

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
14 March 2002 (14.03.2002)

PCT

(10) International Publication Number
WO 02/20041 A2

(51) International Patent Classification⁷: A61K 39/00

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(21) International Application Number: PCT/US01/27601

(22) International Filing Date:
6 September 2001 (06.09.2001)

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(25) Filing Language: English

(26) Publication Language: English

(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(30) Priority Data:
09/658,547 9 September 2000 (09.09.2000) US

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(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

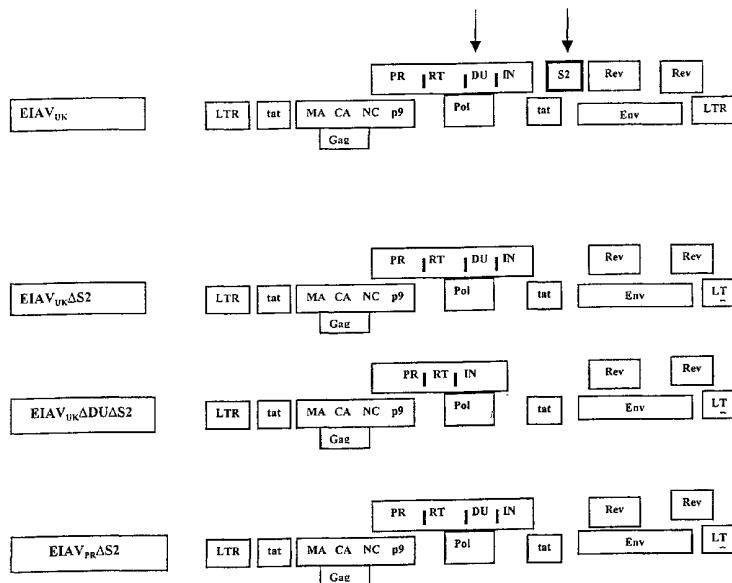
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(54) Title: EIA VACCINE AND DIAGNOSTIC

Schematic diagrams of replication competent EIA viruses and Constructs



(57) Abstract: The invention provides and equine infectious anemia (EIA) vaccine that provides immunity to mammals, especially equines, from infection with equine infectious anemia virus (EIAV) and which allows differentiation between vaccinated and non-vaccinated, but exposed, mammals or equines. Preferably said vaccine encompasses at least one mutation in an EIAV which produces a non-functional gene in the vaccine virus that is always expressed in disease-producing wild-type EIA viruses. Additionally, said EIA vaccine virus cannot cause clinical disease in mammals or spread or shed to other mammals including equines.

WO 02/20041 A2



Published:

- without international search report and to be republished upon receipt of that report
- with sequence listing part of description published separately in electronic form and available upon request from the International Bureau

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

EIA VACCINE AND DIAGNOSTICBACKGROUND OF THE INVENTION

Field of the Invention:

This invention pertains to an EIA vaccine, which provides immunity from disease and/or infection with EIAV, which vaccine allows diagnostic differentiation between vaccinated and non-vaccinated, but exposed or
5 diseased mammals. More specifically, this invention pertains to a vaccine comprising an EIAV wherein an accessory gene has been made nonfunctional and wherein said nonfunctional accessory gene still allows the EIAV to replicate in tissue culture.

Brief Description of the Prior Art:

10 The equine infectious anemia virus is a member of the lentivirus subfamily of retroviruses and causes persistent infection and chronic disease in horses worldwide. As such, it is closely related to human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV) and feline immunodeficiency virus (FIV). As with HIV and SIV, disease caused
15 by EIAV is spread by blood transmission. With EIAV, the blood transmission most often occurs by biting flies and other insects carrying virus particles from one horse to another. The first cycle of disease (clinical episode or first febrile episode) in an infected horse usually occurs within 42 days after transmission of the virus. This first cycle is usually
20 characterized by the acute stage of EIA and manifested by pyrexia, thrombocytopenia, anorexia, depression and high plasma viremia levels. Anemia is not usually detected at this stage. Resolution of this first febrile episode is normally observed after 1 to 5 days and occurs concomitantly with a dramatic drop in the amount of plasma-associated virus. Following
25 the acute stage, some animals may remain clinically normal, while others go on to experience multiple bouts of illness in which severe anemia may accompany pyrexia, thrombocytopenia, edema, and dramatic weight loss, and death. Nucleotide sequence data has revealed a high mutation rate

of this lentivirus genome during persistent infection (Payne et al, Virology, 1987: 161, p. 321-331) incorporated herein by reference. It is generally known that multiple isolates from the field demonstrate similar genomic differences indicating that EIAV, as HIV and FIV, undergoes a continuing
5 mutation process within its various hosts. It is generally thought that neutralizing antibodies aid in the selection of new antigenic virus variants (mutations) during persistent infections. In infections with EIAV, serologically distinct variants emerge possibly through immune selection pressure operating on random viral genome mutations. It is proposed that
10 horses that show no further clinical signs of disease have developed a mature immune response that can contain the virus and its immunologically-recognized mutants.

The disease is significant because horses that demonstrate exposure to EIAV via testing for antibodies in the blood (Coggins Test or
15 similar anti-p26 antibody detecting test) are either required to be destroyed or strictly quarantined. Because of the Coggins Test and its broad use in the world, especially in testing all performance horses that are transferred into and out of the United States, it is critical that vaccinated equines be able to be differentiated from infected equines.

20 The genetic organization of EIAV, as with HIV, SIV and FIV contains only three accessory genes (*S1*, *S2* and *S3*), in addition to the *gag*, *pol* and *env* genes common to all retroviruses. The *S1* open reading frame (ORF) encodes the viral Tat protein, a transcription *trans* activator that acts on the viral long-terminal-repeat (LTR) promoter element to
25 stimulate expression of all viral genes. The *S3* ORF encodes a Rev protein, a post-transcriptional activator that acts by interacting with its target RNA sequence, named the Rev-responsive element (RRE), to regulate viral structural gene expression. The *S2* gene is located in the *pol-env* intergenic region immediately following the second exon of Tat
30 and overlapping the amino terminus of the Env protein (see Figures 1, 2a and 2b). It encodes a 65-amino-acid protein with a calculated molecular mass of 7.2 kDa, which is in good agreement with the size of an *in vitro*

translation product. S2 appears to be synthesized in the late phase of the viral replication cycle by ribosomal leaky scanning of a tricistronic mRNA encoding Tat, S2 protein, and *Env*, respectively. The ORF coding for the S2 protein of EIAV is highly conserved in all published EIAV sequences and contains three potential functional motifs (Figure 2a): GLFG (putative nucleoporin motif), PXXP (putative SH3 domain binding motif) and RRKQETKK (putative nuclear localization sequence). Antibodies to S2 protein can be found in sera from experimentally and naturally infected horses, indicating that S2 is expressed during EIAV replication in vivo. These observations suggest that S2 is likely to perform an important role in the virus life cycle. A discussion of the function of S2 is found in Li et al (J. Virol., Oct. 1998, p 8344-8348), incorporated herein by reference.

A second interesting gene contained within the lentivirus group codes for dUTPase. This enzyme catalyzes the conversion of dUTP to dUMP and pp_i. The gene encoding the dUTPase has been mapped within the *pol* gene for EIAV and FIV. The lentivirus dUTPase gene has been designated *DU*. Studies with *DU* deletion mutants (ΔDU) of EIAV and FIV show that this enzyme is not required for replication of the viruses in fetal equine kidney cells or Crandell cells. However, efficient replication of the EIAV or FIV in monocyte/macrophage cells (typical replication host cell) does require *DU*. The differences indicated have been described in detail in a publication by Lichtenstein et al (J. Virol., May 1995, p 2881-2888), incorporated herein by reference.

Envelope proteins (*env*) are thought to be required for protection from disease and, perhaps, protection from infection. By protection from disease is meant that a mammal exposed to the virus, does not demonstrate clinical signs (fever, lethargy, anemia, etc.) but does carry particles associated with the viral RNA genome (shortened herein to viral particles) in its blood, said particles being detectable by a reverse transcriptase polymerase chain reaction test (RT-PCR). By protection from infection is meant that a mammal exposed to the virus does not demonstrate clinical signs nor does its blood contain RT-PCR-detectable

virus particles as described above. The major envelope proteins of EIAV are gp90 and gp45. These are proposed as the protective antigens of EIAV. By the term protective antigens is meant antigens from EIAV that produce either protection from disease or protection from infection as indicated above.

It would seem obvious to prepare a vaccine by purifying out the *env* proteins, especially gp 90 and gp45. Indeed, preparation of vaccines comprising gp90 and gp45 has been attempted with essentially no success. Issel et al (J. Virol. June 1992, p 3398-3408) report that a gp90/gp45 vaccine protected ponies from infection caused by homologous EIAV (the subunits were derived from the same EIAV strain as was used for challenge), however, these subunits did not protect ponies from either disease or infection when challenged with a heterologous EIAV strain. In fact, the latter produced enhanced disease signs. The subunit enhancement corroborates findings with SIV and FIV subunit vaccines that appear to enhance disease post challenge. These authors conclude that perfecting a subunit vaccine for lentiviruses (e.g., HIV, SIV, EIAV and FIV) poses a significant challenge because of the subunit enhancement effect.

Issel, et al (J. Virol., June 1992, pp 3398-3408) report the prevention of infection by a high-dose whole-virus EIA vaccine. However, vaccination of horses with this vaccine produces horses that are Coggins Test positive (anti-p26 antibody positive) and there is no practical method to demonstrate the difference between vaccinated and infected equines. Due to the previously-mentioned eradication program in effect in the U.S., a whole-virus vaccine is not feasible.

Since there has been no effective and safe method for immunizing mammals against lentiviral diseases, particularly equines against EIAV and since EIAV is such a wide-spread and significant disease world-wide, there remains a long-felt need to prepare such a vaccine.

SUMMARY OF THE INVENTION

The vaccine of this invention provides the first successful vaccine that effectively and safely immunizes mammals, especially equids, from disease and/or infection caused by EIAV wherein vaccinated mammals
5 can be differentiated from wild-type EIAV infected mammals.

This invention describes a vaccine for effectively and safely immunizing mammals, especially equids, from disease caused by EIAV, said vaccine comprising a gene-mutated EIAV wherein said virus lacks the ability to express the mutated gene protein *in vivo* and wherein said
10 lack of expression can be used to differentiate vaccinated from non-vaccinated or infected mammals.

Encompassed within this invention is an EIAV wherein said virus contains a mutation in a gene that allows replication of the virus *in vitro* such that large-scale production can be accomplished.

15 Also encompassed within this invention is an EIAV wherein said virus contains a mutation of the S2 gene or portions thereof ($\Delta S2$), a mutation in the DU gene (ΔDU) or a portion thereof, a mutation in a regulatory gene that inhibits expression of the S2 or DU genes or a combination of types of said mutations ($\Delta S2\Delta DU$). It is expected that
20 further mutations can be made such that the EIAV in the vaccine contains multiple mutations in multiple genes including the $\Delta S2$, ΔDU or both.

It is within the scope of this invention that a diagnostic test can be used to differentiate vaccinated equines from non-vaccinated and/or infected equines by measuring the presence or absence of antibodies to
25 the S2 protein, to the DU protein or to both proteins. Also, a PCR-based diagnostic test could be used to detect the presence or absence of the S2 and/or DU genes or gene sequences in the equine and, thus, detect whether an equine had been infected with EIAV or vaccinated with the composition of this invention.

30 Finally, it is expected that said mutated regions could serve as points for insertion of foreign genes or gene sequences and that said $\Delta S2$

or ΔDU or combination thereof with a foreign gene insert could be useful as a vector for vaccination against diseases of mammals other than EIA. Preferably, the insertion would be placed into the ΔDU region.

BRIEF DESCRIPTION OF THE DRAWINGS

5 Figure 1 is a schematic diagram of replication competent EIAV including the location of the accessory genes of EIAV_{UK}.

Figure 2a is a schematic representation of the EIAV S2 gene and mutant clones derived from EIAV_{UK}.

10 Figure 2b is a schematic representation of the Wild-type EIAV S2 gene compared with the EIAV.2M/X (EIAV_{UK} Δ S2) gene.

Figure 3a is a circular map of biological proviral clone EIAV_{PR}.

Figure 3b is a circular map of molecular infectious clone EIAV_{UK}.

Figure 3c is a circular map of mutant EIAV_{UK} Δ S2.

Figure 3d is a circular map of mutant EIAV_{PR} Δ S2.

15 Figure 3e is a circular map of mutant EIAV_{UK} Δ DU Δ S2

Figure 4 are graphs demonstrating the *in vitro* replication of EIA virus mutant clones.

Figure 5 is a schematic representation of the DU gene location and construction of EIAV Δ DU.

20 DETAILED DESCRIPTION OF THE INVENTION

This invention encompasses a composition for effectively and safely immunizing mammals from disease caused by EIAV, said composition comprising a gene-mutated EIAV wherein said virus lacks the ability to express the mutated gene protein *in vivo* and wherein said
25 lack of expression can be used to differentiate vaccinated from non-vaccinated or infected mammals. It is contemplated that any gene could be mutated from any EIAV as long as the mutated gene would allow large-scale production of EIAV or EIAV particles. It is further contemplated that more than one gene from EIAV could be mutated. It is also understood
30 that the composition of the present invention produces a mature immune

response capable of protecting equines from disease caused by heterologous as well as homologous EIAV strains.

By gene-mutated is meant that one or more deletions or insertions are made in a gene of EIAV which makes the gene non-functional and thus, differentiating between the gene-mutated virus and wild-type virus. The gene mutation can either be produced biologically, by passaging the virus through cells, cell lines or animals until it becomes non-infective and a gene-mutated virus is produced, or molecularly (produced by recombinant techniques). By non-functional is meant that the gene does not express its protein or product at all or is not expressing its normal gene product. By a differentiating gene is meant that the gene product is normally expressed by wild-type EIAV and antibodies to the gene product are found in infected horses but not in horses vaccinated with the vaccine compositions of the present invention. By large-scale production is meant that the gene-mutated EIAV can be grown or replicated *in vitro* such that large quantities (e.g., >1 liter, preferably greater than 10 liters) can be produced for vaccine manufacture. Such large-scale production is accomplished if a virus or virus construct can be produced that is stable, withstands concentration and/or purification, if necessary, is stable to adjuvants and storage as a vaccine for up to 18 months. By deletion is meant that all or a portion of a gene of EIAV is removed thus causing the gene to become non-functional. By insertion is meant that all or part of another gene or a sequence of nucleotides (e.g., a stop codon) is inserted into a gene causing it to express a different protein (e.g., one expressed by the inserted gene) and become non-functional for the normally-expressed protein or become non-functional by the insertion of a stop codon. Encompassed by this invention is an EIAV wherein said virus contains a mutation of the *S2* gene or portions thereof ($\Delta S2$), a mutation in the *DU* gene (ΔDU) or portions thereof, a mutation in a regulatory gene that controls expression of *S2* or *DU*, or a combination of such mutations affecting both genes ($\Delta S2\Delta DU$). Said mutations would produce a non-functional *S2* and/or *DU* gene. Illustratively, it has been demonstrated that

placing a stop codon into the S2 gene, replacing amino acid G⁵ produced a non-functional S2 gene. Additionally, it has been demonstrated that changing the S2 gene's M¹⁶ to T and replacing the G⁵ and G¹⁸ with stop codons produced a non-functional S2 gene. Finally, it has been

5 demonstrated that deletion of the initial 5 nucleotides of S2 produced a non-functional S2 gene. Therefore, mutations in the S2 gene have produced EIAV with non-functional S2 genes. The following is an illustration, but non-limiting description of how to produce the above mutations. Two adjacent fragments were amplified by PCR spanning the

10 whole S2 gene. One of the two resultant PCR products carried the specific substitution or deletion mutations incorporated into a PCR primer. The flanking PCR products were phosphorylated, ligated, and then used as a template for a second round of PCR with the outer primer pair. The final full-length PCR product was digested with NcoI and Bpu1102I, cloned

15 into EIAV_{UK} previously digested with NcoI and Bpu1102I. All plasmid clones were sequenced to verify introduced mutations to ensure the integrity of the PCR-amplified sequence. It is important to note that the above-identified mutant EIAV clones replicated well *in vitro*, especially in fetal equine kidney cells (FEK), in equine blood monocyte-derived

20 macrophage cells (MDM) or an equine dermal cell line (ED). Therefore, these gene-mutated EIAV clones can be produce in large-scale and have been used to prepare a vaccine for safe and effective immunization of horses.

As would be recognized, mutations comprising deletions could be

25 made such that the EIAV contained multiple deletions in genes including the S2 ($\Delta S2$), DU (ΔDU) or both. A gene-mutated EIAV comprising a deletion in the DU (ΔDU) gene was prepared by deleting a StylI restriction fragment containing 80% of the DU coding sequence, including four of the five conserved amino acid motifs, from the proviral clone designated

30 PV19-2-6A (described by Lichtenstein et al, J. Virol. May 1995, p. 2881-2888 and incorporated herein by reference). It has been demonstrated that the above-described deletion in the *DU* gene does not reduce the

ability of this gene-mutated EIAV to replicate in either fetal equine kidney cells (FEK) or in an equine dermal cell line (ED) both considered to be *in vitro* growth. Therefore, it has been demonstrated that this gene-mutated EIAV can be produced in large-scale and vaccine production is possible.

5 In accord with the invention, it has been found that the S2 antibodies can be detected in horses with EIAV infections by using immunoassays comprising recombinant S2 protein or synthetic S2 peptides as the capture antigen. Additionally, it has been determined that the presence of the type of virus found in a mammal can be differentiated
10 between the vaccine virus and the wild-type virus by use of gene probes (PCR-based). It has also been determined that the S2 gene of EIAV is not required for *in vitro* replication in a variety of equine cells including but not limited to fetal equine kidney cells (FEK), equine dermal cell lines (ED) or cultured equine monocytes/macrophages. It has further been determined
15 that the S2 deletion mutant replicates *in vivo* only at very low levels as compared with the wild-type EIAV (Li, et al, Jan. 2000, J. Virol. pp 573-579), incorporated herein by reference. By low levels is meant that the virus produces less than 1×10^5 EIAV particles (as detected by PCR) *in vivo*, preferably less than 1×10^4 . Further, it has been determined that the
20 S2 protein is not a component of purified EIAV particles and that horses immunized with purified EIAV particles do not produce serum antibodies reactive with *in vitro* synthesized S2 protein or peptides. Therefore, even horses vaccinated with purified EIAV particles can be differentiated from wild-type infected horses. These results indicate that the presence of S2
25 specific antibody can be used to identify EIAV-infected horses and to distinguish infected horses from those that have been vaccinated with an inactivated whole virus or an attenuated vaccine in which the S2 gene is mutated so as to make it non-functional. Therefore, it is within the scope of this invention that a diagnostic test can be used to differentiate
30 vaccinated equines from non-vaccinated and/or infected equines by measuring the presence or absence of antibodies to the S2 protein, to the DU protein or to both proteins. Such differentiation can be measured by

developing an immunoassay, an antibody-detecting assay (e.g., indirect fluorescent antibody, immunodiffusion, agar diffusion, electrophoresis) or a PCR-based assay known to the art. An example of an immunoassay is an enzyme linked immunosorbant assay (ELISA) that detects and/or
5 quantitates antibodies to specific proteins in serum, blood or tissues. ELISA technology could also be used to detect the presence or absence of virus-associated antigens in the blood, serum or tissues. By virus associated antigens is meant the presence or absence of a gene expression product such as the *S2* or *DU* proteins in the case of the *S2* or
10 *DU* genes, respectively. Additionally, PCR-based assays have been used to measure the presence or absence of genes or gene sequences in the blood, serum or tissues of an equine, thus indicating that a horse had been infected or vaccinated, as the case may be. For this particular embodiment, an ELISA would detect the presence of antibodies to the *S2*
15 or *DU* proteins. If antibodies were present in horses that were tested it would indicate that the horse had been infected with EIAV. Horses that had been vaccinated with a gene-mutated EIAV construct containing a non-functional *S2* gene would not contain *S2* antibodies in their serum. Horses that had been vaccinated with a gene-mutated EIAV construct
20 containing a non-functional *DU* gene would not contain *DU* antibodies in their serum. Thus, vaccinated horses could be differentiated from infected horses. The PCR-based assays would be used to detect the presence or absence of gene sequences within the horse. For instance, if a horse had been infected with a wild-type EIAV, it would contain the gene sequence
25 for wild-type *S2* or *DU*. However, equines immunized with vaccines comprising a gene-mutated EIAV, particularly one wherein the *S2* or *DU* genes comprised deletions or specific mutations would not contain the gene sequence for wild-type *S2* or *DU* gene products.

As would be recognized from this invention, said mutated (deleted)
30 gene regions could serve as potential points for insertion of foreign genes and that said $\Delta S2$ or ΔDU or a combination thereof, preferably within the ΔDU , with a foreign gene insert could be useful as a vector for vaccination

against diseases of mammals other than EIA and could serve to protect mammals from a second type of viral, bacterial or parasitic disease. For instance, it would be highly advantageous to incorporate a gene for another important equine disease (e.g., equine influenza, equine herpes virus types 1, 2, or 4, *Streptococcus equi*, *Rhodococcus equi*) into the gene-mutated EIAV. When such a vaccine is used to vaccinate horses, the horse would not only be protected from disease caused by EIAV but also from disease caused by the other equine disease organism.

Vaccines of the present invention have been either inactivated or administered live. Inactivated vaccines of the present invention comprise treatment of the live virus, attenuated virus, purified virus particles or whole virus particles with agents that inactivate the virus such that it cannot replicate *in vitro* or *in vivo*. Such agents are selected from the group consisting of formalin, formaldehyde, beta-propiolactone, binary ethyleneimine, ethyleneimine, merthiolate, thimerosal, psoralen and combinations thereof. These agents can be used at concentrations varying from 1 part per billion to 0.5%, depending on the agent. For instance, thimerosal would be used at a concentration of between 1 part per 1,000 and 1 part per billion, preferably between 1 part per 5,000 and 1 part per 100,000. Formalin would be used at a concentration between 0.00001% and 0.5%, preferably between 0.0001% and 0.1%. Ethyleneimine would be used at a concentration between 0.00001M and 0.1M, preferably between 0.0001M and 0.01M. Beta-propiolactone would be used at a concentration similar to that used for ethyleneimine.

Vaccines of the present invention may also include adjuvants in order to enhance the immune response. Adjuvants are chemical agents or extracts of microorganisms that induce an enhanced immune response. When accompanied by an antigen, they enhance the immune response produced by the antigen. In the case of EIAV particles, EIAV purified virus particles, EIAV constructs, attenuated EIAV, EIAV (whole virus) or EIAV subunits, adjuvants may be added to enhance the immune response to the vaccine composition to provide improved protection. It is recognized

that adjuvants would be used according to the present invention at concentrations varying from 0.1% to 50% v/v, preferably from 1% to 20%.

Although any adjuvant will enhance the immune response and can be used with the vaccine compositions of the present invention, it is within
5 the teaching of the present invention that adjuvants selected from the group consisting of polymer-based, oil-based, block copolymer-based, aluminum salt based, organism-based, lipid-based and aqueous-based, surfactants are preferred. Non-limiting examples of surfactants useful as
10 adjuvants include hexadecylamine, octadecylamine, lysolecithin, demethyldioctadecyl ammonium bromide, N,N-dioctadecyl-N'-N-bis (2-hydroxyethylpropane diamine), methoxyhexa-decyl-glycerol and pluronic polyols and saponin, Quil A. Non-limiting examples of polyanions or polycations include pyran, diethylaminoethyl (DEAE) dextran, dextran sulfate, polybrene, poly IC, polyacrylic acid, carbopol, ethylene maleic
15 acid, aluminum hydroxide, and aluminum phosphate. Non-limiting examples of peptide adjuvants include muramyl dipeptide, dimethylglycine and tuftsin. Non-limiting examples of other types of adjuvants include oil emulsions, immunomodulators (interleukin-1, interleukin-2 and interferons) or combinations of any of the foregoing adjuvants. A number of acrylic
20 acid polymers and copolymers of acrylic acid and methacrylic acid and styrene have adjuvant activity. Polyvinyl Chemical Industries (Wilmington, MA) provides such polymers under the trade-name NEOCRYL®, BEOCRYL A640, an aqueous acrylic copolymer with styrene. Other useful NEOCRYL products are 520 and 625, and NEOREZ 966. Ethylene
25 maleic acid, produced from ethylene maleic anhydride is a preferred adjuvant. In order to produce ethylene maleic acid, EMA 31 or EMA 91 (Monsanto Co., St. Louis, MO) is prepared in an aqueous solution at a concentration between 0.1 and 10% (w/v), preferably between 0.5 and 5% (w/v). It is used in product at a concentration of 1 to 50% (v/v). More
30 preferably, Carbopol is used as an adjuvant alone or in combination with tweens, spans and oils. Representatives of this type of adjuvant are HAVLOGEN® and SPUR®. These adjuvants are prepared by mixing

Carbopol 934P at a concentration between 0.5 and 10% (w/v), preferably between 1 and 5% (w/v), more preferably between 2.0 and 4% (w/v). Added to the Carbopol can be detergents such as Tween 80 and Span 20, and an oil for producing an emulsion. The oils can be cottonseed, peanut, mineral, or any other type known to be safe for use in animals. The concentrations of the oil ranges from 0.000001% to 10% (v/v), preferably from 0.00001% to 5% (v/v), more preferably from 0.0001% to 1% (v/v). Other commercially-available adjuvants useful for this vaccine include but are not limited to POLYGEN™, a polymer-based low molecular weight, non-particulate copolymer which can form cross-linkages in solution to become a high molecular weight gel (MVP Laboratories, Inc., Ralston, NE) or EMULSIGEN™ or EMULSIGEN™ PLUS, both oil-in-water adjuvants provided by MVP Laboratories, Inc. Organism-based adjuvants are those utilizing whole microorganisms or extracts of microorganisms, such as Muramyl Dipeptide, RIBI®, whole Parapox viruses or extracts thereof (also known as Baypamun) and *Corynebacterium acne* extracts. Lipid-based adjuvants include but are not limited to BAY R1005, liposomes and ISCOMS. The most preferred adjuvants of the present invention include HAVLOGEN®, POLYGEN™, BAY R1005, Baypamun and ethylene maleic acid-based. Often, two or more adjuvants can be used to formulate with the EIAV constructs of this invention.

In order to better understand the following Examples, the wild-type EIAV is referred to as the Wyoming isolate or EIAVwyo. This virus is termed a primary isolate and it replicates only in equine monocyte-macrophage cell cultures in which the virus is cytopathic for the infected cells by 7-10 days post infection. Thus, EIAVwyo can be produced only in short-term macrophage cultures to obtain infectious virus in cell supernatants or in experimentally infected horses to obtain infectious plasma (Malmquist et al. 1973, Arch. Virol. 42, p 361-370). Either source of the primary isolate EIAVwyo can be used to experimentally infect equids and produce classical EIA disease. To obtain a cell-adapted strain of

EIAVwyo that is able to replicate in other cell types, the primary EIAVwyo isolate was serially passaged in equine cells to produce a stock of EIAV virus that could be grown on various fibroblastic cells (Malmquist et al 1973, Arch Virol. 42, p 361-370; Parekh et al. 1980 Virology 107:520-525). The cell-adapted EIAVwyo was then grown in fetal equine kidney cell cultures to produce larger amounts of virus and thus used to prepare stocks of the cell-adapted virus designated EIAV_{PR} (Montelaro et al. 1982 J. Virology 42:1029-1038). Inoculation of ponies with the avirulent EIAV_{PR} results in 100% infection but does not produce EIA disease, confirming the attenuated avirulent nature of the EIAV_{PR} strain (Orrego et al., 1982 Am. J. Vet. Res. 43:1556-1560). To obtain a reference strain of EIAV that can be grown in fibroblastic cells and produce disease in experimentally-infected equids, the EIAV_{PR} strain was serially passaged in ponies and isolated in the context of infectious plasma after the third serial passage (Orrego et al. 1982 Am. J. Vet. Res. 43:1556-1560). The *in vivo* serial passage restored virulence to the EIAV, but did not cause it to lose its ability to replicate in cells other than equine macrophages. This virus stock in infectious plasma was designated as host-adapted EIAVwyo. Inoculation of ponies with host-adapted EIAVwyo induced 100% infection and clinical EIA disease (Payne et al. 1987 Virology 161:321-333). In a subsequent set of experiments, a host-adapted EIAVwyo was grown in fetal equine cell culture in the presence of neutralizing immune serum from a pony to generate antigenic neutralization escape mutants by antibody selection that were then biologically cloned to obtain a more homogeneous genomic population (Rwambo et al. 1990 Arch. Virol. 111:275-280). Subsequent stocks of this biologically cloned reference virus produced in fetal equine kidney cell culture were termed EIAV_{PV} to indicate "pony virulent". Infection of ponies with the biologically cloned EIAV_{PV} results in 100% infection and disease (Hammond et al. 1997 J Virology 71:3840-3852). Since lentiviruses like EIAV exist in nature as complex genomic mixtures termed quasispecies, primary isolates (EIAVwyo) and biological clones (EIAV_{PV}) contain a variety of genomic species. To obtain genetically

homogenous forms of EIAV, infectious molecular clones were derived from the avirulent EIAV_{PR} (e.g., EIAV 19-2) (Payne et al 1994 J. Gen. Virol. 75:425-429) and pathogenic EIAV_{PV} (Cook et al. 1998 J. Virology 72:1383-1393) reference stocks by standard molecular biology cloning
5 procedures. Inoculation of ponies with infectious virus stocks produced from chimeras with EIAV_{PR} and EIAV_{PV} sequences (e.g., EIAV_{UK}) were shown to produce disease in experimentally-infected horses. The infectious molecular clone EIAV_{UK} was the first reported pathogenic molecular clone.

10 Figure 3b displays the circular map of this infectious molecular clone, EIAV_{UK}. In order to provide further information for the following examples, Figure 3c displays the circular map of EIAV_{UK}ΔS2, Figure 3d displays the circular map of EIAV_{PR} ΔS2, and Figure 3e displays the circular map of EIAV_{UK}ΔDUΔS2.

15 The invention is further illustrated but is not intended to be limited by the following examples in which all parts and percentages are by weight unless otherwise specified.

EXAMPLE 1

20 Several different gene-mutated EIAV constructs were prepared according to the methods of Li et al (J. Virol., October 1998, pp 8334-8348) which are incorporated herein by reference. The basic S2 gene mutations were designed so as not to disrupt the second exon of Tat 10 base pairs (bp) upstream from the S2 initiation sequence, the envelope
25 initiator codon just 23 bp downstream from the S2 start codon sequence, or the putative Rev-response element (RRE) sequences that have been mapped to both the 5' and 3' ends of the *env* gene. A panel of clones with substitutions that introduce one or more premature stop codons (EIAV.2M/X and EIAV.G5/s) or with a deletion of the first 5 nucleotides of
30 the S2 gene to shift the S2 ORF (EIAVΔS2) were produced. These are schematically diagrammed in Figures 2a and 2b. The EIAV proviral DNA

is shown at the top; the complete deduced amino acid sequence of the putative S2 protein is shown in single letter amino acid code at the bottom.

Stop codons (indicated by arrows) were introduced into various positions in the EIAV S2 gene to generate the specific mutant virus strains. As
5 would be recognized, all of the constructs would be considered to be non-functional for S2 and will be referred to herein as Δ S2.

S2 mutant constructs were generated using the PCR-Ligation-PCR (PLP) strategy as previously described (Puffer, et. al., 1997 and Li, et. al., 1998). EIAV_{UK} plasmid DNA was used as the template to perform all PCR
10 reactions for generating S2 mutations except for EIAV_{UK}.2M/X.

EIAV.G5/s was generated using EIAV_{UK} as the template by PCR with Pfu polymerase (Stratagene) by using mutagenic downstream primer mspe3-5' (SEQ ID NO: 1) with upstream primer s2pst (SEQ ID NO: 2). A second flanking fragment was amplified using mutagenic upstream
15 primers mspe5'-3' (SEQ ID NO: 3) and s2sph (SEQ ID NO: 4).

EIAV_{UK} Δ S2 was similarly generated using EIAV_{UK} as the template by PCR with Pfu polymerase (Stratagene) by using downstream primer S2min/35rev (SEQ ID NO: 5) and upstream primer s2pst (SEQ ID NO: 2). A second flanking fragment was amplified using mutagenic upstream
20 primer S2min/53for (SEQ ID NO: 6) and s2sph (SEQ ID NO: 4).

Each of these corresponding two adjacent PCR fragments were gel purified, phosphorylated using T4 polynucleotide kinase (Gibco BRL), and ligated by using T4 DNA ligase (Gibco BRL). After inactivation at 65°C for 15 minutes, the ligation reaction was used for a subsequent amplification
25 using upstream primer s2pst (SEQ ID NO: 2) and downstream primer s2sph (SEQ ID No: 4). This product was gel purified, digested with NcoI and Bpu1102I, and then ligated into the NcoI and Bpyu1102I sites of EIAV_{UK}.

EIAV_{UK}.2M/X, which has its sequence compared with that of EIAV_{UK}
30 in Figure 2b, was generated using the EIAV_{UK}G5/s plasmid DNA as a template with downstream primer 2M35/RE (SEQ ID NO: 7) and upstream primer s2pst (SEQ ID NO: 2). A second flanking fragment was amplified

using mutagenic upstream primer 2M53/For (SEQ ID NO: 8) and downstream primer s2sph (SEQ ID NO: 4). The final cloning procedure was as described above.

For simplification and because all of the EIAV constructs described
5 are non-functional for S2 as demonstrated in tissue culture growth studies (as described in EXAMPLE 2), these EIAV constructs have been redesignated EIAV_{UK}ΔS2.

Standard PCR conditions used for the above-described reactions included, one cycle of denaturation at 95°C for 5 min., followed by 35
10 cycles of denaturation at 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds. The PCR reactions were set up using the following components:

10 μL 10X NEB Thermophilic buffer
1.0 L μ10 mM deoxynucleotide triphosphates dNTPs
15 1.0 μM forward primer (upstream primer)
1.0 μM reverse primer (downstream primer)
10 ng template DNA
x μL double distilled water (ddH₂O) (q.s. to 100 μL volume)
A 10 μL aliquot was run on an 1.0% agarose gel to make sure the
20 correct size product was amplified. The PCR products were then gel isolated and purified with a Qiaex II gel extraction Kit (150)(Qiagen, Cat. # 20021). The Qiaex II protocol is presented below:

1. Cut band from gel and place in a 1.5 mL eppendorf tube.
2. Estimate the volume of agarose gel slice, add 3 volumes of
25 buffer QX1, if the fragment is <4kb, and an additional 2 volumes of ddH₂O if the fragment is >4kb.
3. Vortex the Qiaex II beads and add 10 μL to the agarose slice suspension.
4. Mix well, incubate at 50°C for 5-10 minutes, mixing the tube
30 several times during the incubation period.

5. Centrifuge the sample for 30 seconds and carefully remove the supernatant with a pipette followed by washing the pellet once with 500 μ L of buffer QX1.
6. Wash the pellet twice with 500 μ L of buffer PE, and air dry
5 pellet 15-30 minutes at room temperature.
7. Resuspend the pellet in 20 μ L of ddH₂O, incubate at 55°C for 10 min., spin at full speed for 30 seconds.
8. Pull off supernatant and save to a clean eppendorf tube.
Measure the OD at 260nm for the concentration of the recovered fragment
10 on an agarose gel.
9. Add ddH₂O as needed to resuspend the pellet.

The two adjacent PCR fragments were individually phosphorylated in the following reaction mixture by using T4 polynucleotide kinase (NEB) prior to ligation. The phosphorylation reaction was set up as follows:

- 15 2.0 μ L 10X T4 polynucleotide kinase (PNK) buffer (NEB)
- 2.0 μ L 10 mM ATP (NEB)
- 1.0 μ L T4 PNK (NEB)
- 15 μ L gel purified DNA of each of these two adjacent PCR
 fragments

20 The reaction was incubated at 37°C for 1 hour. Following
 inactivation at 65°C for 10 min. the adjacently phosphorylated PCR
 fragments were then ligated together by using T4 DNA ligase (NEB) under
 the following conditions:

- 1.0 μ L 10X T4 DNA ligase buffer (NEB)
- 25 X μ L (50-100 ng) of each of two adjacent PCR fragments
- 1.0 μ L T4 DNA ligase (NEB)
- X μ L ddH₂O (q.s. to 10 μ L total volume)

After overnight incubation at 16°C the ligation reaction product was used in a second round PCR reaction to amplify the full-length PCR
30 fragment spanning these two adjacent PCR products. The second round PCR reaction was performed as previously described (see below) with the

exception that only upstream primer s2pst (SEQ ID NO: 2) and downstream primer s2sph (SEQ ID NO: 4) were used. Again, a 10 μ L aliquot was run on an agarose gel to make sure the correct product was amplified. The full-length PCR fragments were then gel isolated and
5 purified using the Qiaex II kit (see above). The purified full-length PCR fragment, together with EIAV_{UK}, were then cut with NcoI (Gibco BRL) and Bpu1 102I (Gibco BRL) under the following conditions:

2.0 μ L 10X React2 buffer (Gibco BRL)
1.0 μ L NcoI (Gibco BRL)
10 1.0 μ L Bpu1 102I
X μ L full length PCR product (1.0 μ g) or EIAV_{UK} (500 ng)
X μ L ddH₂O (q.s. to 20 μ L total volume)

The above restriction enzyme digestion mixture was incubated at 37° C for 2 hours. Digested DNA fragments from the full-length PCR
15 product and the EIAV_{UK} plasmid were individually gel isolated and purified using a Qiaex II kit as described above. The digested vector EIAV_{UK} and full length PCR fragment were ligated using T4 DNA ligase using the following procedure:

1.0 μ L 10X T4 DNA ligase buffer (NEB)
20 X μ L (25-50 ng) digested EIAV_{UK}
X μ L (200-400 ng) digested full length PCR fragment
1.0 μ L T4 DNA ligase (NEB)
X μ L ddH₂O (q.s. to 10 L total volume)

The ligation reaction was incubated at 16°C overnight and the
25 ligated products were transformed into *Escherichia coli* DH5 α (Gibco BRL) by heat shock as described below:

1. Thaw 100 μ L of DH5 α competent cells and incubate on ice
2. Add 1 μ L of ligation mixture to cells, mix gently, and incubate
on ice for 30
30 minutes

3. Heat pulse the tube in a 42° C bath for 45 seconds and incubate on ice for 2 minutes.
4. Add 0.9 mL SOC broth (2% bactotryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄ and 20 mM glucose, pH 7.0) and incubate the tubes at 37° C for 1 hour while shaking at 222 rpm.
5. Plate 150 µL of the transformation mixture onto LB-ampicillin (100 µg/mL) plates and incubate at 37° C overnight.

The proviral clones (EIAV_{UK}.2M/X, EIAV_{UK}G5/s and EIAV_{UK}ΔS2) were then screened by automatically sequencing using a Taq Dye Deoxy Terminator Cycle Sequencer Kit (Applied Biosystems) individually using an internal sense primer S40 (SEQ ID NO: 11) and an internal antisense primer S15 (SEQ ID NO: 12). Following the verification for the mutations in the S2 gene by sequencing, the proviral DNA clones were used for various future studies.

The generation of EIAV_{UK}ΔDUΔS2 was based on the modification of the previously studied EIAV_{PR}ΔDU virus in which the deoxyuridinetriphosphatase (dUTPase or *DU*) gene segment was deleted by removing a 330-bp Styl restriction fragment (Lichtenstein, et al., 1995). EIAV_{UK}ΔDUΔS2 was generated by subcloning into the full-length EIAV_{UK}ΔS2 proviral backbone of a SstI-NcoI fragment of EIAV_{PR}ΔDU, which contained a 330-bp deletion in the *DU* gene. EIAV_{PR}ΔS2 was created by subcloning into the full-length EIAV_{PR} proviral backbone of a NcoI-Bpu102I fragment of EIAV_{UK}ΔS2, which contained a S2 gene mutation. All of the various constructs discussed above contain a non-functional S2 gene and could be used in vaccines for immunizing horses against diseases caused by EIAV. The constructs are compared with the wild-type EIAV in Figures 1 and 2. Figures 3d and 3e represent the circular maps of EIAV_{PR}ΔS2 and EIAV_{UK}ΔDUΔS2.

It is expected that each of the gene-mutated EIAV constructs can be used to prepare either live attenuated or inactivated vaccines for safe

and effective immunization of horses from disease caused by EIAV and can be used to differentiate vaccinated horses from infected horses. As indicated previously, it is recognized that inactivation would be produced by adding an appropriate amount of any of the inactivating agents listed previously or others known in the art to be acceptable to lentiviruses. An appropriate amount means the lowest concentration of inactivating agent necessary to inactivate all of the virus particles without damaging the protective antigens (immunogens).

10 EXAMPLE 2

In order to demonstrate that the gene-mutated EIAV constructs from Example 1 could replicate in large-scale, a tissue culture growth study was conducted. One microgram of proviral clone DNA from each of the constructs was used to transfect an ED cell line. The ED cell line (ATCC CRL 6288) was grown in 6 well tissue culture plates seeded with between 2 and 4 x 10⁵ ED cells per well in 2 mL of the complete growth Minimum Essential Media with Earles salts (EMEM) plus 10% fetal calf serum, 100 units/mL of penicillin, 100 µg/mL of streptomycin (Gibco BRL 15140-122) and 2 mM L-glutamine (Gibco BRL 25030-081). The plates were incubated at 37° C in a CO₂ incubator approximately 16 to 24 hours until the cells were between 50 and 80% confluent. For each transfection, 1µg of DNA was diluted into 100 µL of OPTI-MEM I Reduced Serum Medium (Gibco BRL 18324-012) and 10 µL of Lipofectamine reagent (Gibco BRL 18324-012) was added to 100 µL of OPTI-MEM I Reduced Serum Medium (OPTI-MEM RSM). The two solutions were mixed gently and incubated at room temperature for 30 minutes to allow the DNA-liposome complexes to form. During this time, the ED cell cultures were rinsed once with 2 mL of OPTIMEM I RSM. For each transfection, 0.8 mL of OPTI-MEM I RSM was added to the tube containing the DNA-liposome complexes, the tube was mixed gently and the contents were overlaid onto the rinsed cells. No antibiotics were

added during transfection. The DNA-liposome/tissue cultures were incubated for 5 hours at 37° C in a CO₂ incubator. Following incubation, 1 mL of complete growth MEM containing twice the normal concentration of serum was added to the cell culture without removing the transfection mixture. Twenty four hours following the start of transfection the medium was replaced with fresh complete growth medium (EMEM). Starting at 48 to 72 hours post transfection, aliquots of the tissue culture supernatants were taken at periodic intervals and analyzed by using a standard reverse transcriptase (RT) assay as a measure of virus production. Supernatants resulting in RT activity were titrated in an infectivity assay based on cell-ELISA readings as described by Lichtenstein et al, 1995. After titer determination, aliquots of each of the virus construct stocks were frozen at -80°C for further evaluation and use. All of the constructs replicated well in both ED cells and in MDM cells producing RT levels of at least 10,000 CPM/10μL which was the normal level of RT activity observed in wild-type EIAV_{UK} (See Figure 4). Further passaging of the transfected cells in larger vessels was accomplished by use of the same techniques as described and serves as the basis for indicating that the constructs prepared in Example 1 could be produced in large-scale and, therefore, could be used to prepare vaccines.

The tissue culture grown virus construct stocks were molecularly characterized by extracting viral RNA and conducting RT-PCR analyses of the *DU* and *S2* genes using 20% glycerol cushion purified virus construct particles. These sequence analyses confirmed the *DU* and/or *S2* gene mutation in their corresponding virus constructs. The RT-PCR technique was also employed to identify recombinant virus construct stocks. Wild-type EIAV_{UK} generated a RT-PCR product of 592 base pairs (bp). In contrast, virus constructs containing the *DU* deletion (EIAV_{UK}Δ*DU*Δ*S2*) resulted in a RT-PCR fragment of 262 bp. *S2* gene mutant virus constructs identified as EIAV_{UK}Δ*DU*Δ*S2*, EIAV_{UK}Δ*S2* and EIAV_{PR}Δ*S2* were also analyzed by the RT-PCR technique. While creating

the S2 mutation, a SpeI restriction digestion site was created. RT-PCR and restriction digestion analyses of each of EIAV_{UK}ΔS2, EIAV_{UK}ΔDUΔS2, EIAV_{PR}ΔS2 and EIAV_{UK} virus stocks demonstrated that EIAV_{UK} wild-type virus generated a 539 bp RT-PCR fragment that was resistant to digestion
5 by SpeI. Each of the above-listed S2 virus constructs was susceptible to digestion by SpeI, resulting in cleavage of the 539 bp RT-PCR product into 347 and 192 bp fragments.

EXAMPLE 3

10 In order to prove that the constructs prepared and grown in large-scale in the previous examples could protect either ponies or horses from disease produced by EIAV, a vaccine was prepared using proviral clone EIAV_{UK}ΔS2. The EIAV_{UK}ΔS2 virus construct was grown in primary fetal equine kidney cells (FEK), filtered through a 0.45μ filter and frozen in
15 aliquots at -80°C. The titers of these virus construct stocks were 10⁶ infectious center doses (ICD) per mL, as measured by using an EIAV infectious center assay in FEK cells (Lichtenstein, et al, 1995), incorporated herein by reference.

For these studies, the EIAV_{UK}ΔS2 could have been inactivated,
20 preferably, by using agents such as formalin or binary ethylenimine. Additionally, the virus construct could have been adjuvanted with any of several adjuvants, preferably with a Carbopol-based, polymer-based or lipid-based adjuvant. However, for this experiment, the EIAV_{UK}ΔS2 was used without inactivation or adjuvanting so as to determine whether it
25 would replicate *in vivo* as well as it replicated *in vitro*. Thus, this example describes the use of an attenuated live vaccine comprising EIAV_{UK}ΔS2.

The EIAV_{UK}ΔS2 was tested for its ability to protect equines (ponies in this experiment) against an intravenous challenge with pathogenic EIAV_{PV}, a heterologous EIAV. The results of this vaccination/challenge
30 study are shown in Table 1. Each of three ponies was vaccinated once with 1.0 mL of the undiluted EIAV_{UK}ΔS2 virus construct stock. Six months

after vaccination all 3 vaccinated ponies were challenged intravenously with 300 median equine infectious doses (MEID) of pathogenic EIAV_{PV}. All ponies were clinically monitored and maintained in isolation as described by Hammond, et al. (Virology vol: 254, p 37-49). Rectal temperatures and clinical status were recorded daily. Samples of serum, plasma and whole blood were collected from each pony at predetermined intervals. Plasma samples were stored at -80°C until further processed for semi-quantitative viral RNA analyses or identification of the presence of wild-type challenge virus, and serum samples were stored similarly until testing for quantitative and qualitative serological assays could be performed. Whole blood samples were appropriately fractionated for enumeration of platelets or experimentation with PBMCs. Results are shown in Table 1.

During the course of the 6-month immunization, no clinical signs were observed in the vaccinated ponies. This indicates that EIAV_{UK}ΔS2 is avirulent for ponies: To assess virus replication following vaccination, the level of viral RNA in plasma was determined by using a semi-quantitative RT-PCR assay (Li et al, J. Virol, Jan 2000, p. 573-579). EIAV RNA was detected in the plasma of all immunized animals on day 6 after vaccination and unpredictable viremia episodes were observed throughout the course of vaccination. However, the plasma viral RNA levels observed in the vaccinated ponies were 10-6000 fold lower than the levels measured in ponies previously infected with the parental EIAV_{UK} virus over a six month observation period. This finding indicates that the EIAV_{UK}ΔS2 virus construct is highly attenuated due to the absence of the S2 gene and therefore, the vaccine was safe in equids even in live form.

At 6 months post vaccination, the ponies were challenged intravenously with 300 median equid infectious doses of pathogenic EIAV_{PV}. Following challenge of non-vaccinated ponies, clinical signs of EIA are normally apparent in about 16-19 days. Concurrent with the initial EIA-related fever is a rapid decline in quantity of platelets circulating in the blood. In some cases, control ponies recrudescence with more severe clinical manifestations of high fever (>103°F) and extremely low blood platelet

counts of $<105,000/\mu\text{L}$ of blood. Following challenge in these ponies, clinical signs of EIA were not evident at all throughout the 3 months observation period indicating that these ponies were successfully protected from disease.

- 5 In order to determine whether the ponies were protected from infection (sterile protection), genetic analyses of viral RNA present in the plasma was performed by a nested RT-PCR technique used in combination with differential restriction digestion of RT-PCR product for the differentiation of vaccine strain $\text{EIAV}_{\text{UK}\Delta\text{S2}}$ and wild-type challenge
- 10 strain EIAV_{PV} . All of the vaccinated/challenged ponies demonstrated only the presence of the attenuated $\text{EIAV}_{\text{UK}\Delta\text{S2}}$ virus construct. Therefore, all (100%) were protected from infection by wild-type EIAV.

Table 1 Summary of Results of Pony Vaccination/Challenge Study

Group	Pony No.	Febrile Episode Post Challenge	Abnormal Blood Count Post Challenge	Protection From Disease ^a	PCR Detection of Challenge strain EIAV _{PR}	Protection From Infection ^b
EIAV _{UK} ΔS2	94-11	NONE	NONE	YES	Negative	YES
	676	NONE	NONE	YES	Negative	YES
	674	NONE	NONE	YES	Negative	YES

^aAnimals protected from clinical disease did not demonstrate any progression to clinical disease including temperature and platelet count

^bAnimals protected from infection did not demonstrate any level of expression of wild-type challenge virus at the plasma samples of vaccinated horses by a semi-quantitative RT-PCR (Li et al, J. Virol, Jan 2000)

EXAMPLE 4

A vaccination/challenge study similar to that described in Example 3 was conducted with horses using the multiple low dose challenge. This study was conducted in order to demonstrate equivalency
5 between vaccination of ponies or horses as well as demonstration that the multiple low dose EIA equine challenge can serve as a successful model for EIAV infection. The method of Example 2 was used for growth of the EIAV_{UK}ΔS2 construct. Six horses were vaccinated with 1.0 mL of the virus construct. One horse was left unvaccinated to serve as a Control horse.
10 In this study, horses were challenged using the multiple low dose challenge method developed and described in copending patent application Serial No. _____, incorporated herein by reference. In the multiple low dose EIA equine challenge, each horse received three intravenous inoculations of 10 median horse infective doses (MHID) of
15 EIAV_{PV} at two-day intervals. After challenge, the horses were monitored for clinical signs of EIA for about 3 months post challenge. Results of the horse challenge are shown in Table 2.

Table 2 Summary of Results of Horse Vaccination/Challenge Study

Group	Horse No.	Febrile Episode Post Challenge	Abnormal Blood Platelet Count Post Challenge	Protection from Disease	PCR Detection of Challenge strain EIAV _{PR}	Protection From Infection
EIAV _{UK} ΔS2	60	NONE	NONE	Yes	Negative	YES
	971	NONE	NONE	Yes	Negative	YES
	615	NONE	NONE	Yes	Negative	YES
	9791	NONE	NONE	Yes	Negative	YES
	9809	NONE	NONE	Yes	Negative	YES
	9812	NONE	NONE	Yes	Negative	YES
CONTROL	880	YES	YES	No	Positive	NO

These data indicate that a vaccine that protects from both disease and infection in ponies and/or horses produced by EIAV can be prepared from EIAV_{UK}ΔS2. Equines vaccinated with the attenuated EIAV_{UK}ΔS2 construct can be differentiated from infected equines based on the lack of antibody to the S2 protein in vaccinated animals. Such lack of antibody can be determined by any immunological assay known to the art that would demonstrate the presence of S2 antibodies in the blood or serum of infected ponies or horses and the lack of such antibodies in vaccinated ponies or horses. Alternatively, a PCR-based assay known to the art, could be used to detect the presence of the S2 gene sequence in infected horses as compared to the lack of this gene sequence in vaccinated horses. The horse experiment demonstrates that the multiple low dose EIA equine challenge model is effective in both reproducing EIA and in demonstrating that horses can be protected from by a vaccine prepared according to the present invention.

EXAMPLE 5

Live attenuated vaccines were also prepared from the EIAV constructs designated EIAV_{UK}ΔDUΔS2 and EIAV_{PR}ΔS2 according to the methods described in Example 3. Two groups of horses were each inoculated intramuscularly two times (at monthly intervals) with the respective attenuated vaccine. Each vaccine contained approximately 10⁵ infectious-center doses (ICD) in a 1.0 mL dose. Inoculated horses were monitored daily for any clinical signs of EIA post vaccination. Blood samples were taken at weekly intervals for evaluation of vaccine virus replication and for EIA-specific immune responses. At 6 months post vaccination, all 16 vaccinated horses and 2 non-vaccinated control horses were challenged with the multiple low dose EIA equine challenge with EIAV_{PV} pathogenic virus stock as described previously. The multiple low dose challenge involved inoculating each horse three times with 10 median horse infective doses (MHID) at two-day intervals. The horses were monitored for clinical signs of EIA, for seroconversion in commercial

diagnostic assays for p26 and for infection with the challenge virus using RT-PCR for about 3 months post challenge as in EXAMPLE 3. Table 3 summarizes the results of this study.

5 Seven of eight (88%) of the EIAV_{UK}ΔDUΔS2 vaccinated horses remained asymptomatic post challenge, while six of eight (75%) of the EIAV_{PR}ΔS2 vaccinates were protected from disease post challenge. These clinical data indicate that the vaccines were effective in preventing disease post challenge exposure to a pathogenic EIAV_{PV}. However, these vaccines were not as effective as the vaccine tested in Example 3. It is
10 proposed that the reduced protection results from these constructs either being prepared from an avirulent clone of EIA (EIAV_{PR}) or a double deletion mutant of the virulent parent clone (EIAV_{UK}ΔDUΔS2). It is proposed that addition of an adjuvant to the vaccines of this example would improve their immunogenicity (ability to protect horses from
15 disease) and produce a vaccine that is more protective for disease caused by EIA virus.

Surprisingly, not all of the vaccinated horses seroconverted to p26 as measured by testing for positive antibody status using the Coggins Test. This indicates that a normal p26 assay could be run on vaccinated
20 horses. In order to use this vaccine for commercial purposes, any vaccinated equines that were found to be Coggins Test positive could be confirmed with a test for antibodies for the S2 expression product. If S2 antibodies were present, it would be confirmed that the horses had been infected with a field strain of EIAV (wild-type) and not the EIAV vaccine of
25 the present invention.

It is apparent that a vaccine composition for effectively and safely immunizing equines from disease caused by EIAV can be produced and that vaccinated equines can be differentiated from infected equines using the standard Coggins test for antibodies to p26 in addition to a test for
30 antibodies to S2 protein or detection of a gene sequence associated with the S2 gene. Antibodies to both proteins as well as the S2 gene sequence are absent in vaccinated and uninfected equines but present in

infected equines. Additionally, the absence of antibodies to the DU protein and/or the DU gene sequence can serve as a differential diagnostic test for equids vaccinated with the EIAV_{UK}ΔDUΔS2.

It is expected that the attenuated vaccines described in this
5 example were more attenuated than desired. In order to increase their
immunogenicity (ability to protect from disease and infection) an adjuvant
can be added to the attenuated vaccine or the attenuated viruses can be
inactivated as described previously, adjuvanted and administered as
repeat doses (2 to 3) for the vaccination series. It is expected that such a
10 modification would protect completely from disease and infection.

Table 3 Summary of Attenuated EIAV Vaccine Trial

Group	Horse	Febrile Episode	RNA > 10 ⁵	EIAV _{PV} Positive	P26 ANTIBODY Positive
EIAV _{PR} ΔS2	811	X		X	X
	9705			X	X
	9704			X	
	9717			X	X
	9615	X	X	X	X
	9613		X	X	X
	9716			X	X
	9712			X	X
EIAV _{UK} ΔDUΔS2	9708			X	X
	9706			X	X
	673		X	X	X
	677				X
	9711				X
	666	X	X	X	X
	711				
	699				
Control	9714	X	X	X	X
	9720	X	X	X	X

EXAMPLE 6

In order to determine whether a vaccine comprising only a *DU* gene-mutated EIAV would be safe and effective in equines, a *DU* gene-mutated EIAV construct was prepared and tested in a horse

5 vaccination/challenge model for EIAV as described in Examples 3 and 4. The *DU* coding region of EIAV is located within the *pol* open reading frame, positioned between the RT and integrase (IN) genes (See Figure 5). It specifically codes for a dUTPase, an enzyme to convert dUTP to dUMP + pp_i. The predicted amino acid sequence of the EIAV *DU* protein

10 shows a high degree of homology to the dUTPases of other nonprimate lentiviruses and to the human, yeast and *E. coli* enzymes as well. Five conserved amino acid motifs present in all known dUTPase proteins have been recognized and at least one of these motifs has been suggested to be functionally important. Motif 3 contains a highly conserved tyrosine

15 residue, which has been suggested to be involved in catalysis. To construct an EIAV mutant that would be deficient in dUTPase activity, a Styl restriction fragment containing 80% of the *DU* coding sequence, including four of the five conserved amino acid motifs, was deleted from the provirus clone EIAV_{PR}. The deletion left intact the *pol* open reading

20 frame and both protease-processing sites present on either side of the *DU* gene. More specifically, to construct the EIAV_{PR} Δ*DU* that is deficient in dUTPase activity, a 330 bp restriction fragment from a KpnI-PstI *pol* subclone of the proviral clone EIAV_{PR} was deleted. This deleted segment was then subcloned back into a full-length provirus backbone as an SstI-

25 NcoI fragment to create the mutant provirus clone EIAV_{PR} Δ*DU* (see Figure 5). Figure 5 shows the genomic organization of EIAV and the location of the *DU* gene. The position of the two Styl sites used to create the deletion are also shown. The stippled bar represents the approximate positions of five conserved amino acid motifs present in all known

30 DUTPase genes. Nucleotide and amino acid sequences of *DU* flanking the two Styl sites are shown at the bottom. The leucine residue is the first amino acid of mature *DU* protein. A *pol* subclone containing the *DU* gene

was digested with Styl, and the resulting 5' termini were filled in with T4 DNA polymerase and ligated to generate the sequence shown by the arrow. The deleted \sim gene was then inserted back into a full-length proviral clone.

5 The mutant produced as described, was tested for its ability to replicate *in vitro*, a requirement for large-scale vaccine production. FEK cells and the ED cell line were transfected with the EIAV_{PR} Δ DU as described previously in Example 2. It was determined that the RT activity was equal to that of wild-type EIAV_{UK}. However, when equine
10 macrophage cultures were transfected with this construct at a multiplicity of infection (MOI) of 0.01, very little replication (as measured by RT activity) was noted. This suggests that such a construct would replicate poorly if at all in horses. The tissue culture grown proviral construct was confirmed to be EIAV_{PR} Δ DU by RT-PCR. These experiments determined
15 that EIAV_{PR} Δ DU could be produced *in vitro* in large scale in either FEK or ED cells.

 In order to determine whether a vaccine could be prepared and whether such a vaccine would protect horses from disease and/or infection, the ED cell line was transfected and a large quantity of EIAV_{PR} Δ DU was produced. In this study, the proviral construct was inactivated
20 by addition of 0.1% formalin and adjuvanted with a polymer-based adjuvant, specifically with a Carbopol-based adjuvant designated HAVLOGEN®. Two vaccines were formulated. One contained 50 μ g/dose (1.0 mL) while the second contained 10 μ g/dose. Each of three horses
25 received 3 doses of 50 μ g/dose vaccine and each of three horses received 3 doses of 10 μ g/dose vaccine. The interval between vaccinations was one month. Three additional horses were left unvaccinated and served as negative controls. Nine weeks post final vaccination, all horses were challenged with a multiple low dose challenge using EIAV_{PV}, a
30 heterologous strain. This constituted administering 10 MHIDs three times over a 7 day period (days 0, 2 and 5). Horses were monitored for

temperature, platelet count, plasma viremia and seroconversion for 7 weeks post challenge. Results of this vaccination/challenge study are shown in Table 4.

Table 4 Summary of Results of the Vaccination/Challenge Study using an inactivated, Adjuvanted DU gene-mutated EIAV Vaccine

Group	Horse	Febrile Episode	RNA > 10 ⁵	EIAV _{UK} Positive	P26 ANTIBODY Positive
EIAV _{PR} ΔDU 50μg/dose	710	None	Neg	Neg	X
	682	None	Neg	Neg	X
	95-03	None	Neg	Neg	X
EIAV _{PR} ΔDU 10μg/dose	787	X	X	X	X
	785	X	X	X	X
	724	None	Neg	Neg	Neg
Controls	96-08	X	X	X	X
	827	X	X	X	X
	746	X	X	X	X

It is noted from Table 5 that all three horses receiving a 50 μ g/dose of inactivated, adjuvanted vaccine were protected from both disease and infection. These horses demonstrated no clinical signs of disease and did not demonstrate the presence of challenge virus (viremia) as measured by
5 RT-PCR. Even a dose of only 10 μ g was able to protect 1 of 3 horses from both disease and infection. All control horses demonstrated both disease and infection typical of full-blown EIA. This is an extraordinary result, especially since the challenge virus that was administered was heterologous, not homologous to the vaccine constructs. These data
10 prove that the teachings of the present invention can be used to prepare a completely protective vaccine. It also proves that inactivation and adjuvanting do not decrease the immunogenicity of the EIAV vaccines of the present invention.

Although the invention has been described in detail in the foregoing, for the purpose of illustration it is to be understood that such detail is solely for that purpose and that variations can be made therein by those skilled in the art without departing from the spirit and scope of the
5 invention except as it may be limited by the claims.

WHAT IS CLAIMED IS:

1. A vaccine for effectively and safely immunizing mammals from disease caused by EIAV, said vaccine comprising a gene-mutated EIAV wherein said virus lacks the ability to express the mutated gene protein *in vivo* and wherein said lack of expression can be used to differentiate vaccinated from non-vaccinated or infected mammals.
5
2. The vaccine of Claim 1 wherein said gene-mutated EIAV comprises a mutation in at least one accessory gene.
3. The vaccine of Claim 2 wherein said accessory gene is an S2 gene.
10
4. The vaccine of Claim 1 wherein said gene-mutated EIAV comprises a mutation in a *DU* gene.
5. The vaccine of Claim 4 wherein said mutation in the *DU* gene is a deletion.
6. The vaccine of Claim 1 wherein the gene-mutated EIAV comprises at least two mutations.
15
7. The vaccine of Claim 6 wherein the two mutations are in the S2 and *DU* genes.
8. The vaccine of Claim 7 wherein the two mutations in the S2 and *DU* genes are deletions.
20
9. The vaccine of Claim 5 wherein a foreign gene or a foreign gene portion is inserted into the *DU* gene deletion.
10. The vaccine of Claim 7 wherein a foreign gene or a foreign gene portion is inserted into the S2 gene deletion.
11. The vaccine according to Claim 1 wherein the gene-mutated EIAV is treated with an inactivating agent.
25
12. The vaccine according to Claim 11 wherein the inactivating agent is selected from the group consisting of formalin, formaldehyde, beta-propiolactone, binary ethylenimine, ethylenimine, thimerosal, psoralen and heat.
30

13. A vaccine for protecting horses from disease caused by EIAV comprising a gene-mutated EIAV construct, said construct allowing a vaccinated horse to be differentiated from an EIAV infected horse.

5 14. The vaccine of Claim 13, wherein the gene-mutated EIAV construct comprises a deletion in the *S2* and/or the *DU* genes.

15 15. A diagnostic test for differentiating mammals vaccinated with the vaccine of Claim 1 from non-vaccinated mammals or from infected mammals comprising one or more reagents for demonstrating the absence of a normal EIAV gene expression product in mammals vaccinated with the gene-mutated vaccine of Claim 1 and a measurable level of said expression product in infected mammals.

16 16. A diagnostic test for differentiating mammals vaccinated with the vaccine of Claim 1 from non-vaccinated mammals or from infected mammals comprising one or more reagents for demonstrating the absence of a normal gene sequence in mammals vaccinated with the gene-mutated vaccine of Claims 1 and a measureable amount of the normal gene sequence in infected mammals.

20 17. A method of differentiating a vaccinated mammal from a non-vaccinated mammal, said method comprising;
a. obtaining a sample from a test mammal; and
b. analyzing said sample for the presence of a gene expression product normally produced by wild-type EIAV but not produced by an EIAV construct used for vaccinating said test mammal.

25 18. A method of differentiating a vaccinated mammal from a non-vaccinated mammal, said method comprising;
c. obtaining a sample from a test mammal; and
d. analyzing said sample for the presence of a gene sequence normally present in wild-type EIAV but not present in an
30 EIAV construct used for vaccinating said test mammal.

19. A method of immunizing a horse against EIAV comprising vaccinating said horse with an EIAV vaccine comprising a gene-mutated EIAV and an adjuvant.

20. A method of immunizing a horse against EIAV comprising
5 vaccinating said horse with an EIAV vaccine comprising a gene-mutated EIAV, an inactivating agent and an adjuvant

Figure 1. Schematic diagrams of replication competent EIA viruses and constructs

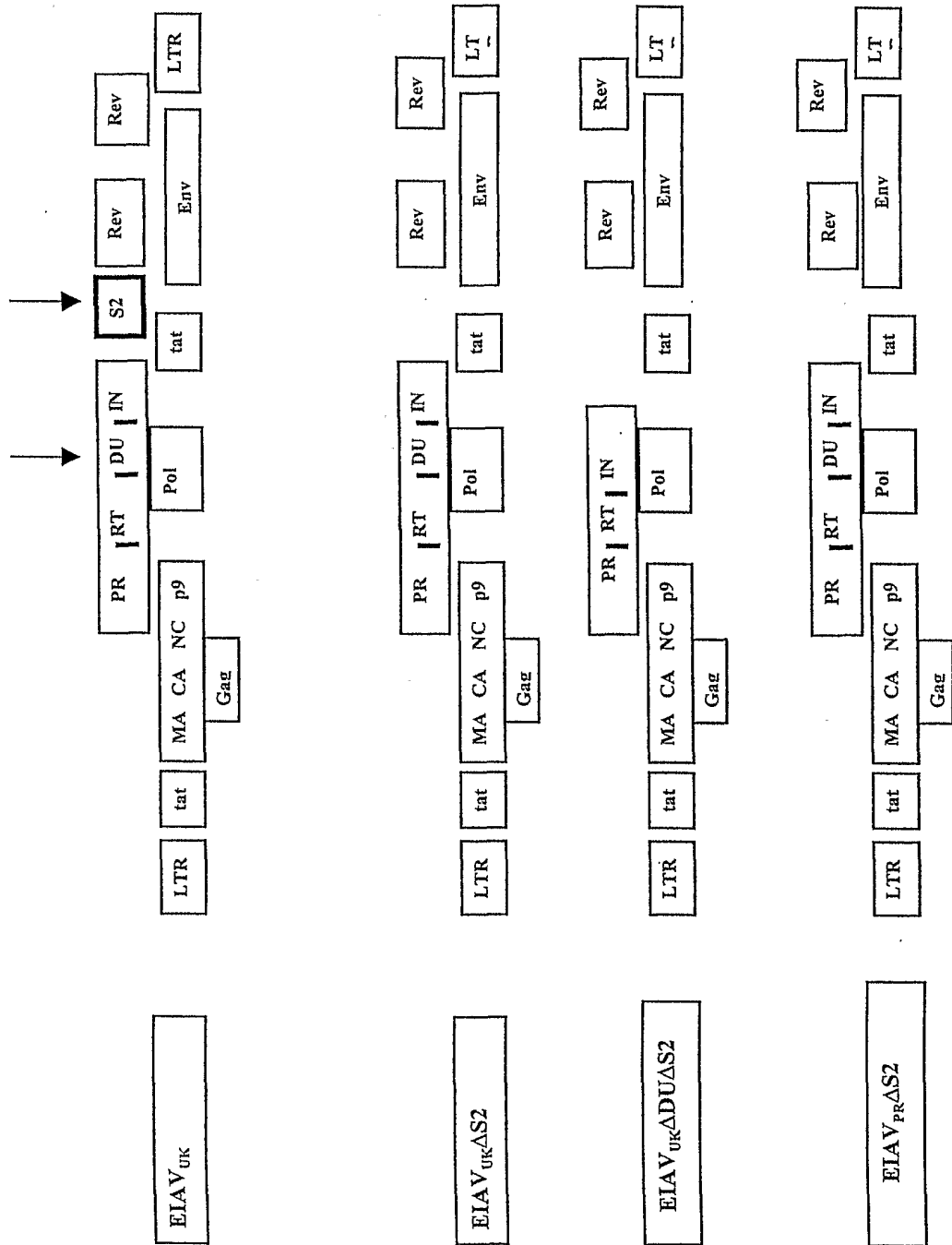
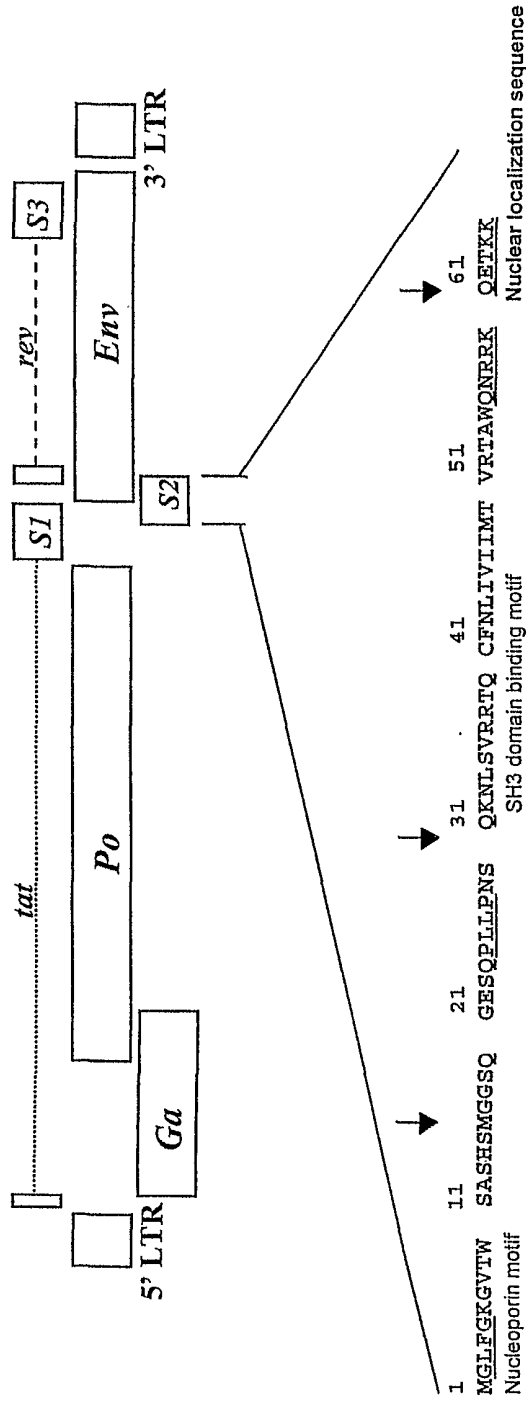


Figure 2a Schematic representation of EIA virus S2 gene and mutant clones derived from EIAV_{UK}. The EIA proviral DNA is shown at the top; the complete deduced amino acid sequence of the putative S2 protein is shown in single letter amino acid code at the bottom. Stop codons (indicated by arrows) were introduced into various positions in the EIA virus S2 gene to generate the specific mutant virus strains.



EIAV.G5/s Stop codon replaced amino acid G⁵

EIAV.2M/X M¹⁶ changed to T
Stop codons replaced G⁵ and G¹⁸

EIAV.ΔS2 Deletion of initial 5 nucleotides of S2

Figure 2b Schematic representation of the Wild-type EIAV S2 gene compared with the Δ S2 gene of EIAV.2M/X (EIAV_{UK} Δ S2)

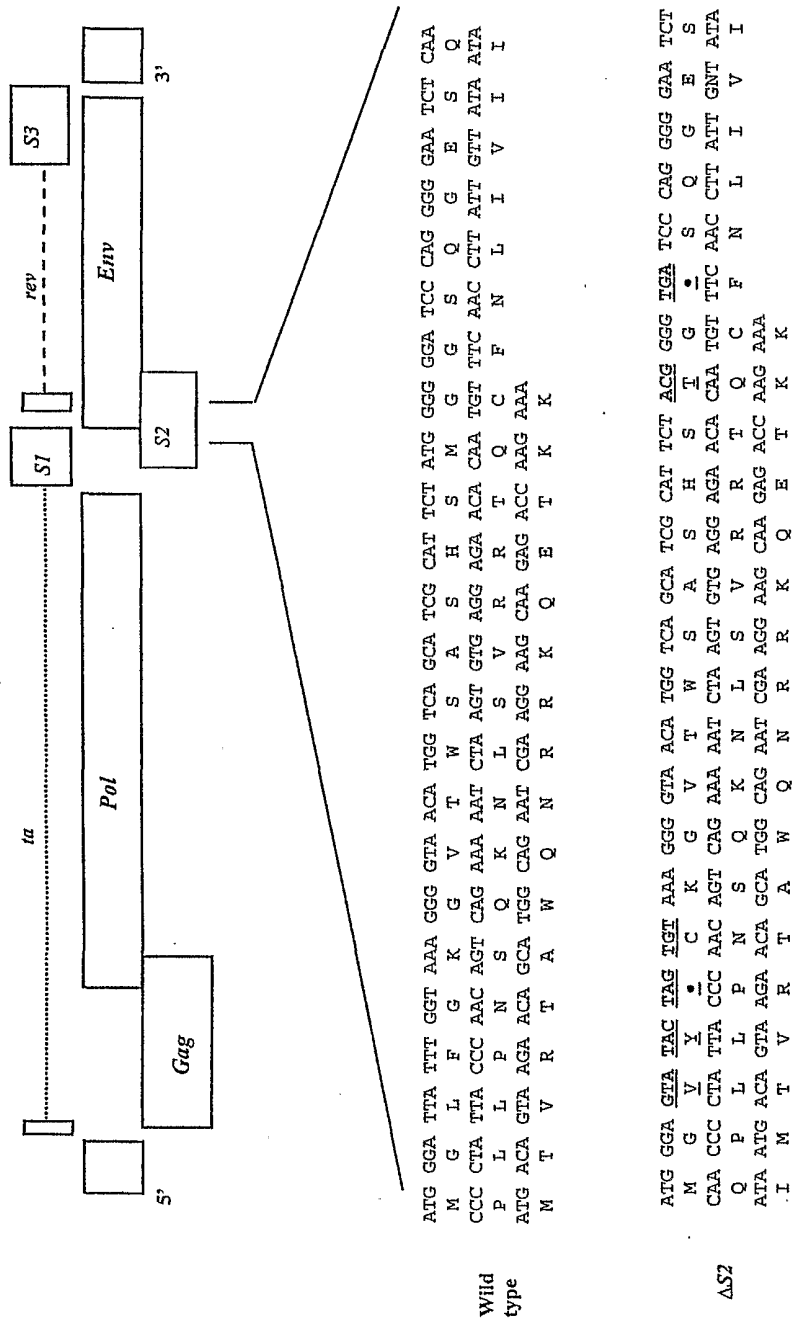


Figure 3a Circular Map of Biological Proviral Clone EIAV_{PV}

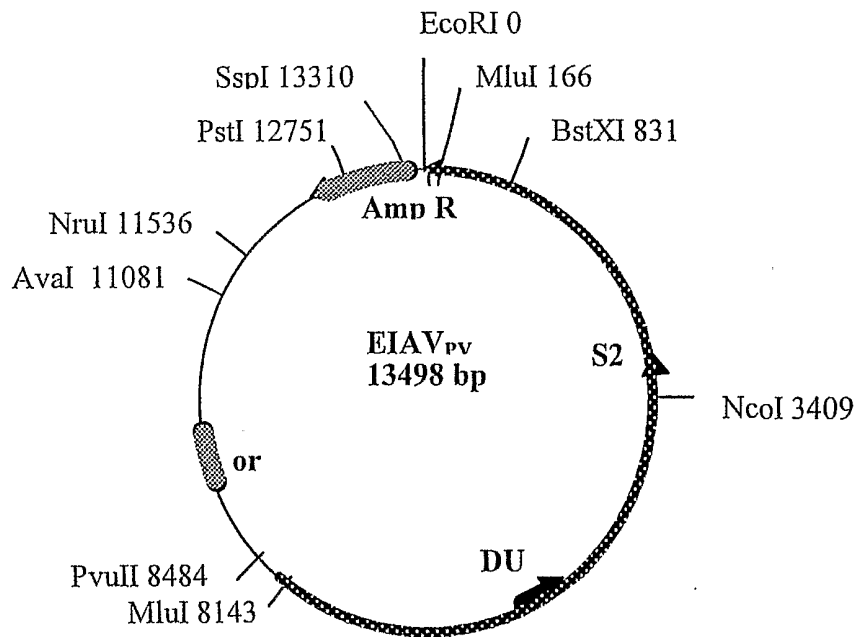


Figure 3b Circular Map of Molecular Infectious Clone EIAV_{UK}

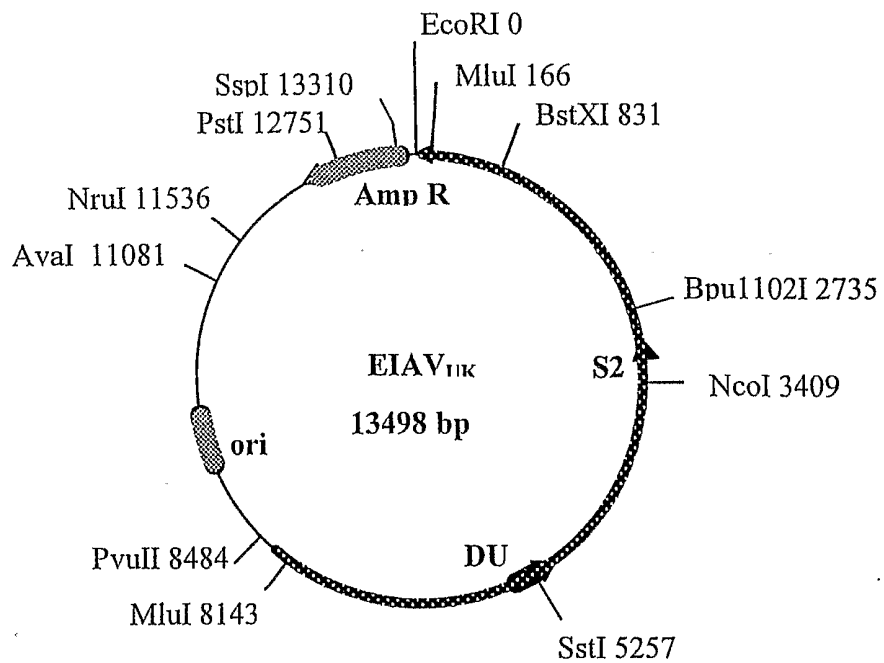


Figure 3c Circular Map of EIAV_{UK}ΔS2

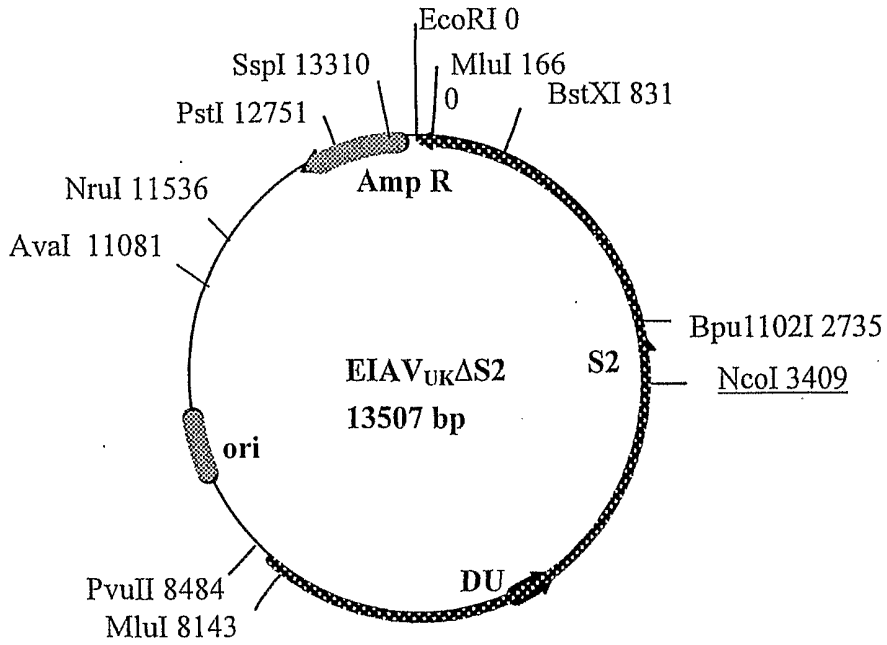


Figure 3d Circular map of EIAV_{PR}ΔS2

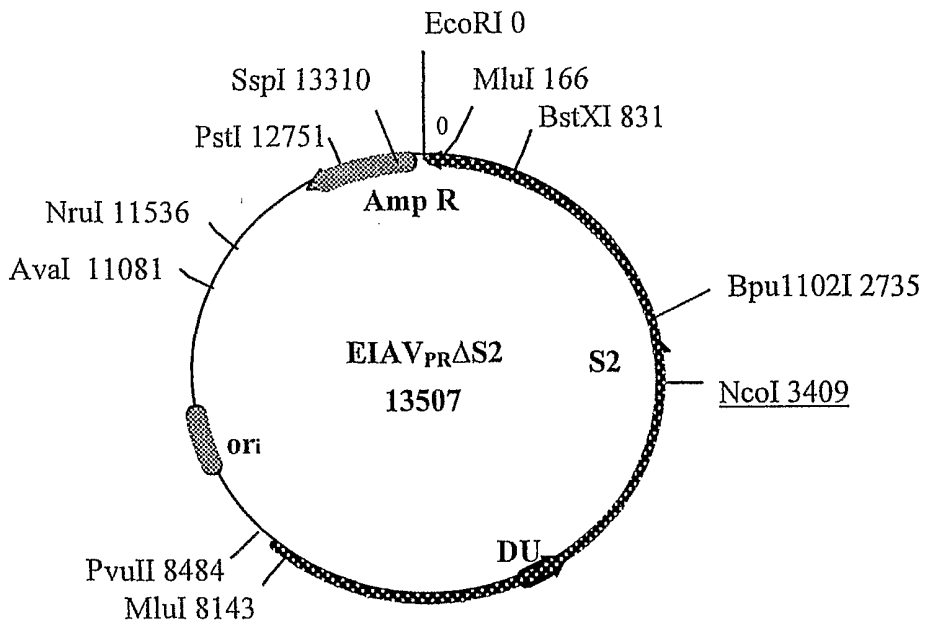


Figure 3e Circular Map of EIAV_{UK} ΔDUΔS2

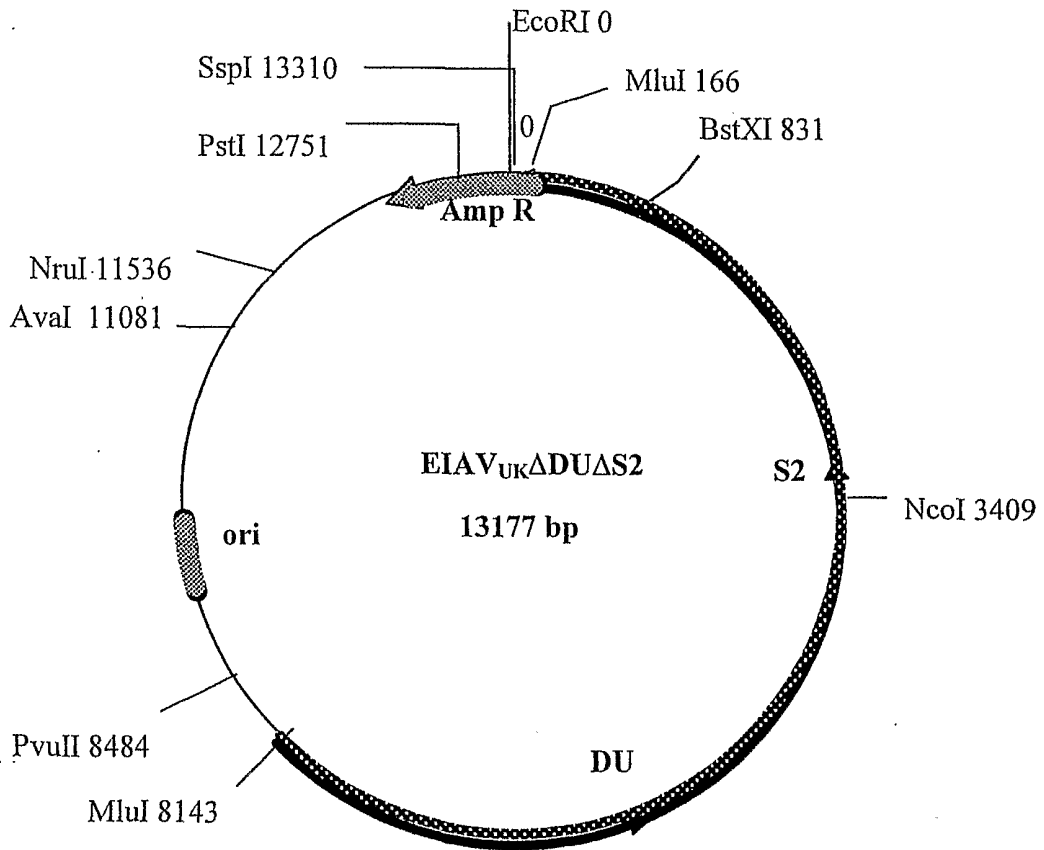


Figure 4 Replication of EIA Virus Mutant Clones *in vitro*

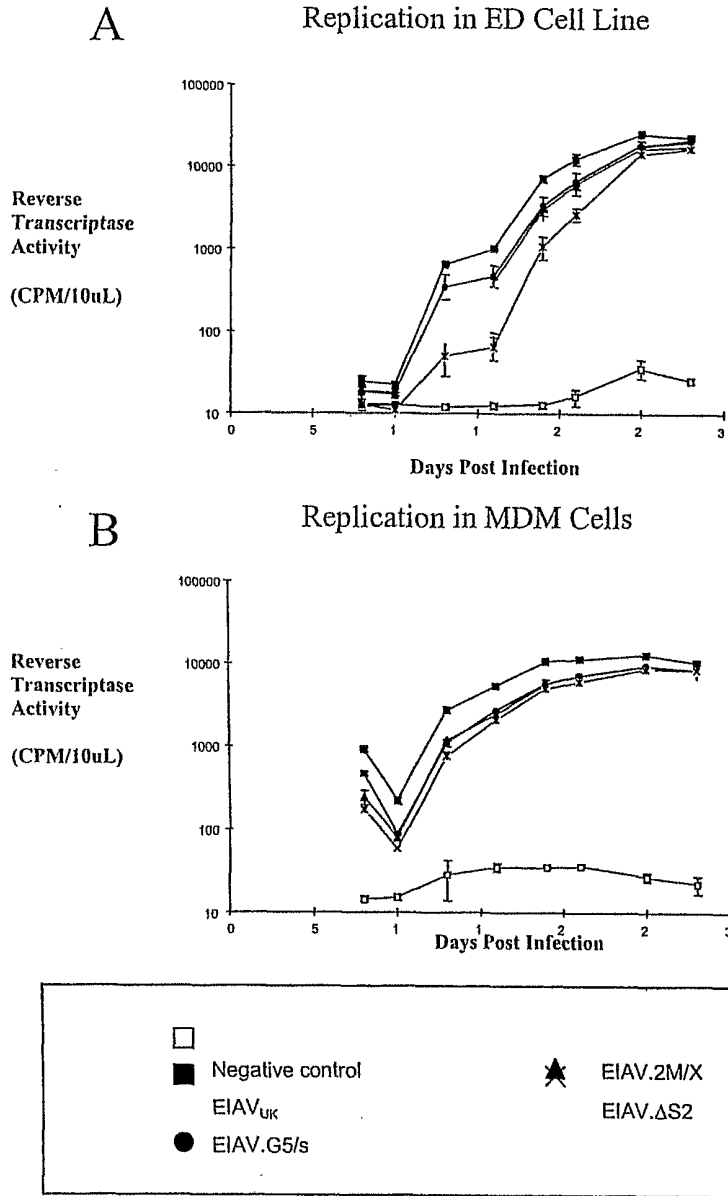
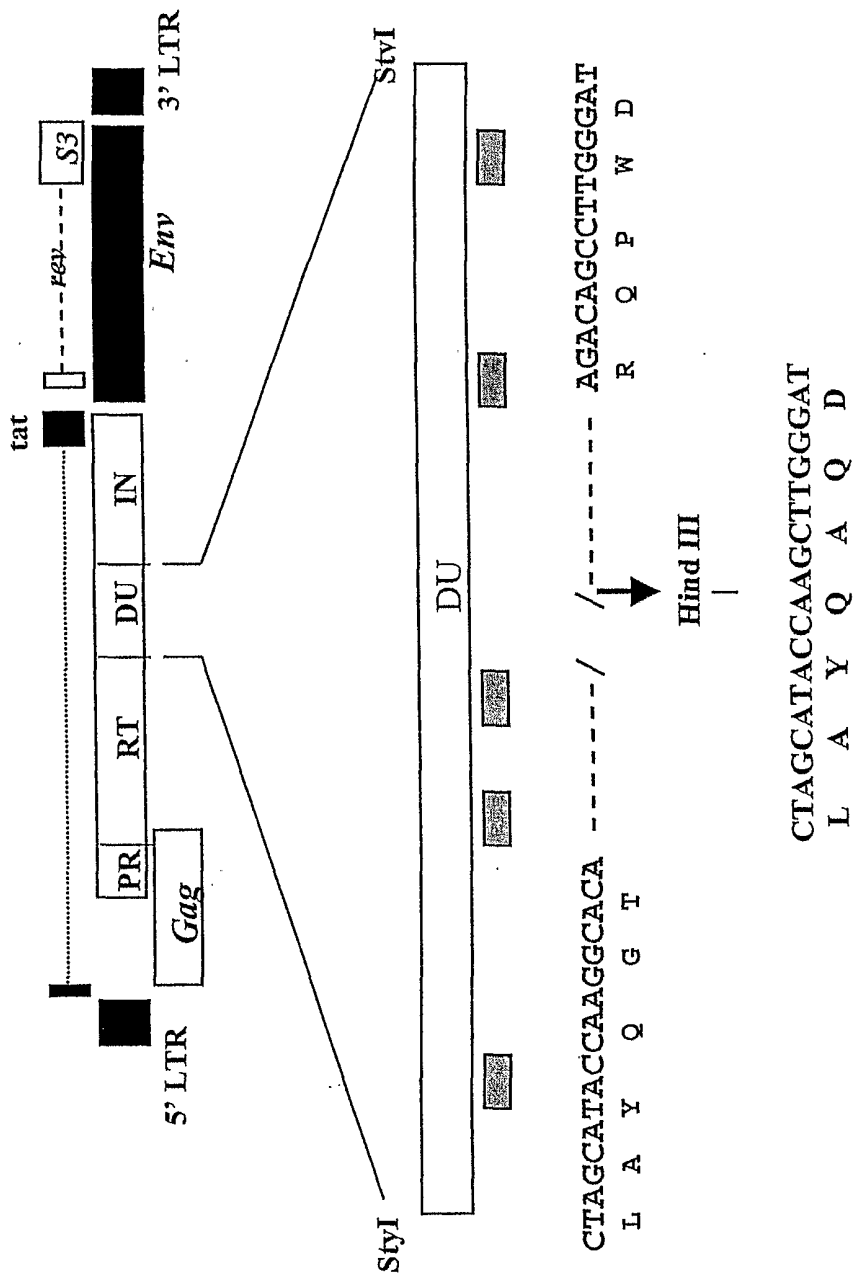


Figure 5 Schematic Representation of the DU gene and construction of ELA VΔDU



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<140> 09/658,547

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