. Alternatively, MgCl₂ or RbCl can be used. Transformation can also be performed by electroporation. When the host is a eukaryote, such methods of transduction of DNA as calcium phosphate coprecipitates, conventional mechanical procedures such as microinjection, electroporation, insertion of a plasmid encased in liposomes, or virus vectors may be used. Eukaryotic cells may also be cotransformed with *L. longipalpis* polynucleotide sequences, and a second foreign DNA molecule encoding a selectable phenotype, such as the herpes simplex thymidine kinase gene. Another method is to use a eukaryotic viral vector (see above), such as a herpes virus or adenovirus (for example, canine adenovirus 2), to transiently transduce eukaryotic cells and express the protein (Gluzman EA). In addition, a transfection agent can be utilized, such as dioleoyl-phosphatidyl-ethanolamine (DOPE).

[0074] Isolation and purification of recombinantly expressed polypeptide may be carried out by conventional means including preparative chromatography (for example, size exclusion, ion exchange, affinity), selective precipitation and ultra-filtration. Examples of state of the art techniques that can be used, but not limited to, may be found in "Protein Purification Applications", Second Edition, edited by Simon Roe and available at Oxford University Press.

Such a recombinantly expressed polypeptide is part of the present disclosure. The methods for production of any polypeptide according to the present invention as described above are also encompassed, in particular the use of a recombinant expression vector comprising a polynucleotide according to the disclosure and of a host cell.

5 [0075] The vaccines containing recombinant viral vectors according to the invention may be freeze-dried, advantageously with a stabilizer. Freeze-drying can be done according to well-known standard freeze-drying procedures. The pharmaceutically or veterinary acceptable stabilizers may be carbohydrates (e.g. sorbitol, mannitol, lactose, sucrose, glucose, dextran, trehalose), sodium glutamate (Tsvetkov T *et al.*; Israeli E *et al.*), proteins such as peptone,
10 albumin, lactalbumin or casein, protein containing agents such as skimmed milk (Mills C K *et al.*; Wolff E *et al.*), and buffers (e.g. phosphate buffer, alkaline metal phosphate buffer). An adjuvant may be used to make soluble the freeze-dried preparations.

[0076] Any vaccine composition according to the invention can also advantageously contain one or more adjuvant.

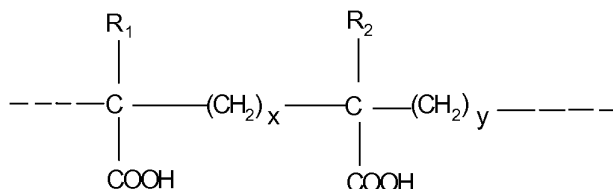
15 [0077] The plasmid-based vaccines may be formulated with cationic lipids, advantageously with DMRIE (N-(2-hydroxyéthyl)-N,N-diméthyl-2,3-bis(tétradécyloxy)-1-propanammonium ; WO96/34109), and advantageously in association with a neutral lipid, for example DOPE (dioleoyl-phosphatidyl-ethanolamine ; Behr J. P.), in order to form DMRIE-DOPE. In one embodiment, the mixture is made extemporaneously, and before its administration it is
20 advantageous to wait about 10 min to about 60 min, for example, about 30 min, for the appropriate mixture. When DOPE is used, the molar ratio of DMRIE/DOPE can be from 95/5 to 5/95 and is advantageously 1/1. The weight ratio plasmid/DMRIE or DMRIE-DOPE adjuvant is, for example, from 50/1 to 1/10, from 10/1 to 1/5 or from 1/1 to 1/2.

[0078] Optionally a cytokine may be added to the composition, especially GM-CSF or cytokines
25 inducing Th1 (e.g. IL12). These cytokines can be added to the composition as a plasmid encoding the cytokine protein. In one embodiment, the cytokines are from canine origin, e.g. canine GM-CSF which gene sequence has been deposited at the GenBank database (accession number S49738). This sequence can be used to create said plasmid in a manner similar to what was made in WO 00/77210.

30 [0079] The recombinant viral vector-based vaccine may be combined with fMLP (N-formyl-methionyl-leucyl-phenylalanine; US 6,017,537) and/or Carbomer adjuvant (Phameuropa Vol. 8,

No. 2, June 1996). Persons skilled in the art can also refer to US 2,909,462, which describes such acrylic polymers cross-linked with a polyhydroxylated compound having at least 3 hydroxyl groups, advantageously not more than 8, the hydrogen atoms of at least three hydroxyls being replaced by unsaturated aliphatic radicals having at least 2 carbon atoms. For example, the radicals are those containing from 2 to 4 carbon atoms, e.g. vinyls, allyls and other ethylenically unsaturated groups. The unsaturated radicals may themselves contain other substituents, such as methyl. The products sold under the name CARBOPOL® (BF Goodrich, Ohio, USA) are appropriate. The products are cross-linked with an allyl sucrose or with allyl pentaerythritol. Among them, there may be advantageously mentioned CARBOPOL® 974P, 934P and 971P.

10 [0080] Among the copolymers of maleic anhydride and alkenyl derivative, the copolymers



EMA® (Monsanto) which are copolymers of maleic anhydride and ethylene, linear or cross-linked, for example cross-linked with divinyl ether, are advantageous. Reference may be made to J. Fields *et al.*

[0081] The polymers of acrylic or methacrylic acid and the copolymers EMA® are formed, for example, of basic units of the following formula in which:

- R₁ and R₂, which are identical or different, represent H or CH₃
- x = 0 or 1, preferably x = 1
- y = 1 or 2, with x + y = 2

For the copolymers EMA®, x = 0 and y = 2. For the carbomers, x = y = 1.

20 [0082] The dissolution of these polymers in water leads to an acid solution, which is neutralized, advantageously to physiological pH, in order to provide the adjuvant solution into which the vaccine itself is incorporated. The carboxyl groups of the polymer are then partly in COO⁻ form.

[0083] In one embodiment, a solution of adjuvant, especially of carbomer (*Pharmeuropa*, vol. 8,

No.2, June 1996), is prepared in distilled water, advantageously in the presence of sodium chloride, the solution obtained being at an acidic pH. This stock solution is diluted by adding it to the desired quantity (for obtaining the desired final concentration), or a substantial part thereof, of water charged with NaCl, advantageously physiological saline (NaCl 9 g/l) all at once
5 in several portions with concomitant or subsequent neutralization (pH 7.3 to 7.4), advantageously with NaOH. This solution at physiological pH is used for mixing with the vaccine, which may be especially stored in freeze-dried, liquid or frozen form.

[0084] The polymer concentration in the final vaccine composition can be from 0.01% to 2% w/v, from 0.06 to 1% w/v, or from 0.1 to 0.6% w/v.

10 [0085] The subunit vaccine may be combined with adjuvants, like oil-in-water, water-in-oil-in-water emulsions based on mineral oil and/or vegetable oil and non ionic surfactants such as block copolymers, TWEEN®, SPAN®. Such emulsions are notably those described in page 147 of "Vaccine Design – The Subunit and Adjuvant Approach", Pharmaceutical Biotechnology, 1995, or TS emulsions, notably the TS6 emulsion, and LF emulsions, notably LF2 emulsion (for
15 both TS and LF emulsions, see WO 04/024027). Other suitable adjuvants are for example vitamin E, saponins, and CARBOPOL® (Noveon; see WO 99/51269; WO 99/44633), aluminium hydroxide or aluminium phosphate ("Vaccine Design, The subunit and adjuvant approach", Pharmaceutical Biotechnology, vol. 6, 1995), biological adjuvants (i.e. C4b, notably murine C4b (Ogata R T *et al.*) or equine C4b, GM-CSF, notably equine GM-CSF (US
20 6,645,740)), toxins (i.e. cholera toxins CTA or CTB, *Escherichia coli* heat-labile toxins LTA or LTB (Olsen C W *et al.*; Fingerut E *et al.*; Zurbriggen R *et al.* Peppoloni S *et al.*), and CpG (i.e. CpG #2395 (see Jurk M *et al.*), CpG #2142 (see SEQ. ID. NO: 890 in EP 1,221,955).

[0086] The composition or vaccine may also be associated with at least one EHV-1 antigen, for example inactivated EHV-1. In a particular embodiment, the EHV-1 strain may be the RacH
25 strain, the RacL strain, the Ab4 strain, the V592 strain, the Kentucky D strain (TACC No. VR-700), the 438/77 strain (ATCC No. VR-2229), the AB69 strain (ATCC No. VR-2581), EHV-1 NY03, or a combination of EHV-1 RacH or RacL strains. These strains of EHV-1 may be inactivated by chemical or physical methods. The chemical methods are notably BPL, formaldehyde. The physical methods may notably be sonication. The inactivated EHV-1 vaccine
30 may be combined with adjuvants, like those described previously for subunit vaccines.

[0087] Another aspect of the present invention relates to methods of vaccinating a host against EHV-1 using the vaccines or compositions disclosed herein.

[0088] The host may be any animals. In one embodiment, the host is an equine.

[0089] The routes of administration may be, for example, intramuscular (IM) or intradermal (ID) or transdermal (TD) or subcutaneous (SC). The means of administration may be, for example, a syringe with a needle, or needle free apparatus, or a syringe with a needle coupled to electrotransfer (ET) treatment, or needle free apparatus coupled to ET treatment.

[0090] For plasmid-based vaccines, advantageous routes of administration may be ID or IM. This administration may be through use of a syringe with a needle or with a needle free apparatus like Dermojet or Biojector (Bioject, Oregon, USA) or Vetjet™ (Merial) or Vitajet™ (Bioject Inc.), see US 2006/0034867. The dosage may be from 50 µg to 500 µg per plasmid. When DMRIE-DOPE is added, 100 µg per plasmid may be utilized. When GM-CSF or other cytokines are used, the plasmid encoding this protein may be present at a dosage of from about 200 µg to about 500 µg and may be 200 µg. The volume of doses can be between 0.01 ml and 0.5 ml, for example, 0.25 ml. Administration may be provided with multiple points of injection.

[0091] Alternatively, plasmid-based vaccines may be administered via the IM route coupled to electrotransfer (ET) treatment. The ET treatment may be performed using an apparatus for electrotransfer and the specifications of the manufacturer (i.e. Sphergen G250 generator (Sphergen SARL, Evry Genopole, France); MedPulser® DNA electroporation system (Innovio Biomedical Corporation, San Diego, California, USA)).

[0092] For recombinant viral vector-based vaccines, the routes of administration may advantageously be SC, IM, TD, or ID. This administration may be made by a syringe with a needle or with a needle free apparatus like Dermojet or Biojector (Bioject, Oregon, USA) or Vetjet™ (Merial) or Vitajet™ (Bioject Inc.). The dosage may be from about 10^4 pfu to about 10^9 pfu per recombinant EHV vector. The volume of doses may be from about 0.01 ml to 0.2 ml, and is advantageously 0.1 ml. Administration may comprise multiple points of injection.

[0093] For the IM route the volume of the vaccine provided may be from 0.2 to 2 ml, in particular from about 0.5 to 1 ml. The same dosages are utilized for any of the vectors of the present invention.

[0094] For subunit vaccines, the route of administration may advantageously be via SC or IM or TD or ID. This administration may be made by a syringe with a needle or with a needle free

apparatus like Dermojet or Biojector (Bioject, Oregon, USA) or Vetjet™ (Merial) or Vitajet™ (Bioject Inc.). The dosage may be from about 50 to about 500 µg, in particular from about 50 to about 150 µg, and more particularly from about 50 to about 100 µg. The volume of the sub-unit vaccine provided is from 0.2 to 2 ml, in particular from about 0.5 to 1 ml.

5 [0095] In one aspect, the present invention relates to a vaccine strategy, which is based on a prime-boost administration regimen, where the prime-administration and the boost-administration utilize a composition comprising a pharmaceutically or veterinary acceptable excipient, diluent, adjuvant, or vehicle and the recombinant EHV-1 of the present invention. In another aspect, the present invention relates to a method of vaccinating a subject or host
10 susceptible to EHV-1 comprising a prime-boost administration regime.

[0096] A prime-boost regimen comprises at least one prime-administration and at least one boost administration using at least one common antigen and/or variants or fragments thereof. The vaccine used in prime-administration may be different in nature from those used as a later booster vaccine. It is further noted that both the prime-administration and the boost-
15 administration may comprise the recombinant EHV-1 of the present invention. The prime-administration may comprise one or more administrations. Similarly, the boost-administration may comprise one or more administrations.

[0097] The routes of administration, doses and volumes are as previously disclosed herein.

[0098] The prime-boost administrations may be carried out 2 to 6 weeks apart, for example,
20 about 4 weeks apart. According to one embodiment, a semi-annual booster or an annual booster is also envisaged.

[0099] In one embodiment, the prime-boost administration regimen comprises at least one prime-administration of a recombinant EHV-1 vaccine or composition of the present invention and at least one boost-administration of an inactivated viral vaccine comprising the EHV-1
25 antigen, or a plasmid-based vaccine expressing the EHV-1 antigen, or a subunit vaccine comprising the EHV-1 antigen, or a combination thereof.

[0100] In another embodiment, the prime-boost administration regimen comprises at least one prime-administration of an inactivated viral vaccine comprising the EHV-1 antigen, or a plasmid-based vaccine expressing the EHV-1 antigen, or a subunit vaccine comprising the EHV-
30 1 antigen, or a combination thereof and at least one boost-administration of a recombinant EHV-1 vaccine or composition of the present invention.

[0101] Another aspect of the present invention relates to a kit for prime-boost vaccination according to the present invention. The kit may comprise at least two vials: a first vial containing a vaccine for the prime-vaccination according to the present invention, and a second vial containing a vaccine for the boost-vaccination according to the present invention. The kit may advantageously contain additional first or second vials for additional prime-vaccinations or additional boost-vaccinations.

[0102] In one embodiment, the kit may comprise two vials, one containing a plasmid-based vaccine for the prime-vaccination according to the present invention, the other vial containing a recombinant viral vector-based vaccine for the boost-vaccination according to the present invention.

[0103] The invention will now be further described by way of the following non-limiting examples.

EXAMPLES

[0104] Without further elaboration, it is believed that one skilled in the art can, using the preceding descriptions, practice the present invention to its fullest extent. The following detailed examples are to be construed as merely illustrative, and not limitations of the preceding disclosure in any way whatsoever. Those skilled in the art will promptly recognize appropriate variations from the procedures both as to reactants and as to reaction conditions and techniques.

[0105] Construction of DNA inserts, plasmids and recombinant viral vectors was carried out using the standard molecular biology techniques described by J. Sambrook *et al.* (Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989). All the restriction fragments used for the present invention were isolated using the "GeneClean" kit (BIO 101 Inc., La Jolla, Calif.).

Example 1 Construction of Cell Line RK13_Pol

Cells and Viruses

[0106] Rabbit kidney (RK13) cell was maintained in Earle's minimum essential medium (EMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 0.1 mg/ml streptomycin (1% Pen/Strep). Equine skin fibroblast cell line (NBL6) was

maintained in EMEM supplemented with 10% FBS, 29mg/ml L-glutamate, 1% Pen/Strep and 1% nonessential amino acids (Gibco BRL). EHV-1 strains RacL11 and NY03 were grown on fresh RK13 cells. NY03, harboring a non-neurological form of DNA polymerase (N752 Pol), was isolated from an aborted foal from a farm in New York in 2003.

5 Construction of cell line RK13_Pol

[0107] To support the growth of Pol-negative RacL11 mutant, a rabbit kidney cell line designated RK13_Pol expressing the non-neurological form of the polymerase (N752) was first generated. The whole polymerase open reading frame of NY03 was amplified from viral genomic DNA, which was extracted from virus infected RK13 cells. The polymerase chain
 10 reaction (PCR) was performed using Accuprime DNA polymerase (Invitrogen, Carlsbad, CA, USA) and primer pair PN1/PN2 (Table 1). The amplicon, flanked with a HindIII restriction site at 5'end and a BamHI site as well as a HA Tag at 3'end, was cloned into plasmid pCDNA3 to generate pCDNA3-Pol. After digestion with PvuI, 4µg of recombinant plasmid pCDNA3-Pol was linearized and transfected into RK13 cells grown in six-well plates using Lipofectamine
 15 2000 (Invitrogen). Transfected cells were propagated in EMEM containing 10% FBS and 1.2 mg/ml G418 (Merck). A single cell clone in which every cell was shown to express DNA polymerase by indirect immunofluorescence assay (IFA) was selected and termed RK13_Pol. By IFA using anti-HA MAb, the expression of polymerase in RK13_Pol could be demonstrated (Fig.3) and shown to be stable in ten times of passages.

20 Table 1 Primer Sequences

Primer Name	SEQ ID NO:	Sequence
PN1	16	5'-CCCAAGCTTgagATGGCGGCGCGGAACAGGCCA -3'
PN2	17	5'-CGCGGATCCTTAAGCGTAGTCTGGGACGTCGTATGGGTA GCTTTGATGGGGAGCTGCTTCT -3'
P1	18	5'-GCCTGCGTGGAGGAGTATTGGG-3'
P2	19	5'-TAATTGATTACTATTAATAACTA TTACACCGGAGGAAGAAAGTCG-3'
P3	20	5'-CAAACATCATCAATGTATCTTAAG GTCTGTGTAAATTTAAAGTGCGA-3'
P4	21	5'-CAAAGGTGCCAGCGTCACATCG-3'

P5	22	5'- <u>ACGACTTTCCTCCTCCGGTGTA</u> TAGTTATTAATAGTAATCAATT-3'
P6	23	5'- <u>CCCTTGCTCACCATGGTGGCGGATCTGACGGTTC</u> ACTAAACC-3'
P7	24	5'- <u>GGTTTAGTGAACCGTCAGATCCGCCACCATGGT</u> GAGCAAGGG-3'
P8	25	5'- <u>CGCACTTTAAATTTACACAGAC</u> CTTAAGATACATTGATGAGTTTG-3'
P9	26	5'- <u>TCCCCGCGGATAACTTCGTATAGCATA</u> CATTATACGAAGTTAT TAGTTATTAATAGTAATCAAT-3'
P10	27	5'-CTAGCTAGCATAACTTCGTATAATGTATGCTATACGAAGTTAT CTTAAGATACATTGATGAGTT-3'
gC-1	28	5'-GACTCTGTCGACGGCCACCGCCGAC-3'
gC-2	29	5'-CCTGGATCCAGACTCTATTCCCATG-3'
ΔgC-1	30	5'- TTGGCCTATGCGGACGACTT-3'
ΔgC-2	31	5'- CCCTTTGGTGCATGGTATGT-3'
Poly1	32	5'- TCTGG AACTA TCGGC GGTGG C-3'
Poly2	33	5'- CGGGT CTTGA GGAGC ATGTC G-3'

Example 2 Plasmids and Viral Mutagenesis

Generation of Virus Mutants

[0108] Conventional homologous recombination strategy was employed for all genetic manipulations. Two 1.7kbp flanking fragments on either side of DNA polymerase were amplified using thermostable *Pfu* polymerase (Promega) and primer pairs P1/P2 and P3/P4 from RacL11 genome. Another two primer pairs P5/P6 and P7/P8 were used to amplify the HCMV (human cytomegalovirus) promoter and EYFP (enhanced yellow fluorescent protein) gene from plasmid pEYFP-N1, separately. The 5'ends of primers P2 and P5, P3 and P8 as well as P6 and P7 carry homologous sequences of 21-23bp. With an overlapping PCR using P5 and P8, HCMV promoter and EYFP gene were fused and cloned into plasmid pCR2.1-Topo (Invitrogen) resulting in pCES1. For the construction of shuttle plasmid pCR-Topo-P1P4, the above two homolog arms and EYFP expressing cassette in pCES1 were combined by overlapping PCR using primers P1/P4 and cloned into pCR2.1-Topo. Using pCES1 as template and primer pair P9/P10, EYFP cassette flanked with *Sac*II and *Nhe*I restriction sites as well as *LoxP* sequences upstream and downstream was amplified and cloned into pCR2.1-Topo resulting in plasmid

pCES2. To generate plasmid pCR-Topo-gC, a 5.87kbp gC-containing fragment, which was amplified from RacL11 genome using primers gC-1 and gC-2, was cloned into pCR2.1-Topo. After digestion with SacII and NheI, EYFP cassette was released from pCES2 and transferred to pCR-Topo-gC to replace gC gene and the recombinant shuttle plasmid was termed Topo-ΔgC-EYFP. Plasmids pCES1, pCES2, pCR-Topo-P1P4 and Topo-ΔgC-EYFP were identified by digestion and sequencing. The cloning scheme is depicted in Figure 2.

[0109] For the mutation of DNA polymerase, 1μg of RacL11 viral DNA and 10μg of plasmid pCR-Topo-P1P4 were co-transfected into RK13_Pol cells in a 6-well plate by calcium phosphate precipitation. Two days post transfection, green plaques were observed under a fluorescence microscope (Axiovert 25, Zeiss). The whole virus was harvested after 2 cycles of freeze-thaw and grown on fresh RK13_Pol cells that were overlaid with 1.5% methylcellulose in EMEM-2% FBS at 1 hour post infection. After 3 times of purification, homogenous Pol-negative RacL11 mutant, termed L11_ΔPol EYFP, was generated and identified using restriction fragment length polymorphism (RFLPs) analyses. To restore N752 Pol, a 7kbp fragment, termed p1p4pol, was amplified from EHV-1 NY03 genome using Accuprime Tag polymerase and primers P1/P4. By co-transfection of 1μg of L11_ΔPol EYFP viral DNA and 4μg of p1p4pol PCR product into RK13 cells, homologous recombination was used again to introduce N752 Pol variant and RacL11 mutant L11_D752N was obtained. The D752 to N752 mutation in polymerase was confirmed (Fig. 2D) by sequencing a 780bp Pol fragment that was amplified from L11_D752N using sequencing primers poly1 and poly2.

[0110] Next steps were to delete gC open reading frame from L11_D752N mutant. Briefly, 1μg of L11_D752N viral DNA and 10μg of transfer plasmid Topo-ΔgC-EYFP were co-transfected into RK13 cells. The SacII/NheI fragment (1.2kbp in length) in gC gene was replaced with EYFP cassette leading to the generation of recombinant virus L11_D752N ΔgC EYFP. The EYFP selection marker was excised by expressing Cre recombinase upon co-transfection of L11_D752N ΔgC EYFP and plasmid pCAGGS-NLS/Cre into RK13 cells. White plaques were purified and a gC-negative, non-neurological RacL11 mutant L11_D752N ΔgC was finally engineered. By the homologous recombination between L11_D752N ΔgC EYFP viral DNA and plasmid pCR-Topo-gC, gC gene was repaired, resulting in a gC revertant L11_D752N ΔgC rev (Fig.2). The deletion of gC gene and the absence of gC protein were confirmed by PCR identification using primer pairs gC-1/gC-2 and ΔgC-1/ΔgC-2, sequencing, as well as indirect

immunofluorescence assay (IFA).

[0111] All the primers used for the construction of plasmids and sequencing were listed in Table 1.

Indirect Immunofluorescence Assay (IFA)

5 [0112] For the detection of DNA polymerase that was expressed in RK13_Pol cells, monoclonal antibody (MAb) directed against HA Tag (H3663, Sigma) was used. RK13_Pol cells grown in a 6-well plate were washed with phosphate-buffered saline (PBS) and fixed in 3.5% paraformaldehyde in PBS for 1h at room temperature (RT), followed by a 5min incubation in PBS containing 30mM glycine and another 5min permeation in 0.1% Triton X-100 in PBS. After
10 washing with PBS, cells were blocked with PBS-3% bovine serum albumin (BSA) for 30min at RT. Cells were then incubated with the primary antibody at a 1:10000 dilution in PBS-3%BSA for 1h at RT and extensively washed with PBS. The secondary antibody (Alexa Fluor568-conjugated goat anti-mouse IgG, Invitrogen) was added with a dilution of 1:2000 in PBS-3% BSA and incubated for 1h at RT. After thorough washing for 3 times of 10min, fluorescence
15 signal was inspected under the inverted fluorescence microscope (Axiovert 25, Zeiss).

[0113] To confirm the absence of gC protein, RK13 cells were seeded in a 6-well plate and infected with wild type RacL11, mutant L11_D752N ΔgC or gC revertant L11_D752N ΔgC rev at a multiplicity of infection (MOI) of 0.0001. One hour post infection, viruses were removed and infected cells were overlaid with 1.5% methylcellulose in EMEM-2% FBS. After 48h of
20 incubation at 37°C, cells were fixed and blocked as described above. MAb 1G4 directed against EHV-1 gC protein and B8 against EHV-1 gM protein were used as the primary antibodies with a dilution of 1:100 and 1:200, separately. After incubation with the secondary antibody (Alexa Fluor568-conjugated goat anti-mouse IgG) for 1h, cells were washed and plaques were observed.

Characterization of Virus Mutants

25 [0114] A Pol-negative virus mutant L11_ΔPol EYFP was generated by replacing the authentic polymerase gene with EYFP via co-transfection of RacL11 viral DNA and shuttle plasmid pCR-Topo-P1P4 into RK13_Pol. L11_ΔPol EYFP was found to be able to grow only in RK13_Pol but not in non-complementing RK13 cells (Fig.4), which confirms that DNA polymerase is essential for virus growth of EHV-1 in vitro. The mutation from neurological D752 to non-
30 neurological N752 genotype was then achieved by restoring the non-neurological polymerase gene of EHV-1 NY03 into L11_ΔPol EYFP. The single amino acid variation was confirmed by

sequencing a 780bp amplicon from the mutant L11_D752N. Based on L11_D752N, the SacII/NheI region representing a 1222bp fragment (from nucleotide position 94 to 1315) in gC gene was replaced with EYFP, resulting in a gC-negative intermediate L11_D752N ΔgC EYFP. By the expression of Cre, EYFP cassette was finally excised, leaving one copy of LoxP sequence (34bp) between the SacII and NheI restriction sites in the engineered mutant L11_D752N ΔgC. To confirm the deletion in gC gene, PCR was performed. While from the parental mutant L11_D752N, 5.87kbp and 1.6kbp fragments could be amplified using primer pair gC-1/gC-2 and ΔgC-1/ΔgC-2, from L11_D752N ΔgC, however, 4.68kbp and 410bp fragments were amplified (Fig.2), suggesting that a deletion of 1.2kbp in gC gene was present. By IFA using anti-EHV-1 gC MAb, it could be shown that gC protein was detectable in cells infected with either RacL11 or the revertant L11_D752N ΔgC rev, but in cells infected with L11_D752N ΔgC, the expression of gC protein was abolished (Fig.5). A gC-negative and non-neurological RacL11 mutant was thus successfully generated.

Example 3 Characterization of *in vitro* Virus Growth

Virus attachment assay

[0115] Virus attachment assay was carried out as described in Sun *et al.* (J Gen Virol 77, 493-500, 1996) with slight modifications. Monolayers of RK13 cells seeded in 6-well plates were cooled for 1h at 4°C and infected with RacL11, L11_D752N, L11_D752N ΔgC or L11_D752N ΔgC rev with an MOI of 400 plaque-forming units (PFU) per well. The viruses were left to attach for various length of time (0, 15, 30, 60, 120, 240min) at 4°C. At various time points, infected cells were washed three times with PBS and overlaid with EMEM containing 2% FBS and 1.5% methylcellulose. After incubation for 3 days at 37°C, cells were fixed with 10% formaldehyde and stained with 0.3% crystal violet. Plaques were counted and the percentage of virus attachment at each time point was calculated, relative to time point 240min that was set to 100% attachment.

Plaque size and single-step growth kinetics

[0116] To compare the plaque sizes, RK13 cells grown in 6-well plates were infected with RacL11 wild type and the mutants L11_D752N, L11_D752N ΔgC or L11_D752N ΔgC rev at an MOI of 0.0001 and overlaid with 1.5% methylcellulose in EMEM containing 2% FBS at 2hpi. Three days post-infection, plaques were visualized by IFA using anti-EHV-1 gM MAb B8. For

each virus, 50 plaques were photographed and average plaque areas were measured by using ImageJ software (<http://rsb.info.nih.gov/ij/>). Plaque sizes induced by RacL11 were set to 100%. Mean percentage and standard deviation were calculated from three independent experiments. For single-step growth kinetics assays, RK13 cells seeded in 24-plates were infected at an MOI of 3. The viruses were allowed to attach for 1h at 4°C, followed by a penetration step of 1.5h at 37°C. After washing twice with PBS, the infected cells were treated with ice-cold citrate buffered saline (CBS, pH 3.0) for 3 min to remove virus on the surface. At different time points (0, 4, 8, 12, 24, 36, 48hpi), supernatants and cells were collected separately. Extracellular and cell-associated viral titers were determined using conventional plaque assays. Single-step growth curves were computed from three independent experiments.

In vitro growth properties

[0117] The *in vitro* growth properties of various virus mutants in cultured cells were analyzed.

To investigate the impact of gC deletion on binding ability of L11_D752N ΔgC to target cells, attachment assay was performed. As expected, L11_D752N ΔgC was shown to bind less efficiently to RK13 cells when compared with either wild type RacL11, parental virus L11_D752N or the revertant L11_D752N ΔgC rev (Fig.6). The ability of L11_D752N ΔgC to spread from cell to cell was determined by comparing plaque sizes. Whereas no significant difference could be observed between wild type, parental virus and gC revertant, the relative plaque area formed by L11_D752N ΔgC, however, was 10% larger ($p < 0.0001$) than those of others (Fig.7), indicating that with the absence of gC, the cell-to-cell spread of EHV-1 is even more efficient. With respect to growth kinetics, the *in vitro* replication of L11_D752N ΔgC was less effective as demonstrated by extracellular and intracellular titers that were reduced by approximately 20-fold and 10-folds, respectively, relative to wild type, parental virus or gC revertant (Fig.8). The virus titers in cell culture supernatants infected with wild type RacL11, parental virus L11_D752N or the repaired L11_D752N ΔgC rev reached the value of intracellular titers at approximately 12 hours post-infection. In L11_D752N ΔgC infected cells, however, a delayed release of infectious progeny was observed by that the extracellular titer crossed intracellular titer at 16-18 hours post-infection. From these results, it could be concluded that the *in vitro* growth of L11_D752N ΔgC was impaired in virus binding and egress though it could form slightly larger plaques.

Example 4 Vaccination of Mice

[0118] Wild type RacL11 as well as the mutants L11_D752N, L11_D752N ΔgC and L11_D752N ΔgC rev were purified by ultracentrifuge for 1h at 27,000rpm. The pellets were suspended in PBS, titrated on fresh RK13 cells, aliquoted and stored at -70°C until use. Three-week-old female BALB/c mice (Harlan) were randomly allocated into five groups of 16 mice each and left to acclimate to each other for one week in the Biosafety Level-2 facility. On day 0, all mice were weighted, anesthetized with 0.1mL/10g Xylazine/Ketamine and four groups were inoculated intranasally (IN) with each virus at a dose of 1×10^5 PFU in 20μL of PBS. The last group was used as a negative control and received 20μL of PBS. Bodyweights of each mouse was inspected until day 14 post inoculation (p.i.). Three mice from each group were euthanized on day 2 and day 4. The lung was removed, part of which was homogenized and used for determination of virus titers by standard titration on RK13 cells, and the rest was fixed with 10% formaldehyde and processed for histopathological analysis. On day 28, mice were challenged with wild type RacL11 at a dose of 1×10^5 PFU by the intranasal route. Data of individual bodyweights were collected for two weeks. On day 2 and day 4 post challenge (p.c.), three mice of each group were euthanized to remove the lung. Virus titers in the lung tissues were determined on fresh RK13 cells after homogenization.

[0119] On day 2 p.i., mean virus titers in lungs infected with RacL11, L11_D752N or L11_D752N ΔgC rev reached 5366 PFU/mg, 3450 PFU/mg and 4800 PFU/mg, respectively (Fig.9), between which no statistically significant difference was observed ($p \geq 0.442$). This result indicated that the single amino acid variation from D752 to N752 in DNA polymerase does not impair the *in vivo* growth of EHV-1 in mice, which was consistent with previous findings (Goodman *et al.*, 2007). In contrast, the *in vivo* replication of the gC-negative mutant L11_D752N ΔgC was significantly less effective ($p < 0.001$), with a mean virus titer of only 0.45 PFU/mg in lung tissues. On day 4 p. i., no virus could be recovered from L11_D752N ΔgC infected mice. With respect to histopathological changes on day 2 p. i., the lungs of mice infected with either wild type RacL11 or the polymerase mutant L11_D752N showed mild suppurative pneumonia accompanied by neutrophilic infiltration, increased perivascular edema and increased number of inflammatory cells (predominantly lymphocytes) in lymph vessels and perivascular area, whereas no abnormality was detected in lungs infected with L11_D752N ΔgC and PBS (Fig.10). As a result of the inflammatory response, continuous body weight loss of mice infected

with RacL11, L11_D752N or the rescuant virus L11_D752N ΔgC rev was observed till day 3 p. i. The body weight of mice infected with L11_D752N ΔgC, however, did not exhibit apparent reduction. On day 28 p. i., all the mice were challenged intranasally with RacL11. Two days post challenge (p. c.), RacL11 replicated to a titer of 2100 PFU/mg in lungs of mock-inoculated mice, but only 18.8 PFU/mg in L11_D752N ΔgC group (Fig.9). The virus titers in lungs of mice inoculated with RacL11, L11_D752N or L11_D752N ΔgC rev were even lower (0.5, 0.9 and 1.3 PFU/mg, Fig.9) on day 2 p. c., which was, however, at the expense of high growth efficiency and pathogenicity in vivo. On day 4 p. c., reisolation of virus from L11_D752N ΔgC group was not successful. On the basis of these results we concluded that the gC-negative, nonneurological mutant L11_D752N ΔgC is severely attenuated and apathogenic for mice, but can confer protective immunity.

Example 5 Vaccination of Horses

[0120] Horses (6 to 8 month old foals) were grouped into three groups A, B and C. Group A

was treated with live attenuated EHV-1 virus having gC gene deleted (L11_D752N ΔgC).

Group B was treated with live attenuated EHV-1 virus (RacL11). Group C was control group.

Vaccination and samplings were done according to the schedule shown in Table 2.

Table 2

Group	Vaccination 6.3 log ₁₀ DIC ₅₀	Samples for vaccine shedding	Challenge	Post- challenge examination	Blood sampling	Nasal swabbing
A n=8	Intramuscular route D0: 2.4 ml D28: 2.0 ml	D-1, D3, D4, D5, D6, D7, D10 and D13. D27, D31,	D42 EHV1 strain 10 ^{5.0} TCID ₅₀ /nostril	Daily from D42 to D56	D-1, D7,	D-1,
B n=8	D0 and D28 Intramuscular route Dose = 1.5 ml	D32, D33, D34, D35, D38 and D41			D13, D27,	D41,
C n=8	Not vaccinated	D-1 and D27			D41, D43,	daily
					D44, D45,	from
					D46, D47,	D43 to
					D48, D49,	D49,
					D52 and	D52 and
					D56	D56

[0121] Both vaccines were well tolerated by the foals. The vaccine virus strains could not be detected in any sample by virus isolation. Control horses did not seroconvert up to the time of challenge as evidenced by the absence of SN and CF antibody titers to EHV-1. Horses from group A and B mounted a SN response to first vaccination which was boosted after second vaccination. CF antibody titers in both groups of horses remained low/undetectable after first vaccination and increased after second vaccination, but never reached high levels (Figure 11).

[0122] Clinical signs after challenge in the control horses included a tri-phasic fever response and respiratory disease characterized by moderate to severe nasal discharge. In contrast, hyperthermia was only sporadically found in both vaccinated groups of horses and nasal discharge was reduced in severity and duration. A notable effect of vaccination was the complete absence of viremia after challenge in 5/8 horses from both group 1 and 2 (Figure 12). Also the duration of viremia was reduced in the vaccinated compared to the control horses. Figure 13 shows that group A had significantly reduced virus shedding in the nasal swabs, whereas nasal shedding in group B horses was more variable, but reduced compared to the control horses.

Example 6 Sequencing of RacL11 gC gene

[0123] The gC gene of RacL11 was sequence and is represented by SEQ ID NO:34 and SEQ ID NO:35 for DNA and protein sequence, respectively.

[0124] It will be apparent that the precise details of the methods described may be varied or modified without departing from the spirit of the described disclosure. We claim all such modifications and variations that fall within the scope and spirit of the claims below.

[0125] All documents cited or referenced herein ("herein cited documents"), and all documents cited or referenced in herein cited documents, together with any manufacturer's instructions, descriptions, product specifications, and product sheets for any products mentioned herein or in any document incorporated by reference herein, are hereby incorporated herein by reference, and may be employed in the practice of the invention.

Claims

1. An immunogenic composition comprising a recombinant Equine Herpesvirus -1 (EHV-1), wherein the EHV-1 comprises a mutated glycoprotein C (gC) gene which does not encode functional gC protein, wherein the entire gC gene is deleted and wherein the recombinant EHV-1 comprises a DNA polymerase (Pol) comprising an asparagine (N) at the amino acid position 752 when compared to SEQ ID NO: 2 or 37 from the starting methionine.
2. The composition of claim 1, wherein the EHV-1 is derived from the RacL strain.
3. The composition of claim 1, further comprising a pharmaceutically or veterinarily acceptable vehicle, diluent, adjuvant, or excipient.
4. The composition of claim 2, further comprising a pharmaceutically or veterinarily acceptable vehicle, diluent, adjuvant, or excipient.
5. A recombinant EHV-1 comprising a mutated gC gene, wherein the entire gC gene is deleted, and wherein the recombinant EHV-1 comprises a DNA polymerase (Pol) gene encoding a Pol comprising an asparagine (N) at the amino acid position 752 when compared to SEQ ID NO: 2, 4, 6, or 37 from the starting methionine.
6. The recombinant EHV-1 of claim 5, wherein the EHV-1 is derived from the RacL strain.