West Nile Virus Vaccine

Background

West Nile Virus ("WNV") is in the family Flavivirade. Infection is usually contracted through a mosquito vector transferred through the insect's bite. West Nile infects all types of animals and birds across the globe. This virus was first discovered in the North American region in 1999 with the first diagnosis occurring in Canadian horses. Presently, West Nile Virus has become endemic in the United States affecting birds, humans, and animals of all types. In 2002, over 14,700 confirmed cases of West Nile Virus were reported in 43 states.

The spread of WNV has been influenced by several factors. Since the mosquito is the vector for the virus and perpetuates WNV, the ecological conditions conducive to growth and development of mosquito populations have had an impact of the spread of the WNV. There are several tactics that have been utilized to control populations of mosquitoes in an effort to prevent the spread of WNV. These tactics include the use of pesticides, repellants, physical barriers preventing contact between mosquitoes and animals, eliminating environments that perpetuate breeding of mosquitoes, and the use of immunizations. Typical signs of WNV include various symptoms affecting the central nervous system. Symptoms of encephalitis are often seen and include viremia, histopathologic lesions of the central nervous system, anorexia, depression, fever, weakness, abnormal gait, paralysis of hind limbs, impaired vision, ataxia, aimless wandering, convulsions, inability to swallow, coma, and death.

A few vaccines directed towards WNV have been introduced which are undesirable for various reasons. For example, one vaccine was produced from a canarypox-vectored West Nile Virus. Another set of vaccines were produced from a recombinant chimeric protein of West Nile Virus, wherein the chimeric protein vaccine was designed by fusing a modified version of bacterial flagellin (STF2 Delta) to the EIII domain of the WNV envelope protein. Another vaccine included an inactivated early North American West Nile strain that required a metabolizable oil as an adjuvant. Finally, a live, attenuated chimeric vaccine was produced from an infectious clone of yellow fever 17D virus in which the pre-membrane and envelope proteins have been replaced by the corresponding genes of WN(4).

There are several problems inherent in vaccines described above. Vaccines containing live viral organisms have the risk of infecting an animal with the virus through vaccination leading to sickness and even death. Chimeric protein vaccines, recombinantly expressed vaccines, and some subunit vaccines have the problem of limited immunological activity and effect related to the number of proteins included in the vaccine composition. The efficacy of these types of vaccines is usually limited and the risk of infection by the virus or reversion to wild type virus is prevalent. In addition, some of the adjuvants utilized in common vaccines are comprised of metabolizable oils which are removed relatively rapidly from the body and limit the duration during which the immune system of the vaccinated animal may respond to the immunogenically active composition. Other adjuvants can cause allergic reactions and unfavorable effects in the vaccinated animals. Additionally, these vaccines do not include antigens for stimulating immunity to other pathogens besides WNV, so they fail to protect animals against several diseases with both convenience and safety. Also, all previous vaccines were derived from an early isolate of WNV that is no longer present in the environment, and hence, can no longer infect animals and cause disease.

Accordingly, what is needed in the art is a vaccine that is safe for administration to animals of all ages, including pregnant animals, that includes adjuvants suitable for aiding the immunogenic effect and duration of the vaccine, and that is prepared from contemporary or dominant isolates of WNV that remain present in the natural environment and cause disease against which such vaccines would afford protection. What is further needed is a vaccine that reduces the incidence and/or severity of up to and including the elimination or prevention of clinical signs associated with the disease or infection by West Nile Virus. Additionally what is needed is a vaccine against West Nile virus, which includes West Nile Virus antigens in combination with antigens from other equine pathogens, thereby providing further protection by reducing the incidence of or severity of clinical signs of disease from both West Nile Virus and the other pathogen(s).

Summary

The present disclosure overcomes the problems inherent in the prior art and provides a distinct advance in the state of the art. More particularly, the present disclosure provides for a vaccine or immunogenic composition comprising an immunogenically active antigenic component comprised of one or more strains or isolates of West Nile Virus. In a first aspect,

there is provided an immunogenic composition comprising one or more strains of killed or inactivated West Nile Virus or any antigen thereof, wherein said immunogenic composition further comprises at least one antigen selected from the group consisting of Eastern Equine Encephalomyelitis Virus, Western Equine Encephalomyelitis Virus and Venezuelan Equine Encephalomyelitis Virus, Tetanus Toxoid, and combinations thereof, and wherein said West Nile Virus is one of the strains selected from the group consisting of Horse Origin 2005, deposited with the ATCC under accession number PTA-9409; NAEE159, deposit at the United States Department of Agriculture Isolate under accession number 405330; NY2002Nassau; NY2002Clinton; NY2002Queens; GA20021; GA20022; TX20021; TX20022; IN2002; NY2003Albany; NY2003Suffolk; NY2003Chatauqua; CO20031; CO20032; TX2003; TX2003Harris4; TX2003Harris6; TX2003Harris7; TX2003Harris10; AZ2004; TX2004Harris4; and combination thereof. In some preferred

embodiments, the composition further comprises an adjuvant, preferably a carbomer, and a pharmaceutically acceptable carrier. Preferably, the West Nile Virus antigen is killed or inactivated. This composition induces an immunogenic response in animals susceptible to contraction of West Nile Virus and provides for a safe vaccine for animals of any age.

The present disclosure additionally provides for a vaccine composition, which is immunogenically active, and which overcomes the limitations of those previously described. The present disclosure provides an inactivated vaccine thereby providing unique safety for the vaccinated animals, including pregnant females. Additionally, the immunogenic composition of the present disclosure overcomes interference from passively acquired maternal immunity and stimulates active immunity in vaccinated animals. Advantageously, the present disclosure provides a broad and effective immunogenically active composition containing many or all relevant antigenic components and proteins of pathogenic WNV. The immunogenic composition of the present disclosure is unique in that it includes antigens of contemporary isolates or epidemiologically dominant isolates of WNV in the composition, providing protective immunogenic responses by reducing the incidence of and/or severity of clinical signs of WNV infection up to and including immunity against the most prevalent isolates seen in animals, including horses, today. In a preferred embodiment, those contemporary isolates of WNV include those isolates that are part of the North American West Nile Virus isolates or North American Dominant West Nile Virus isolates For purposes of the present disclosure, WN02 is a representative example of a WNV strain that can be referred to as a North American Dominant West Nile Virus strain or isolate. Specifically, North American Dominant strains and isolates are those having at least 1 nucleotide change resulting in an amino acid change from the WN99 isolates. Strain NY99 (GenBank accession no. AF196835) serves as a reference strain for determining if a strain or isolate is North American Dominant. In addition, these strains or isolates may have one or more silent amino acid changes. In a preferred embodiment, the nucleotide change results in an amino acid change in an envelope protein of the strain or isolate and, more preferably, the nucleotide change results in an amino acid change from valine to alanine. Preferably, this amino acid change is associated with a greater ability to replicate in the intermediate host, namely, the mosquito. More preferably, North American Dominant strains include either (and preferably both) a U to C mutation and a C to U mutation at positions 1442 and 2466 (in comparison to a North American strain, e.g. NY 99 and SEQ ID NO.23),

respectively. Still more preferably, North American Dominant strains or isolates further include a mutation in the nucleotide sequence encoding the E protein and the C to U mutation at position 9352 in the sequence encoding the NS5 protein (again in comparison to a North American strain, e.g. NY 99 and SEQ ID NO. 23). These preferred mutations are shown in Example 10 and in Phylogenetic Analysis of North American West Nile Virus Isolates, 2001-2004: Evidence For the Emergence of a Dominant Genotype, C. Todd Davis, et. al, Virology 342, p. 252-265 (2005), the teaching and content of which is hereby incorporated by reference herein.

The present disclosure also provides for a method of making the immunogenic composition of the present disclosure. The method generally comprises the steps of combining a West Nile Virus antigen and an excipient or pharmaceutically or veterinary acceptable carrier. A preferred embodiment further comprises the step of adding one or more additional equine antigens. In another embodiment, the method further comprises the step of adding a suitable adjuvant to the composition.

In one preferred embodiment, the present disclosure includes WNV antigens and a non-metabolizable oil adjuvant, preferably mineral oil, to extend the duration during which the immune system of the vaccinated animal may respond to the immunogenically active composition. The non-metabolizable oil is understood to be an oil that, when administered with an antigen, does not metabolize in the body after administration. A preferred non-metabolizable oil is mineral oil. In other preferred forms, both a carbomer adjuvant and non-metabolizable oil (preferably mineral oil) are present in addition to the WNV antigens. The adjuvant(s) can be used in any of the compositions described herein.

In an additional embodiment, the composition of the present disclosure contains WNV antigens, preferably an inactivated or killed WNV from a North American dominant strain, and essentially no oil or oil-based adjuvants. In such an embodiment, other adjuvants, preferably carbomer, can be included.

In another embodiment, a vaccine composition comprised of WNV antigens in combination with other antigens from equine microbial pathogens is provided in order to confer a broad scope of protection to the animal. In such embodiments, the WNV antigens are in any form as described above.

In one preferred embodiment, the present disclosure provides a vaccine composition comprising WNV antigens as described above in combination with one or more immunologically

effective amounts of antigenic components selected from the group consisting of Venezuelan Equine Encephalomyelitis (VEE), Eastern Equine Encephalomyelitis (EEE), Western Equine Encephalomyelitis (WEE), Tetanus toxoid (T), Equine herpes viruses (EHV) including types 1 and 4, Equine influenza viruses (EIV), and combinations thereof, along with a pharmaceutically acceptable carrier. Preferably such embodiments will include an adjuvant, preferably carbomer, and a pharmaceutically acceptable carrier. Additionally, a non-metabolizable oil, preferably mineral oil, may be present, however, such an oil is not required.

Preferred embodiments also include WNV antigens, as described above, in combination with: Eastern Equine Encephalomyelitis; Western Equine Encephalomyelitis; Venezuelan Equine Encephalomyelitis; Tetanus Toxoid; Eastern Equine Encephalomyelitis and Western Equine Encephalomyelitis; Eastern Equine Encephalomyelitis and Venezuelan Equine Encephalomyelitis; Eastern Equine Encephalomyelitis and Tetanus Toxoid; Eastern Equine Encephalomyelitis, Western Equine Encephalomyelitis, and Venezuelan Equine Encephalomyelitis; Eastern Equine Encephalomyelitis, Western Equine Encephalomyelitis, and Tetanus Toxoid; Eastern Equine Encephalomyelitis, Western Equine Encephalomyelitis, Venezuelan Equine Encephalomyelitis and Tetanus Toxoid; Western Equine Encephalomyelitis and Venezuelan Equine Encephalomyelitis; Western Equine Encephalomyelitis and Tetanus Toxoid; Western Equine Encephalomyelitis, Venezuelan Equine Encephalomyelitis, and Tetanus Toxoid; Venezuelan Equine Encephalomyelitis and Tetanus Toxoid; and Eastern Equine Encephalomyelitis, Venezuelan Equine Encephalomyelitis and Tetanus Toxoid. The most preferred combination of these specified combinations includes WNV antigens in combination with antigens or antigenic components of Eastern Equine Encephalomyelitis, Western Equine Encephalomyelitis, Venezuelan Equine Encephalomyelitis, and Tetanus Toxoid. In each such specified combination, an adjuvant or combination of adjuvants can be used, with carbomer, and even more preferably carbopol, being particularly preferred. In the most preferred forms of the combination of WNV and Eastern Equine Encephalomyelitis, Western Equine Encephalomyelitis, Venezuelan Equine Encephalomyelitis and Tetanus Toxoid, no oil (metabolizable or non-metabolizable) is present. The NJO strain of Eastern Equine Encephalomyelitis, the Fleming strain of Western Equine Encephalomyelitis strain, and the TC-83 strain of Venezuelan Equine Encephalomyelitis strain are all representative strains of these vaccine components.

Further preferred embodiments of the present disclosure can be made using each of the specified combination vaccines listed above and adding in antigens from Equine Herpesvirus, preferably type 1, type 4, (EHV1 and/or EHV4) or combinations thereof.

Still further variations of each of the specified combination vaccines listed above, including those that include EHV1 and/or EHV4 can be made by adding in antigens from Equine influenza virus (EIV). Preferred embodiments incorporating Equine influenza virus include: West Nile Virus, at least one strain of Equine Influenza Virus, and Tetanus Toxoid; West Nile Virus, at least one strain of Equine Influenza Virus, Tetanus Toxoid, and Eastern Equine Encephalomyelitis; West Nile Virus, at least one strain of Equine Influenza Virus, Tetanus Toxoid, Eastern Equine Encephalomyelitis, and Western Equine Encephalomyelitis; West Nile Virus, at least one strain of Equine Influenza Virus, Tetanus Toxoid, Eastern Equine Encephalomyelitis, Western Equine Encephalomyelitis; and Venezuelan Equine Encephalomyelitis; West Nile Virus, at least one strain of Equine Influenza Virus, and Eastern Equine Encephalomyelitis; West Nile Virus, at least one strain of Equine Influenza Virus, and Western Equine Encephalomyelitis; West Nile Virus, at least one strain of Equine Influenza Virus, and Venezuelan Equine Encephalomyelitis; West Nile Virus, at least one strain of Equine Influenza Virus, Eastern Equine Encephalomyelitis, and Western Equine Encephalomyelitis; West Nile Virus, at least one strain of Equine Influenza Virus, Eastern Equine Encephalomyelitis, and Venezuelan Equine Encephalomyelitis; West Nile Virus, at least one strain of Equine Influenza Virus, Western Equine Encephalomyelitis, and Venezuelan Equine Encephalomyelitis; West Nile Virus, at least one strain of Equine Influenza Virus, Western Equine Encephalomyelitis, and tetanus toxoid; West Nile Virus, at least one strain of Equine Influenza Virus, Venezuelan Equine Encephalomyelitis, and tetanus toxoid; West Nile Virus, at least one strain of Equine Influenza Virus, Venezuelan Equine Encephalomyelitis, Western Equine Encephalomyelitis, and tetanus toxoid; and West Nile Virus, at least one strain of Equine Influenza Virus, Venezuelan Equine Encephalomyelitis, Eastern Equine Encephalomyelitis, and tetanus toxoid. In each specified embodiment any one or more strains or isolates of Equine Influenza may be present. Preferred strains of Equine Influenza virus include Influenza A/equine –2/Ohio/03, Influenza A/equine-2/New Market/2/93, Influenza A/equine-2/Kentucky/95, and combinations thereof. In all of the combinations listed above, it is preferred to use at least two strains of Equine Influenza and still more preferred to use at least 3 strains of Equine Influenza.

Preferred embodiments incorporating Equine Herpes Virus include: West Nile Virus, at least one strain of Equine Influenza Virus, Tetanus Toxoid, and Equine Herpes Virus; West Nile Virus, at least one strain of Equine Influenza Virus, Tetanus Toxoid, Eastern Equine Encephalomyelitis, and Equine Herpes Virus; West Nile Virus, at least one strain of Equine Influenza Virus, Tetanus Toxoid, Eastern Equine Encephalomyelitis, Western Equine Encephalomyelitis, and Equine Herpes Virus; West Nile Virus, at least one strain of Equine Influenza Virus, Tetanus Toxoid, Eastern Equine Encephalomyelitis, Western Equine Encephalomyelitis; Venezuelan Equine Encephalomyelitis, and Equine Herpes Virus; West Nile Virus, at least one strain of Equine Influenza Virus, and Eastern Equine Encephalomyelitis; West Nile Virus, at least one strain of Equine Influenza Virus, Western Equine Encephalomyelitis and Equine Herpes Virus; West Nile Virus, at least one strain of Equine Influenza Virus, Venezuelan Equine Encephalomyelitis, and Equine Herpes Virus; West Nile Virus, at least one strain of Equine Influenza Virus, Eastern Equine Encephalomyelitis, Western Equine Encephalomyelitis, and Equine Herpes Virus; West Nile Virus, at least one strain of Equine Influenza Virus, Eastern Equine Encephalomyelitis, Venezuelan Equine Encephalomyelitis, and Equine Herpes Virus; West Nile Virus, at least one strain of Equine Influenza Virus, Western Equine Encephalomyelitis, Venezuelan Equine Encephalomyelitis, and Equine Herpes Virus; West Nile Virus, at least one strain of Equine Influenza Virus, Western Equine Encephalomyelitis, Tetanus Toxoid, and Equine Herpes Virus; West Nile Virus, at least one strain of Equine Influenza Virus, Venezuelan Equine Encephalomyelitis, tetanus toxoid, and Equine Herpes Virus; West Nile Virus, at least one strain of Equine Influenza Virus, Venezuelan Equine Encephalomyelitis, Western Equine Encephalomyelitis, Tetanus Toxoid, and Equine Herpes Virus; and West Nile Virus, at least one strain of Equine Influenza Virus, Venezuelan Equine Encephalomyelitis, Eastern Equine Encephalomyelitis, Tetanus Toxoid, and Equine Herpes Virus. In all of the combinations listed above, it is preferred to use at least two strains of Equine Influenza and still more preferred to use at least 3 strains of Equine Influenza. Additionally, in all combinations above, the "at least one" strain of Equine Herpesvirus is preferred to be selected from the group consisting of EHV-1 and EHV-4. In some preferred forms, both strains, EHV-1 and EHV-4, will be included in the immunogenic composition. In other preferred forms, just EHV-1 will be included. The WNV component of the combination will preferably be an inactivated or killed North American dominant strain as described herein.

The vaccine composition can be administered in any immunogenically effective dose. In a preferred embodiment, the vaccine composition is administered as a single dose. Preferably, the dose has a total volume between about 0.5ml and 2.5ml, more preferably between about 0.6ml and 2.0ml, even more preferably between about 0.7ml and 1.75ml, still more preferably between about 0.8ml and 1.5ml, even more preferably between about 0.9ml and 1.25ml, with a single 1.0 ml dose being the most preferred.

In another embodiment, the vaccine is administered with a first dose being administered prior to the administration of a second (booster) dose. Preferably, the second dose is administered at least 15 days after the first dose. More preferably, the second dose is administered between 15 and 28 days after the first dose. Even more preferably, the second dose is administered at least 17 days after the first dose. Still more preferably, the second dose is administered between 17 and 25 days after the first dose. Even more preferably, the second dose is administered at least 19 days after the first dose. Still more preferably, the second dose is administered between 19 and 23 days after the first dose. Most preferably the second dose is administered at least 21 days after the first dose. In a preferred embodiment, both the first and second doses of the vaccine are in the same amount. Preferably, each dose is in the preferred amounts specified above, with a dose of 1 ml for the first and second dose being most preferred. In addition to the first and second dose regimen, an alternate embodiment comprises further subsequent doses. For example, a third, fourth, or fifth dose could be administered in these embodiments. Preferably, subsequent third, fourth, and fifth dose regimens are administered in the same amount as the first dose, with the time frame between the doses being consistent with the timing between the first and second doses mentioned above.

In an additional preferred embodiment, in each dose of the composition of the present disclosure, the WNV antigen comprises at least $10^{2.0}TCID_{50}$/dose. More preferably, the WNV antigen comprises between about $10^{2.0}TCID_{50}$/dose to $10^{10.0}TCID_{50}$/dose. Still more preferably, the WNV antigen comprises at least $10^{2.5}TCID_{50}$/dose. Even more preferably, the WNV antigen comprises between about $10^{2.5}TCID_{50}$/dose to about $10^{9.5}TCID_{50}$/dose. Still more preferably, the WNV antigen comprises at least $10^{3.0}TCID_{50}$/dose. Even more preferably, the WNV antigen comprises between about $10^{3.0}TCID_{50}$/dose to about $10^{9.0}TCID_{50}$/dose. Still more preferably, the WNV antigen comprises at least $10^{3.5}TCID_{50}$/dose. Even more preferably, the WNV antigen comprises between about $10^{3.5}TCID_{50}$/dose to about $10^{9.0}TCID_{50}$/dose. Most preferably, the

WNV antigen comprises between $10^{7.0}$TCID$_{50}$/dose and $10^{9.0}$TCID$_{50}$/dose. The TCID$_{50}$ values of an inactivated WNV vaccine or any other inactivated vaccine refer in general to the antigen content in the final vaccine that however is equivalent to the antigen content calculated for the vaccine composition prior to the inactivation of its antigen. Preferably, the immunogenic composition of the present disclosure stimulates serum neutralizing antibodies to WNV at a titer of 1:4 or higher when determined in a commercial available detection assay or using the procedures known to those of skill in the art with a representative example provided herein. In a preferred embodiment, in each dose of an embodiment of the present disclosure that comprises additional equine antigen, the amount of Eastern Equine Encephalomyelitis or Venezuelan Equine Encephalomyelitis in any dose is preferably at least $10^{5.5}$TCID$_{50}$/dose. Even more preferably, the dose is between about $10^{5.5}$TCID$_{50}$/dose and $10^{9.5}$TCID$_{50}$/dose. Still more preferably, the dose is at least $10^{6.0}$TCID$_{50}$/dose. Still more preferably, the dose is between about $10^{6.0}$TCID$_{50}$/dose and $10^{9.0}$TCID$_{50}$/dose. Even more preferably, the dose is at least $10^{6.5}$TCID$_{50}$/dose. Still more preferably, the dose is between about $10^{6.5}$TCID$_{50}$/dose and $10^{9.5}$TCID$_{50}$/dose. Even more preferably, the dose is at least $10^{7.0}$TCID$_{50}$/dose. Most preferably, the dose is between $10^{6.7}$TCID$_{50}$ and $10^{9.2}$TCID$_{50}$/dose.

Preferably, the Western Equine Encephalomyelitis antigen, when present in the composition of the present disclosure, is in an amount of at least $10^{6.2}$PFU/ml. Even more preferably, the amount is between $10^{6.2}$PFU/ml and $10^{10.2}$PFU/ml. Still more preferably, the amount is at least $10^{6.7}$PFU/ml. Even more preferably, the amount is between $10^{6.5}$PFU/ml and $10^{9.7}$PFU/ml. Still more preferably, the amount is at least $10^{7.2}$PFU/ml. Even more preferably, the amount is between about $10^{7.2}$PFU/ml and $10^{9.2}$PFU/ml. Still more preferably, the amount is at least $10^{7.7}$PFU/ml with at between $10^{6.5}$ PFU/dose and $10^{9.0}$PFU/ml being the most preferred.

In another preferred embodiment, the amount of tetanus toxoid, if present in the composition of the present disclosure, is in an amount of at least 3 CPU, more preferably, between about 3 CPU and 20CPU, still more preferably, at least 4 CPU, and most preferably, at least 5 CPU but not more than 20 CPU.

In an alternate embodiment, where one or more strains of Equine Influenza Virus is present, the amount of Equine Influenza present in the composition is in an amount of at least $10^{5.0}$ TCID$_{50}$/mL. More preferably, the Equine Influenza is in an amount of between about $10^{5.0}$ TCID$_{50}$/mL to $10^{9.0}$ TCID$_{50}$/mL, and, more preferably, at least $10^{6.0}$ TCID$_{50}$/mL. Still more

preferably, the amount is between about $10^{6.0}$ $TCID_{50}$/mL to $10^{8.0}$ $TCID_{50}$/mL and, more preferably, the amount is at least $10^{6.5}$ $TCID_{50}$/mL. Still more preferably, the amount is between about $10^{6.5}$ $TCID_{50}$/mL to $10^{7.0}$ $TCID_{50}$/mL, with the most preferred amount being between about $10^{6.7}$ $TCID_{50}$/mL to $10^{7.0}$.

In an embodiment that comprises Equine Herpes Virus, the amount of Equine Herpes Virus in each dose is at least $10^{6.0}$ $TCID_{50}$/mL. More preferably, Equine Herpes Virus is present in the composition in an amount of between $10^{6.0}$ $TCID_{50}$/mL to $10^{9.5}$ $TCID_{50}$/mL and, more preferably, in an amount of about $10^{7.0}$ $TCID_{50}$/mL. Still more preferably, Equine Herpes Virus is present in an amount between $10^{7.5}$ $TCID_{50}$/mL to $10^{9.0}$ $TCID_{50}$/mL and, more preferably, in an amount of about $10^{8.0}$ $TCID_{50}$/mL. Still more preferably, Equine Herpes Virus is present in an amount of between $10^{8.0}$ $TCID_{50}$/mL to $10^{9.0}$ $TCID_{50}$/mL and, most preferably, in an amount of about $10^{8.50}$ $TCID_{50}$/mL.

In yet another preferred embodiment, a vaccine composition comprising the chronologically contemporary and epidemiologically prevalent strains of WNV is provided. Such a composition will generally improve the efficacy of the composition. Preferably, such a prevalent strain is isolated from the tissues of a horse. Such a source is a preferred source of WNV for preparing vaccine seed virus for an immunological composition for a species for which a comprehensively safe and effective WNV vaccine is particularly needed, namely, the horse. Further, the present disclosure discloses a vaccine composition comprising an inactivated low passage strain of WNV from the tissues of a horse, thereby overcoming the limitations of previous vaccines with the inappropriate limited repertoire of protein antigens found in either high passage attenuated vaccines, subunit vaccines, or other compositions produced by recombinant technology that express less than the full complement of proteins. This inactivated low passage WNV strain, isolated from horse tissues, overcomes deficiencies inherent in previous vaccines and provides a broad number of immunogenic proteins of most relevance by virtue of being produced from a highly virulent equine strain of low passage, thereby comprising a uniquely and comprehensively effective, yet safe, immunogenic composition not previously available for vaccination of the horse. Additionally, preferred chronologically contemporary and epidemiologically prevalent strains of WNV are North American dominant WNV strains, as defined herein.

The present disclosure provides for a broader scope of protection than traditional immunogenic or vaccine compositions, as the present disclosure provides protection against a broad range of isolates of a particular antigen. The challenge model used to evaluate the efficacy of the composition of the present disclosure utilized a heterologous challenge strain, evidencing the composition's ability to provide protection to isolates and strains outside of the particular strain or isolate used to vaccinate the animal. This is a unique feature of the present disclosure.

The present disclosure additionally provides for a method of reduction of the incidence and/or severity of clinical signs associated with West Nile Virus infection in an animal, preferably a horse, when compared to wild type infection. Such methods generally comprise the step of administering a vaccine composition comprising a killed or inactivated isolate of West Nile virus, preferably a North American dominant WNV strain, and a pharmaceutically acceptable carrier. In some preferred embodiments of the present application, an adjuvant is added to the composition, and in other preferred forms, no adjuvant is provided. In an alternate preferred embodiment, the method comprises administering a vaccine composition comprising one or more killed or inactivated isolate(s) of West Nile virus in combination with immunologically effective amounts of antigenic components from other equine pathogens. Preferably those isolates are selected from the group consisting of Eastern Equine Encephalomyelitis antigen, Western Equine Encephalomyelitis antigen, Venezuelan Equine Encephalomyelitis antigen, tetanus toxoid, and combinations thereof, and more preferably being those combinations described above. In another preferred embodiment, the vaccine of the present disclosure is combined with a suitable adjuvant, diluent, or pharmaceutically acceptable carrier.

The present disclosure provides for reduction of the incidence and/or severity of clinical symptoms associated with West Nile Virus infection in a herd, when compared to wild type infection. In a second aspect, there is provided the use of an immunogenic composition as defined above in the manufacture of a medicament for reducing the incidence or lessening the severity of clinical symptoms associated with or caused by West Nile Virus, wherein said medicament is to be administered to an animal or a herd of animals in need thereof. Preferably, the severity and/or incidence of clinical symptoms in animals receiving the immunogenic composition of the present disclosure are reduced at least 10% in comparison to animals not receiving such an administration when both groups (animals receiving and animals not receiving the composition) are challenged with or exposed to wild type infection by WNV.

More preferably, the incidence or severity is reduced at least 20%, even more preferably, at least 30%, still more preferably, at least 40%, even more preferably, at least 50%, still more preferably, at least 60%, even more preferably, at least 70%, still more preferably, at least 80%, even more preferably, at least 90%, still more preferably, at least 95%, and most preferably, at

least 100%, wherein the animals receiving the composition of the present disclosure exhibit no clinical symptoms. Preferably, the WNV strain is a North American dominant strain of WNV. Advantageously, the present disclosure also provides protection from heterologous strains (relative to the strain used in the composition) of pathogens.

The present disclosure further provides a method of stimulating serum neutralizing or serum hemagglutination antibodies to a pathogen selected from the group consisting of WNV, WEE, VEE, EEE, EHV, EIV, and combinations thereof by administering a composition in accordance with the present disclosure described herein. Preferably the compositions of the present disclosure stimulate serum neutralizing antibodies to WNV at a titer of 1:4 or higher, thereby preventing or reducing WNV viremia.

The immunogenic composition of the present disclosure provides an extended duration of immunity against all antigens present in the vaccine. Preferably, the duration of immunity against West Nile is at least 1 month, more preferably, the duration of immunity is at least 2 months, still more preferably, the duration of immunity is at least 3 months, even more preferably, the duration of immunity is at least 4- 24 months, still more preferably, the duration of immunity is at least 6 -24 months, even more preferably, the duration of immunity is at least 7-24 months, still more preferably, the duration of immunity is at least 8-24 months, even more preferably, the duration of immunity is at least 9-24 months, still more preferably, the duration of immunity is at least 10-24 months, and most preferably, the duration of immunity is at least 12-24 months.

Preferably, the duration of immunity against EIV is at least 1 month, more preferably, the duration of immunity is at least 2 months, still more preferably, the duration of immunity is at least 3 months, even more preferably, the duration of immunity is at least 4- 24 months, still more preferably, the duration of immunity is at least 6 -24 months, even more preferably, the duration of immunity is at least 7-24 months, still more preferably, the duration of immunity is at least 8-24 months, even more preferably, the duration of immunity is at least 9-24 months, still more preferably, the duration of immunity is at least 10-24 months, and most preferably, the duration of immunity is at least 12-24 months.

Preferably, the duration of immunity against EHV is at least 1 month, more preferably, the duration of immunity is at least 2 months, still more preferably, the duration of immunity is at least 3 months, even more preferably, the duration of immunity is at least 4- 24 months, still

more preferably, the duration of immunity is at least 6 -24 months, even more preferably, the duration of immunity is at least 7-24 months, still more preferably, the duration of immunity is at least 8-24 months, even more preferably, the duration of immunity is at least 9-24 months, still more preferably, the duration of immunity is at least 10-24 months, and most preferably, the duration of immunity is at least 12-24 months.

Preferably, the duration of immunity against Western Equine Encephalomyelitis is at least 1 month, more preferably, the duration of immunity is at least 2 months, still more preferably, the duration of immunity is at least 3 months, even more preferably, the duration of immunity is at least 4- 24 months, still more preferably, the duration of immunity is at least 6 -24 months, even more preferably, the duration of immunity is at least 7-24 months, still more preferably, the duration of immunity is at least 8-24 months, even more preferably, the duration of immunity is at least 9-24 months, still more preferably, the duration of immunity is at least 10-24 months, and most preferably, the duration of immunity is at least 12-24 months.

Preferably, the duration of immunity against Eastern Equine Encephalomyelitis is at least 1 month, more preferably, the duration of immunity is at least 2 months, still more preferably, the duration of immunity is at least 3 months, even more preferably, the duration of immunity is at least 4- 24 months, still more preferably, the duration of immunity is at least 6 -24 months, even more preferably, the duration of immunity is at least 7-24 months, still more preferably, the duration of immunity is at least 8-24 months, even more preferably, the duration of immunity is at least 9-24 months, still more preferably, the duration of immunity is at least 10-24 months, and most preferably, the duration of immunity is at least 12-24 months.

Preferably, the duration of immunity against Venezuelan Equine Encephalomyelitis is at least 1 month, more preferably, the duration of immunity is at least 2 months, still more preferably, the duration of immunity is at least 3 months, even more preferably, the duration of immunity is at least 4- 24 months, still more preferably, the duration of immunity is at least 6 -24 months, even more preferably, the duration of immunity is at least 7-24 months, still more preferably, the duration of immunity is at least 8-24 months, even more preferably, the duration of immunity is at least 9-24 months, still more preferably, the duration of immunity is at least 10-24 months, and most preferably, the duration of immunity is at least 12-24 months.

Preferably, the duration of immunity against Tetanus Toxoid is at least 1 month, more preferably, the duration of immunity is at least 2 months, still more preferably, the duration of

immunity is at least 3 months, even more preferably, the duration of immunity is at least 4- 24 months, still more preferably, the duration of immunity is at least 6 -24 months, even more preferably, the duration of immunity is at least 7-24 months, still more preferably, the duration of immunity is at least 8-24 months, even more preferably, the duration of immunity is at least 9-24 months, still more preferably, the duration of immunity is at least 10-24 months, and most preferably, the duration of immunity is at least 12-24 months.

Preferably, the duration of immunity of at least 12 months further relates to any combination of antigens forming the immunogenic composition of the present disclosure.

In another preferred embodiment comprising EIV and/or EHV antigen, as described above, the immunogenic composition ameliorates shedding of infectious EIV or EHV to prevent spread of the virus to other susceptible animals.

In yet another preferred embodiment, compositions in accordance with the present disclosure described herein overcome interference from passively acquired maternal immunity and stimulates active immunity and a reduction in the incidence of or severity of clinical signs of EIV infection in vaccinated animals against EIV.

In another preferred embodiment of the present disclosure, an immunogenic composition comprising VEE, WEE, EEE, tetanus, WNV, equine rhinopneumonitis and equine influenza, all as described herein, demonstrates efficacy against VEE, WEE, EEE, tetanus, WNV, equine rhinopneumonitis and equine influenza after administration in accordance with the present disclosure. Preferably, such a composition will further include an adjuvant, preferably mineral oil and/or a carbomer, and a veterinary acceptable carrier. In preferred forms, the composition will be administered in a single, 1 ml dose.

Each of the immunogenic compositions described herein that include WNV antigen can be administered as described such that they reduce the incidence of or lessen the severity of clinical symptoms associated with West Nile Virus.

Each of the immunogenic compositions described herein that include EIV antigen can be administered as described such that they reduce the incidence of or lessen the severity of clinical symptoms associated with Equine Influenza.

The present disclosure also provides a method for reducing the incidence of or lessening the severity of clinical symptoms associated with Equine Herpes virus comprising the step of

administering any one of the immunogenic compositions described above, that includes an Equine Herpes virus antigen, to an animal.

The present disclosure also provides a method for reducing the incidence of clinical symptoms associated with West Nile Virus comprising the step of administering any one of the immunogenic compositions that includes West Nile Virus antigen, as described herein, to an animal.

The present disclosure also provides a method for reducing the incidence of clinical symptoms associated with Equine Influenza Virus comprising the step of administering any one of the immunogenic compositions described above, that includes an Equine Influenza antigen, to an animal.

The present disclosure further provides a method for reducing the incidence of clinical symptoms associated with Equine Herpes Virus comprising the step of administering any one of the immunogenic compositions described above that includes an Equine Herpes virus antigen, to an animal.

The present disclosure also provides a method of reducing the incidence of clinical symptoms associated with Equine Influenza Virus comprising the step of administering any one of the immunogenic compositions described above to an animal, wherein the reduction in clinical signs, compared to animals not receiving the immunogenic composition, is at least a 10% reduction in clinical signs.

The present disclosure provides a method of reducing the incidence of infection in a herd comprising the step of administering any one of the immunogenic compositions described above to an animal.

The present disclosure provides a method of reducing the incidence of infection in a herd comprising the step of administering any one of the immunogenic compositions described above to an animal, wherein the reduction of incidence of infection, compared to herds not receiving the immunogenic composition, is from about 10% - 50% reduction.

The present disclosure provides a method of reducing the incidence and severity of clinical symptoms of EHV in a herd, wherein the clinical symptoms are selected from the group consisting of respiratory disease, abortion, reproductive complications, neurological disease, central nervous system disease, and combinations thereof.

The present disclosure provides a method for reducing the incidence of or lessening the severity of clinical symptoms associated with Equine Herpes Virus comprising the step of administering any one of the immunogenic compositions described above, that includes an Equine Herpes Virus antigen, to an animal.

The present disclosure provides a method for reducing the severity of or lessening the severity of clinical symptoms associated with Equine Influenza in a herd, comprising the step of administering any one of the immunogenic compositions described above, that includes an Equine Influenza antigen, to an animal.

The present disclosure provides a method for reducing the incidence of or lessening the severity of clinical symptoms associated with West Nile Virus in a herd, comprising the step of administering any one of the immunogenic compositions described above, that includes a West Nile Virus antigen, to an animal.

The present disclosure provides a method for reducing the incidence of or lessening the severity of clinical symptoms associated with Eastern Equine Encephalomyelitis in a herd, comprising the step of administering any one of the immunogenic compositions described above that includes an Eastern Equine Encephalomyelitis antigen to an animal.

The present disclosure further provides a method for reducing the incidence of or lessening the severity of clinical symptoms associated with Western Equine Encephalomyelitis in a herd, comprising the step of administering any one of the immunogenic compositions described above, that includes an Western Equine Encephalomyelitis antigen, to an animal.

The present disclosure further provides a method for reducing the incidence of or lessening the severity of clinical symptoms associated with Venezuelan Equine Encephalomyelitis in a herd, comprising the step of administering any one of the immunogenic compositions described above, that includes a Venezuelan Equine Encephalomyelitis antigen, to an animal.

In a third aspect, there is provided the use of an immunogenic composition as defined above in the manufacture of a medicament for reducing the incidence or lessening the severity of clinical symptoms associated with or caused by one or more of the pathogens selected from the group consisting of West Nile Virus, Eastern Equine Encephalomyelitis Virus, Western Equine Encephalomyelitis Virus, Venezuelan Equine Encephalomyelitis Virus and Clostridium tetani, wherein said medicament is to be administered to an animal or a herd of animals in need thereof.

In a fourth aspect, there is provided the use of an immunogenic composition as defined above in the manufacture of a medicament for reducing the incidence or lessening the severity of clinical symptoms associated with or caused by one or more of the pathogens selected from the group consisting of: Eastern Equine Encephalomyelitis Virus, Western Equine Encephalomyelitis Virus, Venezuelan Equine Encephalomyelitis Virus, Equine Herpes Virus and Clostridium tetani, wherein said medicament is to be administered to an animal or a herd of animals in need thereof.

In a fifth aspect, there is provided the use of an immunogenic composition as defined above in the manufacture of a medicament for reducing the incidence or lessening the severity of clinical symptoms associated with or caused by one or more of the pathogens selected from the group consisting of: West Nile Virus, Eastern Equine Encephalomyelitis Virus, Western Equine Encephalomyelitis Virus, Venezuelan Equine Encephalomyelitis Virus, Equine Herpes Virus, Equine Influenza Virus and Clostridium tetani, wherein said medicament is to be administered to in an animal or a herd of animals in need thereof.

The present disclosure also provides a method of making any one of the immunogenic composition of the present disclosure as described above and herein, comprising the steps of combining a West Nile Virus antigen with a suitable excipient or pharmaceutical carrier. In preferred forms, this method further comprises the step of adding one or more equine antigens. A preferred group of equine antigens are selected from the group consisting of Western Equine Encephalomyelitis, Eastern Equine Encephalomyelitis, Venezuelan Equine Encephalomyelitis,

Tetanus Toxoid, EHV, EIV, and combinations thereof. In some preferred forms, the methods described herein can further comprise a filtration step, wherein the final product is in a purified form.

"Adjuvants" as used herein, can include aluminum hydroxide and aluminum phosphate, saponins e.g., Quil A, QS-21 (Cambridge Biotech Inc., Cambridge MA), GPI-0100 (Galenica Pharmaceuticals, Inc., Birmingham, AL), non-metabolizable oil, mineral and/or plant/vegetable and/or animal oils, polymers, carbomers, surfactants, natural organic compounds, plant extracts, carbohydrates, cholesterol, lipids, water-in-oil emulsion, oil-in-water emulsion, water-in-oil-in-water emulsion. The emulsion can be based in particular on light liquid paraffin oil (European Pharmacopeia type); isoprenoid oil such as squalane or squalene; oil resulting from the oligomerization of alkenes, in particular of isobutene or decene; esters of acids or of alcohols containing a linear alkyl group, more particularly plant oils, ethyl oleate, propylene glycol di-(caprylate/caprate), glyceryl tri-(caprylate/caprate) or propylene glycol dioleate; esters of branched fatty acids or alcohols, in particular isostearic acid esters. The oil is used in combination with emulsifiers to form the emulsion. The emulsifiers are preferably nonionic surfactants, in particular esters of sorbitan, of mannide (e.g. anhydromannitol oleate), of glycol, of polyglycerol, of propylene glycol and of oleic, isostearic, ricinoleic or hydroxystearic acid, which are optionally ethoxylated, and polyoxypropylene-polyoxyethylene copolymer blocks, in particular the Pluronic products, especially L121. See Hunter et al., The Theory and Practical Application of Adjuvants (Ed.Stewart-Tull, D. E. S.). John Wiley and Sons, NY, pp51-94 (1995) and Todd et al., Vaccine 15:564-570 (1997). In a preferred embodiment the adjuvant is at a concentration of about 0.01 to 50%, preferably at a concentration of about 2% to 30%, more preferably at a concentration of about 5% to 25%, still more preferably at a concentration of about 7% to 22%, and most preferably at a concentration of 10% to 20% by volume of the final product. Of the possible adjuvants used in combination with the present invention, it is preferred to not use a metabolizable oil. In a preferred embodiment, the adjuvant is at least a non-metabolizable oil, preferably mineral oil. In an alternate preferred embodiment, the vaccine composition contains essentially no oil-based adjuvants. In a most preferred embodiment the vaccine composition contains both a non-metabolizable oil, preferably mineral oil, and carbomer as adjuvants.

In addition, the immunogenic and vaccine compositions of the present invention can include one or more veterinary-acceptable carriers. As used herein, "a veterinary-acceptable carrier" includes any and all solvents, dispersion media, coatings, adjuvants, stabilizing agents, diluents, preservatives, excipients, antibacterial and antifungal agents, antimicrobic agents, isotonic agents, adsorption delaying agents, and the like. In some preferred embodiments, and especially those that include lyophilized immunogenic compositions, stabilizing agents for use in the present invention include stabilizers for lyophilization or freeze-drying.

"Diluents" can include water, saline, dextrose, ethanol, glycerol, and the like. Isotonic agents can include sodium chloride, dextrose, mannitol, sorbitol, and lactose, among others. Stabilizers include albumin and alkali salts of ethylendiamintetracetic acid, among others.

In a preferred embodiment, the immunogenic composition of the present invention is prepared comprising a preservative and a stabilizer; and, more preferably, the immunogenic composition of the present invention is prepared comprising gentamycin, EDTA, Glycerol, and combinations thereof.

An "immunogenic or immunological composition" refers to a composition of matter that comprises at least one antigen, which elicits an immunological response in the host of a cellular and/or antibody-mediated immune response to the composition or vaccine of interest. Usually, an "immunological response" includes but is not limited to one or more of the following effects: the production or activation of antibodies, B cells, helper T cells, suppressor T cells, and/or cytotoxic T cells and/or gamma-delta T cells, directed specifically to an antigen or antigens included in the composition or vaccine of interest. Preferably, the host will display either a therapeutic or protective immunological response such that resistance to new infection will be enhanced and/or the clinical severity of the disease reduced. Such protection will be demonstrated by either a reduction or lack of clinical signs normally displayed by an infected host, a quicker recovery time and/or a lowered duration or bacterial titer in the tissues or body fluids or excretions of the infected host.

The term "in need of such administration" or "in need of such administration treatment", as used herein means that the administration/treatment is associated with the boosting or improvement in health or any other positive medicinal effect on health of the animals which receive the immunogenic composition in accordance with the present invention.

The term "West Nile Virus" antigen means, but is not limited to the components of the WNV virion that are immunogenic when present in an animal, and most particularly protein components, such as envelope and non-structural proteins, of the WNV that provoke humoral or cellular immune responses when present in an animal. Such antigens can include DNA, protein subunits, modified live virus, and killed or inactivated virus. In preferred forms of the invention, the WNV antigen or antigens comprise inactivated or killed, and even more preferably, North American dominant, WNV strains.

The term "North American West Nile Virus (strains)" refers to, but is not limited to any West Nile Virus strain or isolate that has ever been discovered on the North American continent. Preferably, a North American West Nile Virus strain has a sequence identity to the NY99 strain (GenBank accession no. AF196835 or NCBI reference sequence NC_00942.1 (SEQ ID No. 23) of at least 97%, even more preferably, at least 98%, still more preferably, at least 98.5%, more preferably, at least 99%, even more preferably, at least 99.2%, and, most preferably of at least 99.4%.

"Sequence Identity" as it is known in the art refers to a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, namely a reference sequence and a given sequence to be compared with the reference sequence. Sequence identity is determined by comparing the given sequence to the reference sequence after the sequences have been optimally aligned to produce the highest degree of sequence similarity, as determined by the match between strings of such sequences. Upon such alignment, sequence identity is ascertained on a position-by-position basis, e.g., the sequences are "identical" at a particular position if at that position, the nucleotides or amino acid residues are identical. The total number of such position identities is then divided by the total number of nucleotides or residues in the reference sequence to give % sequence identity. Sequence identity can be readily calculated by known methods, including but not limited to, those described in Computational Molecular Biology, Lesk, A. N., ed., Oxford University Press, New York (1988), Biocomputing: Informatics and Genome Projects, Smith, D.W., ed., Academic Press, New York (1993); Computer Analysis of Sequence Data, Part I, Griffin, A.M., and Griffin, H. G., eds., Humana Press, New Jersey (1994); Sequence Analysis in Molecular Biology, von Heinge, G., Academic Press (1987); Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M. Stockton Press, New York (1991); and Carillo, H., and Lipman, D., SIAM J. Applied Math., 48: 1073

(1988), the teachings of which are incorporated herein by reference. Preferred methods to determine the sequence identity are designed to give the largest match between the sequences tested. Methods to determine sequence identity are codified in publicly available computer programs which determine sequence identity between given sequences. Examples of such programs include, but are not limited to, the GCG program package (Devereux, J., et al., Nucleic Acids Research, 12(1):387 (1984)), BLASTP, BLASTN and FASTA (Altschul, S. F. et al., J. Molec. Biol., 215:403-410 (1990). The BLASTX program is publicly available from NCBI and other sources (BLAST Manual, Altschul, S. et al., NCVI NLM NIH Bethesda, MD 20894, Altschul, S. F. et al., J. Molec. Biol., 215:403-410 (1990), the teachings of which are incorporated herein by reference). These programs optimally align sequences using default gap weights in order to produce the highest level of sequence identity between the given and reference sequences. As an illustration, by a polynucleotide having a nucleotide sequence having at least, for example, 85%, preferably 90%, even more preferably 95% "sequence identity" to a reference nucleotide sequence, it is intended that the nucleotide sequence of the given polynucleotide is identical to the reference sequence except that the given polynucleotide sequence may include up to 15, preferably up to 10, even more preferably up to 5 point mutations per each 100 nucleotides of the reference nucleotide sequence. In other words, in a polynucleotide having a nucleotide sequence having at least 85%, preferably 90%, even more preferably 95% identity relative to the reference nucleotide sequence, up to 15%, preferably 10%, even more preferably 5% of the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or a number of nucleotides up to 15%, preferably 10%, even more preferably 5% of the total nucleotides in the reference sequence may be inserted into the reference sequence. These mutations of the reference sequence may occur at the 5' or 3' terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence. Analogously, by a polypeptide having a given amino acid sequence having at least, for example, 85%, preferably 90%, even more preferably 95% sequence identity to a reference amino acid sequence, it is intended that the given amino acid sequence of the polypeptide is identical to the reference sequence except that the given polypeptide sequence may include up to 15, preferably up to 10, even more preferably up to 5 amino acid alterations per each 100 amino acids of the reference amino acid sequence. In other

words, to obtain a given polypeptide sequence having at least 85%, preferably 90%, even more preferably 95% sequence identity with a reference amino acid sequence, up to 15%, preferably up to 10%, even more preferably up to 5% of the amino acid residues in the reference sequence may be deleted or substituted with another amino acid, or a number of amino acids up to 15%, preferably up to 10%, even more preferably up to 5% of the total number of amino acid residues in the reference sequence may be inserted into the reference sequence. These alterations of the reference sequence may occur at the amino or the carboxy terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in the one or more contiguous groups within the reference sequence. Preferably, residue positions which are not identical differ by conservative amino acid substitutions. However, conservative substitutions are not included as a match when determining sequence identity.

The term "North American Dominant West Nile Virus" strains and isolates refers to those strains or isolates defined as such in Phylogenetic Analysis of North American West Nile Virus Isolates, 2001-2004: Evidence For the Emergence of a Dominant Genotype, C. Todd Davis, et. al, Virology 342, p. 252-265 (2005), the teaching and content of which is hereby incorporated by reference herein. As noted therein, North American Dominant WNV strains or isolates have at least 1 nucleotide change resulting in an amino acid change from the WN99 isolates. Strain NY99 (GenBank accession no. AF196835), an example of which is provided in SEQ ID. NO. 23, serves as a reference strain for determining if a strain or isolate is North American Dominant. In a preferred embodiment, the nucleotide change results in an amino acid change in an envelope protein of the strain or isolate and, more preferably, the nucleotide change results in an amino acid change from valine to alanine at position 159 in the critical envelope protein or "E159". Preferably, this amino acid change is associated with a greater ability to replicate in the intermediate host, namely, the mosquito. In addition, these strains or isolates may have one or more silent amino acid changes. Preferably, North American Dominant strains also include either (and preferably both) a U to C mutation and a C to U mutation at positions 1442 and 2466 (in comparison to a North American strain, e.g. NY 99 and SEQ ID NO.23), respectively. Still more preferably, North American Dominant strains or isolates further include a mutation in the nucleotide sequence encoding the E protein and the C to U mutation at position 9352 in the sequence encoding the NS5 protein (again in comparison to a North American strain,

e.g. NY 99 and SEQ ID NO. 23). These preferred mutations are shown in detail for specific regions in Example 10 and Figs. 10-17. Representative North American Dominant WNV strains are listed in this application. Additionally, for purposes of the present invention North American Dominant and WN02 are used interchangeably.

For purposes of the present invention, Horse Origin 2005 strain North American Equine E159, E159 (Horse Origin), NAEE159, United States Department of Agriculture Isolate 405330 (USDA 2005) Horse Origin, and E159 strain are used interchangeably. For purposes of the present invention, Donkey Origin 2004 strain, United States Department of Agricultures Isolate 292206 (USDA 2004) Donkey Origin, E159 (Donkey Origin), and North American Donkey E159 (NADE159) are used interchangeably. E159 indicates that the amino acid change in the envelope protein from valine to alanine occurs at position 159, as described above.

West Nile Virus strains or isolates, for purposes of the present invention, are not limited to horse and equine West Nile Virus strains but encompass, while not being limited to, those West Nile Virus strains of bird origin, donkey origin, pig origin, human origin, mammal origin, and equine origin.

For purposes of the present invention the terms "strain" and "isolate" have the same meaning and are used interchangeably.

As used herein, "a pharmaceutically" or "veterinary acceptable carrier" or "pharmaceutical carrier" includes any and all solvents, growth media, dispersion media, coatings, adjuvants, stabilizing agents, diluents, preservatives, antibacterial and antifungal agents, isotonic agents, adsorption delaying agents, and the like.

An "immunogenic or immunological composition" refers to a composition of matter that comprises at least one antigen which elicits an immunological response in the host of a cellular and/ or antibody-mediated immune response to the composition or vaccine of interest. Usually, an "immunological response" includes but is not limited to one or more of the following effects: the production or activation of antibodies, B cells, helper T cells, suppressor T cells, and/or cytotoxic T cells and/or gamma-delta T cells, and/or virus neutralizing antibodies directed specifically to an antigen or antigens included in the composition or vaccine of interest. Preferably, the host will display either a therapeutic or protective immunological response such that resistance to new infection will be enhanced and/or the clinical severity of the disease reduced. Such protection will be demonstrated by either a reduction or lack of clinical signs

normally displayed by an infected host, a quicker recovery time and/or a lowered duration of clinical disease or higher viral antibody titer in the tissues or body fluids or excretions of the infected host, or lessened viremia in the blood, or lessened gross or histopathological lesions due to infection.

In addition, the immunogenic and vaccine compositions of the present invention can include one or more veterinary-acceptable carriers. As used herein, "a veterinary-acceptable carrier" includes any and all solvents, dispersion media, cell culture media and cell culture constituents, coatings, adjuvants, stabilizing agents, diluents, preservatives, antibacterial and antifungal agents, isotonic agents, adsorption delaying agents, and the like. "Diluents" can include water, saline, buffered saline, dextrose, ethanol, glycerol, and the like. Isotonic agents can include sodium chloride, dextrose, mannitol, sorbitol, and lactose, among others. Stabilizers include albumin and alkali salts of ethylendiamintetracetic acid, among others.

"Clinical signs" of West Nile Virus, for purposes of this invention, include, but are not limited to, symptoms or lesions associated with encephalitis, viremia, anorexia, depression, fever, weakness, abnormal gait, paralysis of hind limbs, impaired vision, ataxia, aimless wandering, convulsions, inability to swallow, coma, posterior weakness, paralysis, poor coordination, depression and related behavior, tremors, convulsions, paddling of the limbs, neurological problems, swelling of the central nervous system, death, and combinations thereof. The clinical signs exhibited by an infected animal vary depending on the severity of infection

"Clinical Signs" of Equine Herpes virus, for purposes of this invention include, but are not limited to, abortion, neurological deficiencies, respiratory disease, reproductive system deficiencies and failure, and symptoms relating to the central nervous system. Additionally, clinical symptoms of EHV 1 include, but are not limited to, the phenomenon of foals infected with EHV1, exhibiting respiratory complications, passing the virus to the older members of the herd who then exhibit reproductive deficiencies, including abortion, and neurological deficiencies, normally exhibited in the central nervous system.

"Clinical Signs" of Eastern Equine Encephalomyelitis, Western Equine Encephalomyelitis, and Venezuelan Equine Encephalomyelitis, for purposes of the present invention are those symptoms normally known to be associated with encephalomyelitis, including, but are not limited to fever, nervous signs such as sensitivity to sound, periods of excitement, and restlessness, brain lesions, drowsiness, drooping ears, circling, abnormal gait,

paralysis, loss of appetite, depression, head pressing, lack of coordination, long-term disability, brain damage, death, and combinations thereof. "Safety" as used herein, refers to the absence of adverse consequences in the vaccinated animal following vaccination, including but not limited to, potential reversion of vaccine virus to virulence and clinically significant side effects, such as persistent systemic illness or unacceptable inflammation at the site of vaccine administration.

"Reduction of the incidence and/or severity of clinical signs" or "reduction in the incidence and/or severity of clinical symptoms", as referred to herein, means reducing the number of infected animals in a group, reducing or eliminating the number of animals exhibiting clinical signs of infection, or reducing the severity of any clinical signs that are present in the animals, in comparison to wild-type infection. For example, in the experiments herein, such clinical signs included viremia, fever, antibody response, and histopathology. Preferably, these are reduced in animals receiving the composition of the present invention by at least 10% in comparison to animals not receiving the vaccination which may become infected. More preferably, clinical signs are reduced in animals receiving the composition of the present invention by at least 20%, more preferably by at least 30%, even more preferably by at least 40%, and even more preferably by at least 50%.

"Duration of Immunity," as used herein, refers to the minimum number of days during which an animal produces an immunogenic response such that the animal will be relatively immune from contracting a virus and/or benefit from reduction of incidence and/or severity of clinical signs, as described herein.

The terms "strain" and "isolate", when used herein, are meant to be used interchangeably.

The terms "vaccine" and "immunogenic composition", when used herein, are meant to be used interchangeably.

Any West Nile Virus strain(s) or isolate(s) can be used in accordance with the present invention. In a preferred embodiment, the isolate is selected from one or more of the following: New York (Northeastern North American) Isolate (WN-NY 99), Horse Origin, 1999, New York (Northeastern North American) Isolate (WN-NY 99), Crow Origin, 1999, United States Department of Agricultures Isolate 292206 (USDA 2004), Donkey Origin, United States Department of Agriculture Isolate 405330 (USDA 2005), Horse Origin, North American Isolate (WN-Texas-2002/2003), Southeast Texas Coastal Isolate 2002, Mexico (Tabasco) Isolate 2003, and combinations thereof, and in a more preferred embodiment the isolate is selected from one or

more of the following: United States Department of Agricultures Isolate 292206 (USDA 2004), Donkey Origin, United States Department of Agriculture Isolate 405330 (USDA 2005), Horse Origin, North American Isolate (WN-Texas-2002/2003), Southeast Texas Coastal Isolate 2002, Mexico (Tabasco) Isolate 2003, and combinations thereof. In a most preferred embodiment, the isolate is United States Department of Agriculture Isolate 405330 (USDA 2005), Horse Origin singularly or in combination with one or more isolates as listed above. In an additionally preferred embodiment, those isolates which are part of the North American West Nile Virus isolates are included. In yet another preferred embodiment North American Dominant West Nile Virus isolates are included. In addition to those listed above, specific isolates include, but are not limited to, WN02 and isolates which have at least 1, preferably at least 2, and even more preferably at least 3 nucleotide changes resulting in at least one amino acid change from the WN NY99 isolates, and most preferred are strains with the amino acid change from valine to alanine at position 159 of the envelope protein. Most preferred North American Dominant strains include, but are not limited to: NY2002Nassau, NY2002Clinton, NY2002Queens, GA20021, GA20022, TX20021, TX20022, IN2002, NY2003Albany, NY2003Suffolk, NY2003Chatauqua, CO20031, CO20032, TX2003, TX2003Harris4, TX2003Harris6, TX2003Harris7, TX2003Harris10, AZ2004, and TX2004Harris4, and combinations thereof. The strains of West Nile Virus useful in the vaccine or immunogenic composition of the present invention can be any strain or isolate. In a preferred embodiment, the North American Dominant West Nile Virus strain used is either E-159 (Horse Origin) or E-159 (Donkey Origin). A representative strain of such a North American Dominant WNV strain includes the Horse Origin 2005 strain deposited with the ATCC (ATCC Accession No. PTA-9409), located at 10801 University Boulevard, Manassas, VA, 20110-2209, on August 14, 2008, under the provisions of the Budapest Treaty. Equine Influenza strains useful in the vaccine or immunogenic composition of the present invention can be any strain or isolate. Representative strains include Equi-2/Ohio/03, deposited as ATCC Accession No. PTA-9522, Equi-2/Kentucky/95, deposited as ATCC Accession No. PTA-9523, and Equi-2/New Market/2/93, deposited as ATCC Accession No. PTA-9524. Representative strains ATCC Accession Nos. PTA-9522, PTA-9523, and PTA-9524 were each deposited with the ATCC at 10801 University Boulevard, Manassas, VA, 20110-2209 on September 23, 2008, under the provisions of the Budapest Treaty.

Equine Herpes Virus ("EHV") strains useful in the vaccine or immunogenic composition of the present invention can be any strain or isolate. Representative strains include EHV Subtype 1, deposited as ATCC Accession No. PTA-9525, and EHV Subtype 4, deposited as ATCC Accession No. PTA-9526. Representative strains ATCC Accession Nos. PTA-9525 and PTA-9526 were each deposited with the ATCC at 10801 University Boulevard, Manassas, VA, 20110-2209 on September 23, 2008, under the provisions of the Budapest Treaty.

Western Equine Encephalomyelitis strains useful in the vaccine or immunogenic composition of the present invention can be any strain or isolate. A representative strain includes the Fleming Strain, deposited with the ATCC (ATCC Accession No. PTA-9410), located at 10801 University Boulevard, Manassas, VA, 20110-2209, on August 14, 2008, under the provisions of the Budapest Treaty.

Venezuelan Equine Encephalomyelitis strains useful in the vaccine or immunogenic composition of the present invention can be any strain or isolate. A representative strain includes the TC-83 strain, deposited with the ATCC (ATCC Accession No. PTA-9411), located at 10801 University Boulevard, Manassas, VA, 20110-2209, on August 14, 2008, under the provisions of the Budapest Treaty.

Eastern Equine Encephalomyelitis strains useful in the vaccine or immunogenic composition of the present invention can be any strain or isolate. A representative strain includes the NJO strain, deposited with the ATCC (ATCC Accession No. PTA-9412), located at 10801 University Boulevard, Manassas, VA, 20110-2209, on August 14, 2008, under the provisions of the Budapest Treaty.

Tetanus toxoid strains useful in the vaccine or immunogenic composition of the present invention can be any strain or isolate. A representative strain is that taken from a master seed of Clostridium tetani from The Massachusetts Department of Public Health Institute of Laboratories in Boston, Massachusetts.

The vaccine of the present invention is safe for administration in WNV susceptible species, particularly equidae, at any age and at any stage of reproduction, including pregnant females. In a preferred embodiment, the present invention is safe for administration to foals 12 months of age or older, more preferably, it is safe for administration to foals 10 months of age or older, more preferably, it is safe for administration to foals 8 months or older, more preferably, it is safe for administration to foals 6 months of age or older, more preferably, is safe for

administration to foals 4 months of age or older, more preferably, it is safe for administration to foals 2 months of age or older, more preferably, it is safe for administration to foals 1 month of age or older, even more preferably, it is safe for administration to foals between 1 day and 1 month of age, and, most preferably, it is safe for administration to foals 1 day of age or older.

The composition of the present invention can be administered in any conventional manner. Examples of administration methods include any that afford access by cells of the immune system to the immunogenic composition including oral, transdermal/intradermal, intravenous, subcutaneous, intramuscular, intraocular, intraperitoneal, intrarectal, intravaginal, intranasal, intragastrical, intratracheal, intrapulmonarial, or any combination thereof. In a preferred embodiment, the vaccine is administered parenterally, preferably intranasally, subcutaneously, or intramuscularly, and in the most preferred embodiment the vaccine is administered intramuscularly.

Brief Description of the Drawings

Figure 1 is a graphical representation of the Mean Total Clinical Scores;
Fig. 2 is a graphical representation of the Proportion Shedding;
Fig. 3 is a graphical representation of the Nasal Discharge Score;
Fig. 4 is a graphical representation of Proportion Virus Shedding;
Fig. 5 is a graphical representation of Conjunctivitis Score;
Fig. 6 is a graphical representation of Serum Neutralization Titers;
Fig. 7 is a graphical representation of Proportion Positive for EHV-1;
Fig. 8is a graphical representation of Mean White Blood Cell Count;
Fig. 9 is a graphical representation of Proportion Positive (pyrexic);
Fig. 10 is a nucleotide alignment of the HE region of WNV isolates;
Fig. 11 is a nucleotide alignment of the DE region of WNV isolates;
Fig. 12 is a nucleotide alignment of the D NS5 region of WNV isolates;
Fig. 13 is a nucleotide alignment of the H NS5 region of WNV isolates;
Fig. 14 is a nucleotide alignment of the H WN05 E NS5 region of WNV isolates;
Fig. 15 is a nucleotide alignment of the H WN05 region of WNV isolates;
Fig. 16 is a nucleotide alignment of the NS5 region of WNV isolates; and

Fig. 17 is a nucleotide alignment of the E region of WNV isolates.

Detailed Description

Examples

The following examples are set forth below to illustrate specific embodiments of the present invention. These examples are merely illustrative and are understood not to limit the scope or the underlying principles of the present invention.

Example 1

This example illustrates a preferred vaccine composition in accordance with the present invention.

Materials and Methods

For preparation of working cell stock, the Master Cell Stock (MCS), consisting of the Vero Cell Line known to propagate West Nile Virus, which was tested for purity, identity, and karyology, was thawed and used to inoculate a range of T25 up to T150 cm^2 vessels or 1050 cm^2 roller bottles, or bioreactors or other suitable sterile vessels. Thawed cells were suspended in growth medium at a rate of 0.0015 mL to 5.0 L per vessel, depending on vessel volume. Cells were then incubated at 36-38°C for up to seven days. Cultures planted from frozen stock were re-fed with medium, if needed, within thirty-six hours after planting to remove residual DMSO. Cultures were re-fed with medium, if needed, during the growth period to remove excessive debris, or to stimulate the growth of cultures which have not reached confluence, or to maintain viability of confluent cultures.

Cells were passaged 1-20 times by decanting the spent medium and then by adding 5-500 mL of 0.25% Trypsin-EDTA Solution to each vessel, depending upon vessel volume. The vessels were agitated gently until the cells slough from the surface. The cells were then removed from the vessels by rinsing with growth medium and pooled together. Prior to inoculation, cell growth medium was decanted from Vero Working Cells that are at least 55% confluent. Virus growth medium described was added to each vessel at 0.15 to 0.4 mL per cm^2 surface area. A Multiplicity of Infection (MOI) of 0.000001- 0.0002 was used for infection as determined by

performing a cell count of at least two representative vessels. Roller bottle cultures infected were incubated at 36-38°C for two to five days at 0.1 - 0.8 rpm.

During the growth period, cultures were checked for typical CPE microscopically and for gross contamination macroscopically. Unsuitable cultures were discarded after sterilization. Cultures may be attenuated using standard techniques or may be used without attenuation.

The microorganisms were then harvested for production purposes. Virus fluids were harvested when CPE reached 85% or greater. Roller bottles were swirled to remove loose cells, and fluids and then pooled into sterile 2 – 20 L glass, plastic, or PETG bottles, 20 L sterile polypropylene containers or 2 - 500L sterile stainless steel tanks containers appropriate for clarification.

Next, the product was prepared. Clarified fluids were inactivated with Formaldehyde Solution, USP, 0.2% by volume, or another effective inactivating agent, transferred to a secondary container, and held at 20-25°C (room temperature) with agitation for forty-eight hours. A sample of at least 12 mL of the inactivated fluids was taken for inactivation assurance testing (described below) prior to concentration. After inactivation was completed, inactivated lot material was held at 2-7°C for up to sixty days prior to concentration. A number of suitable adjuvants may be added to the vaccine formulation, most preferably a non-metabolizable oil, preferably mineral oil, and/or a carbomer. Typical processing steps may be employed such as mixing, blending, microfluidization, and emulsification, of the adjuvant and/or the harvested virus antigens with other ingredients.

The product was then standardized. Sufficient volumes of clarified, inactivated, concentrated (optional) lots were combined to provide a calculated titer of at least $10^{4.0}$ $TCID_{50}$ per dose of each strain in the final product. Multiple lots may be blended to achieve the titer requirements per dose.

The product was then assembled to final formulation. Based on the desired final serial volume, the amounts of antigenic components, adjuvant, stabilizer and diluent were calculated as follows:

a. West Nile Virus, Horse Origin 2005 (ATCC No.PTA-9409): minimum $10^{4.0}$ $TCID_{50}$/dose

b. Adjuvant: The total adjuvant concentration, preferably a non metabolizable oil, and more preferably mineral oil and/or a carbomer, in a serial is at least 10% v/v and is added at time of serial batching/assembly.

c. Diluent: An appropriate volume of phosphate buffered saline (PBS) is added to bring the final volume to the desired volume.

d. Additional Formalin: An appropriate volume of 37% Formalin is added to maintain an appropriate level.

e. Gentamicin Sulfate

The required amounts of adjuvant and PBS were combined in a sterile vessel. The pH of this mixture was adjusted to approximately 4.9-5.1 with 10N NaOH or 5N HCl if necessary. Clarified, killed, concentrated West Nile Virus, as well as Gentamicin, and Formalin were added and the pH adjusted to 6.9 to 7.1. This was mixed at 2-6°C for at least 8 hours, not to exceed 48 hours.

The vaccine was given by typical hypodermic injection, with booster vaccinations if desired. Most preferably, the initial dose and the booster doses were 1mL volume administered intramuscularly at 21-day intervals. The vaccination regimen of initial and booster dose was given at the most preferred 1mL dose volume to horses, other equidae, and other WNV susceptible species to reduce the incidence of and or severity of clinical signs of WNV infection, and preferably to prevent infection by WNV as well as to prevent disease due to West Nile Virus infection for a sustained period following vaccination.

Results and Discussion

The vaccine was given by various appropriate parenteral routes, dose volumes, and dosing regimens to animals of varying immunological status for WNV, including naive and those with passive antibody, and provided for long duration of immunity up to and exceeding at least 2 years following vaccination. The vaccine was safe for administration in WNV susceptible species, particularly equidae, at any age and at any stage of reproduction, including pregnant females.

Example 2

This investigation was carried out to obtain an efficacy evaluation of a vaccine to protect horses from challenge with West Nile Virus (WNV).

Materials and Methods

A total of 30 horses were randomly divided into groups of 15 horses each. A total of 20 horses received 2 doses of vaccine at 21-day intervals and 10 horses were used for control. Each group of horses, Block 1 and Block 2, contained 10 vaccinated horses and 5 control horses. The vaccine was a combination including WNV antigen, specifically an inactivated or killed North American Dominant Strain of WNV, Horse Origin 2005 (ATCC Deposit No. PTA-9409) as well as antigenic components of Venezuelan Equine Encephalomyelitis, TC-83 strain (ATCC Deposit No. PTA-9411) Eastern Equine Encephalomyelitis, NJO strain (ATCC Deposit No.PTA-9412) Western Equine Encephalomyelitis, Fleming strain (ATCC Deposit No. PTA-9410) and Tetanus toxoid formulated approximately as follows:

Eastern Equine Encephalomyelitis	$10^{6.7}$-$10^{9.2}$ $TCID_{50}$/mL
Western Equine Encephalomyelitis	$10^{6.7}$-$10^{9.2}$ PFU/mL
Venezuelan Equine Encephalomyelitis	$10^{6.7}$-$10^{9.2}$ $TCID_{50}$/mL
West Nile Virus	$10^{7.0}$ -10^{9} $TCID_{50}$/mL
Tetanus Toxoid	5-10 CPU/mL
Adjuvant	100-200 µl/mL
Diluent – DMEM containing	q.s.
Gentamycin (30 µg/mL of diluent volume)	
Formaldehyde (0.1% of diluent volume)	

All groups were challenged with intrathecal inoculation of 1 ml PBS containing approximately10^5 pfu of a heterologous strain of WNV (NY99, 4132, crow isolate). The challenge was conducted under ketamine-xylazine anesthesia.

Horses were monitored for a maximum of 14 days, then humanely euthanized. Those that developed severe disease prior to 14 days were euthanized prematurely.

The following data were collected to assess the effectiveness of the vaccine:

- Basic clinical evaluation
- Body temperature

- Assay for viremia
- Histopathology: two sections of brainstem were evaluated by a board-certified veterinary pathologist.

Sera collected on appropriate days were evaluated for characterization of serologic responses to challenge.

Results and Discussion

Viremia after challenge and serum neutralization titers were considered the primary outcome variables in this study. The first block of horses that had been vaccinated were 100% protected from viremia after challenge in this study. In comparison, 4 of the 5 control horses demonstrated viremia for 4-5 days post-challenge and 1 of 5 control horses demonstrated viremia for 1 timepoint. In addition, serum neutralization titers of vaccinated horses were statistically significantly higher than those of control horses at each time point examined after vaccination. Furthermore, the data establish that a WNV vaccine that provides a serum neutralization titer of 1:4 or higher is effective in preventing WNV viremia. The serum titers and viremia after challenge for Block 1 is summarized in Table 1 below:

TABLE 1: Serum Titers and Viremia for Block 1

		Serum Titer	Viremia After Challenge
Horse Number	Treatment	Day of Challenge	Highest Titer
1	Control	<2	390
2	Vaccinate	12	<5
3	Vaccinate	12	<5
4	Control	<2	65
5	Control	<2	1475
6	Vaccinate	6	<5
7	Vaccinate	97	<5
8	Vaccinate	10	<5
9	Vaccinate	21	<5
10	Vaccinate	35	<5
11	Vaccinate	10	<5
12	Vaccinate	24	<5
13	Vaccinate	4	<5
14	Control	<2	235
15	Control	<2	165

Viremia after challenge and serum neutralization titers were also considered the primary outcome variables in the second block of horses in this study. In the second block of horses only one vaccinate group horse displayed any timepoints of viremia throughout the challenge period. That horse had 3 separate timepoints on 3 mornings (not those same evenings) with minimal value readings of 5 (where <5 is negative). All control horses in the study (with the exception of one horse which exited the study prematurely but displayed definitive WNV histopathology and was excluded from evaluation) showed high levels of viremia for 1-8 timepoints after challenge.

Since viremia is a prerequisite before virus can cross the blood-brain barrier to cause WNV encephalitis, viremia is well justified as the primary parameter for evaluation of protection in an experimental study of this type.

This study demonstrated that 2 doses of the experimental combination vaccine administered to foals 4 to 5 months of age reliably and effectively stimulated protective serological serum neutralization titers. In addition the data confirm that post vaccination SN titers as low as 1:4 resulting from vaccination using an effectively batched antigen amount of West Nile Virus in this experimental combination vaccine protected vaccinated horses from viremia, clinical disease, and histopathology after a severe intrathecal challenge with a heterologous strain of West Nile Virus.

Histopathology also was different between the two groups with the likelihood of lesions in vaccinates being 40% less in Block 2 and 100% less in Block 1 than the likelihood of lesions in control animals challenged with virulent West Nile virus.

In addition a Control Group horse became weak on his hind legs on Day 9 post-challenge and got progressively worse until he was no longer able to stand. Histopathology of the pons and medulla from this horse showed severe encephalitis and myelitis consistent with WNV pathology that was more prevalent than signs of disease from any other horse in this study.

Two Block 2 control horses in this study displayed 3 days each of clinical signs relating to infection with West Nile Virus. One other control horse had a single timepoint of weakness due to disease. Another control horse did not display any timepoints of clinical signs, although it had multiple days of viremia. Although several vaccinated Block 2 horses in the study had mild to moderate histopathological changes in tissue as a result of the intrathecal challenge of WNV, only very mild clinical disease (mild head tremors) was noted for one vaccinate on one day of

the study as compared to multiple days of clinical disease in 2 control horses and a single day of clinical disease in a third control horse.

The results demonstrated that the vaccine is effective and that an immunogenic reaction is induced in the animals that were administered the vaccine. The effectiveness of the vaccine was evidenced in this example by reduction in WNV viremia, by stimulation of high serum neutralization titers to WNV, and by prevention of WNV related clinical signs and histopathology in the brain and meninges. Because this vaccine is comprised of unique constituents, including a long lasting non-metabolizable adjuvant, it was formulated in a low 1mL dose volume to provide a high degree of safety as a highly immunogenic low passage whole inactivated virus WNV isolate of recent origin and high epidemiological prevalence, and a WNV isolated from the tissues of an infected horse, it provides more comprehensive safety and effectiveness than other vaccines currently available. Additionally, it has the effect of providing a safe vaccine when administered to animals.

Example 3

This example illustrates the efficacy of the immunogenic composition of the present invention against infection by EHV-4

Materials and Methods

Thirty-seven (37) horses, 4-5 months of age, were used in this study. Horses were randomly assigned to either vaccinate or control groups by random number generator and then vaccinated. Twenty-four (24) horses served as vaccinates and thirteen (13) horses were mock-vaccinated control horses. All horses had low (≤ 1:14, avg. =1:7) EHV-4 serum neutralization (SN) titers prior to initiation of the study, indicative of horses susceptible to infection. The vaccine used was an experimental vaccine and had the following components:

The final formulated vaccine contains the following ingredients per 1 mL dose:

EHV-1 (PTA-9525)	$10^{7.0-9.0}$ $TCID_{50}$/mL
Influenza A2/Ohio/03 (PTA-9522)	$10^{6.0-9.5}$ $TCID_{50}$/mL
Influenza A2/KY/95 (PTA-9523	$10^{6.0-9.5}$ $TCID_{50}$/mL
Influenza A2/NewMarket/2/93 (PTA-9524)	$10^{6.0-9.5}$ $TCID_{50}$/mL
Tetanus Toxoid	5-10 CPU

Eastern Equine Encephalomyelitis, (ATCC Deposit No. PTA-9412)$10^{6.7-9.2}$ $TCID_{50}$/mL

Western Equine Encephalomyelitis, (ATCC Deposit No. PTA-9410) PFU/mL	$10^{6.7-9.2}$
Venezuelan Equine Encephalomyelitis, (ATCC Deposit No. PTA-9411) $^{9.2}TCID_{50}$/mL	$10^{6.7-}$
West Nile Virus, Horse Origin 2005 (ATCC Deposit No. PTA-9409) $TCID_{50}$/mL	$10^{7.0-9.0}$
Adjuvant	100-200 µl
Glycerol	100-200 µl
EDTA 240 mM solution	10-20 µl
Diluent – DMEM containing Gentamicin (30 µg/mL of diluent volume) Formaldehyde (0.1-0.2% of diluent volume)	q.s.

The Experimental Vaccine was administered intramuscularly in a 1 mL dose volume to each of 24 horses in the vaccinate group. Thirteen horses in the control group received a 1 mL dose of adjuvanted DMEM (Lot 004) containing excipients used in the 9-way vaccine (Gentamicin and formaldehyde) but no antigens. Challenge inoculation of virulent EHV-4 HRA005 strain virus was performed 15 days post-booster vaccination.

Each of the vaccinated and control horses was challenged with an EHV-4 strain of virus (HRA005). The titer of the dilute challenge virus was sufficient to provoke disease due to EHV infection in the non-vaccinated horses.

Sedivet® (romifidine hydrochloride), a sedative and analgesic, was administered intravenously to each horse prior to challenge at a dosage of 50µg/kg of body weight. Each horse was then challenged with the EHV-4 strain HRA005 virus. The challenge virus was administered intranasally as an aerosol produced by a nebulizer into an Equine AeroMask (Trudell Medical International, Ontario, Canada).

Daily morning rectal temperatures were recorded for each of the 37 vaccinated and control horses on Day of Challenge and for 14 days post challenge by means of a calibrated, electronic thermometer (GSA Electronics) probe. The daily rectal temperatures were recorded in degrees Fahrenheit (°F).

COMPLETE BLOOD CELL COUNTS

Venous blood from each of the 37 vaccinated and control horses was collected daily on the Day of Challenge and for 14 days post-challenge directly into a Vacutainer Disodium EDTA tube for Complete Blood Counts.

NASAL EXUDATE EVALUATION

All nasal exudate observations were made prior to collection of nasopharyngeal swabs. On the Day of Challenge and for 14 days post challenge, the nasal passages and muzzle of each of the 37 vaccinated and control horses were examined and graded using the grading and scoring description listed below.

The scoring grades of 0 through 6 were assigned on the basis of the severity of the disease indicated by each of the following classifications:

TABLE 2: Scores for Clinical Symptoms

Score	Description of symptoms	Score sheet designation
0	Essentially normal indicates the horse was clean and essentially free of nasal exudate	EN
1	Slight clear serous discharge that may be frequently observed in both diseased and normal horses	C-1
2	Moderate clear serous discharge is indicative of a definite increase in volume over that normally observed	C-2
3	Copious clear serous discharge that is generally observed only in diseased horses	C-3
1.5	Very slight mucopurulent discharge indicates that mucus was definitely present in small amounts in either one or both nostrils	VSM
2	Slightly mucopurulent is a discharge easily observed in one or both nostrils	SM
4	Moderately mucopurulent indicates that mucoid discharges were present in large quantities in both nostrils	MM
6	Heavy mucopurulent indicates that copious amounts of a mucoid discharge filled both nostrils	HM

NASOPHARYNGEAL VIRAL ISOLATION METHODS

On each observation test day each nasal passage of each vaccinated and control was sampled deeply by means of sterile swabs. On collection, each of two swabs were immediately placed in a single tube containing 4 mL of chilled transport medium (Dulbecco's Minimal

Essential Medium (DMEM) supplemented with 2% FBS, 2X Pen/Strep, 2X Gentamicin, and 2X Amphotericin B).

For isolation of virus, the tubes were mixed, the swabs aseptically removed, and the medium centrifuged at 1500 rpm for 10 minutes to remove particulates. Medium was filtered through a 0.2μ syringe filter prior to inoculation on tissue culture cells. One mL of the clarified transport medium was used to inoculate a 2 cm^2 one day old monolayer of ED cells grown in a 24 well tissue culture plate from which the growth medium had been aseptically removed. Following inoculation, the inoculum was allowed to adsorb on the cell monolayer for one hour at 37° C in a humidified incubator containing a 5% CO_2 atmosphere. After the adsorption period, an additional 1 mL of re-feed medium (DMEM containing 2-5% fetal bovine serum (FBS), 2mM L-glutamine and 3X Gentamicin and 2X Amphotericin B) was added to each well. Following addition of re-feed media the plates were then incubated at 37° C in a CO_2 incubator. Each test and control tissue culture well was examined microscopically for 7 days for signs of cytopathic effect (CPE) typical of the EHV-4 challenge virus. Wells that were negative at the end of the 7 day observation period were subcultured onto fresh cells and observed for an additional 7 days.

SERUM NEUTRALIZATION TESTING PROCEDURE

A standard microtiter serum neutralization test was employed in this study. All sera were tested in sterile flat bottom microtiter plates using 5 wells per dilution and an 8 well dilution series for each of the 5 test wells. Each of the 5 test wells contained 25 μl of serum dilution mixed with 25 μl of the indicator virus and 150 μl of a freshly planted ED cell suspension containing approximately 5 X 10^4 cells. The test indicator virus used was EHV-4 HRA005 Lot 033106 SN Stock Virus. In all tests the indicator virus back titration titers ranged between 68-149 $TCID_{50}$/25 μl. Serum neutralizing antibody titers are expressed as Reed-Muench ID_{50} titers.

For performance of the test, two-fold dilutions of each test serum was made in a sterile flat bottom microtiter plate using five replicate wells per test serum and an 8 well dilution series. Dilutions were made with an adjustable volume single or multi-channel pipetting instrument using sterile microtiter tips. The volume of serum added each of 5 wells of the first row was 50 μl. All other wells contained 25 μl of DMEM (no FBS). Following serial dilution down the plate, 25 μl was discarded from the last row. 25 μl of a pre-determined dilution of the indicator virus was added to each test well. Plates were then mixed and incubated for one hour at 37°C in

5% CO_2. On conclusion of the incubation period, 150 µl of a suspension containing 5 x 10^4 ED cells was added to each test and cell control well. The plates were incubated at 37° C in a CO_2 incubator for 5-7 days, at which time plates were microscopically examined for CPE typical of EHV-4. However, any other commercial available test or any test described in the prior art could be used for this purpose.

Results and Conclusion

NASAL EXUDATE EVALUATION

The vaccination group by day interaction was statistically significant for the nasal discharge scores ($P < 0.05$, Table 1). Statistically significant group effects were seen on Days 6 – 10 and Day 14 post-challenge (lower nasal scores in the vaccinated group).

When the daily scores were summed over the post-challenge period, horses in the vaccinated group had lower total scores than those in the control group ($P < 0.05$, Table 1). The mitigated fraction was estimated to be 0.824 (95% ASE CI: 0.629, 1.000).

TABLE 3: Nasal Discharge Score

	Control	Vaccinate	P-value	Mitigated fraction (95% ASE CI)
Cumulative nasal discharge score	28.9	13.6	< 0.0001	0.824 (0.629, 1.000)

CONJUNCTIVITIS

The vaccination group by day interaction was statistically significant for the conjunctivitis scores. Statistically significant group effects were seen on Days 6, 7, 9, 10, 13 and 14 post-challenge (lower scores in the vaccinated group on 5 of the 6 days, $P < 0.05$, Figure 2).

SEROLOGICAL STUDIES

Titers were log transformed prior to the statistical analysis. The vaccination group by day interaction was statistically significant for SN titers. Statistically significant group effects were seen on Day 0 (pre-vaccination; control group titers > vaccinated group titers), Days 35 (the day of challenge) and 7 and 14 days post-challenge (study days 42 and 49). Horses in the vaccinated group had higher titers on Days 35, 42 and 49 than those in the control group ($P < 0.05$, Table 4).

TABLE 4: Titers

Study day	Control	Vaccinated	P-value
0	8.31	5.74	**0.0303**
21	8.25	6.51	0.1639
35 (day of challenge)	6.12	8.56	**0.0495**
42	4.57	7.27	**0.0069**
49	4.87	13.12	**< 0.0001**

WHITE BLOOD CELL COUNTS (WBC) AND LYMPHOCYTE COUNTS

The vaccination group by day interaction was statistically significant for WBC and lymphocyte counts. Statistically significant group effects were seen on Days 4 – 6 (WBC) and Days 4 and 5 (lymphocytes) post-challenge. Horses in the vaccinated group were protected from leucopenia due to EHV4 disease and had higher WBC and lymphocyte counts than those in the control group ($P < 0.05$).

DISCUSSION AND CONCLUSIONS

In this study, moderate and adequate clinical signs of EHV-4 infection were seen after challenge. Significantly fewer clinical signs of nasal exudate were seen in vaccinated horses on Days 6-10 and Day 14 post-challenge. Conjunctivitis scores were significantly lower in vaccinated horses on Days 7, 9, 10, 13, and 14 post-challenge. Despite the adequate display of clinical signs following challenge, virus shedding in nasal swab samples was infrequent following this EHV-4 challenge. Nasal swabs were examined by virus isolation in cell culture.

Significant group effects for WBCs and lymphocytes were seen on Days 4-6 (WBC) and Days 4-5 (lymphocytes) with vaccinated animals showing higher WBC and lymphocyte counts than control horses. These values establish that control horses did succumb to the immunosuppression brought on by infection with Herpesvirus, and also demonstrate that vaccination with a cross-protective strain of EHV-1 allowed vaccinated horses to be more refractive to the immunosuppressive properties of Herpesvirus infection. Additionally, horses in the vaccinated group had higher serum neutralization titers on Days 35, 42 and 49 than those in the control group

Data from this study confirm that horses vaccinated with a multi-component vaccine containing EHV-1 demonstrate cross-protective immunity when challenged with a heterologous EHV-4 challenge organism.

Example 4

This example is to illustrate the efficacy of the combination vaccine of the present invention as well as duration of immunity.

Materials and Methods

The influenza viral antigen used in the vaccine evaluated in this study was produced on Madin Darby Canine Kidney (MDCK) cells. Following growth, viral fluids were filtered, formalin inactivated, and concentrated. The inactivated viral fluids were tested for residual live virus after inactivation. On completion of satisfactory residual live virus testing the inactivated viral fluids were then used to formulate a vaccine which also contained inactivated Venezuelan, TC-83 strain (ATCC Accession No. PTA-9411), Eastern, NJO strain (ATCC Accession No.PTA-9412), and Western, Fleming strain (ATCC Accession No.PTA-9410), equine encephalomyelitis viruses, inactivated EHV-1 (ATCC Accession No. PTA-9525), inactivated influenza A/equine-2/Kentucky/95 (ATCC Accession No. PTA-9523) and influenza A/equine-2/NewMarket/2/93 (ATCC Accession No. PTA-9524) viruses, inactivated West Nile Virus, Horse Origin 2005 (ATCC Accession No. PTA-9409), and tetanus toxoid.

Vaccine was formulated to appropriate specifications for all antigens included in the product. Influenza A/equi-2/Ohio/03 (ATCC Accession No. PTA-9522) antigen was added to the vaccine at a pre-inactivation titer of $10^{6.7}$ $TCID_{50}$/mL.

The final formulated vaccine contains the following ingredients per 1 mL dose:

EHV-1	$10^{7.0-9.0}$ $TCID_{50}$/mL
Influenza A2/Ohio/03	$10^{6.7-9.5}$ $TCID_{50}$/mL
Influenza A2/KY/95	$10^{6.7-9.5}$ $TCID_{50}$/mL
Influenza A2/NewMarket/2/93	$10^{6.7-9.5}$ $TCID_{50}$/mL
Tetanus Toxoid	5-10 CPU
Eastern Equine Encephalomyelitis	$10^{6.7-9.2}$ $TCID_{50}$/mL
Western Equine Encephalomyelitis	$10^{6.7-9.2}$ PFU/mL
Venezuelan Equine Encephalomyelitis	$10^{6.7-9.2}$ $TCID_{50}$/mL
West Nile Virus	$10^{7.0--9.0}$ $TCID_{50}$/mL
Adjuvant (preferably mineral oil)	100-200 µl
Glycerol	100-200 µl

EDTA 240 mM solution	10-20 µl
Diluent – DMEM containing	q.s.
Gentamicin (30 µg/mL of diluent volume)	
Formaldehyde (0.1-0.2% of diluent volume)	

Twenty-six (26) horses, 4-5 months of age, were used in this study. Fifteen horses served as vaccinates and eleven horses were mock-vaccinated control horses.

Vaccine was administered intramuscularly in a 1 mL dose volume to each of 15 horses in the vaccinate group. Eleven horses in the control group received a 1 mL dose of adjuvanted DMEM (Lot 004) containing excipients used in the 9-way vaccine (Gentamicin and formaldehyde) but no antigens. Challenge inoculation of virulent influenza A/equi-2/Ohio/03 strain virus was performed 4 months post-booster vaccination.

Serum samples for serological evaluation were collected from the vaccinated and control horses prior to initial vaccination, at 21 days post first dose vaccination (day of booster vaccination), on the day of challenge, and at 7 and 14 days post challenge. Body temperature, whole blood samples, and nasal swabs were obtained from each horse on the day of challenge, and daily throughout the 10 day post-challenge observation period for a total of 11 observation days. Clinical data was also recorded daily for each horse for the 11-day observation period.

Challenge

The challenge virus seed of Influenza A/equi-2/Ohio/03 was produced in eggs. Challenge virus was diluted on the morning of challenge 1:20 with tissue culture media to affect a titer sufficient to cause clinical influenza in the non-vaccinated challenged horses.

Sedivet® (romifidine hydrochloride), a sedative and analgesic, was administered intravenously to each horse prior to challenge at a dosage of 50µg/kg of body weight. Each horse was then challenged with influenza A/equi-2/Ohio/03 virus. The challenge virus was administered intranasally as an aerosol produced by a nebulizer into an Equine AeroMask (Trudell Medical International, Ontario, Canada) by the following method:

Four milliliters of challenge virus were placed into the nebulizer cup in the AeroMask device. A pressure hose was fitted from an air compressor to the inlet port of the nebulizer. The outlet tube was then inserted into the AeroMask attached to the head of the horse being challenged and air pressure was applied to the inlet port. During this time approximately two

milliliters of challenge virus fluid was aerosolized directly into the nostrils of the horse being challenged.

TEMPERATURE

Daily rectal temperatures were recorded for each of the 26 vaccinated and control horses on Day of Challenge and for 10 days post challenge by means of a calibrated, electronic thermometer (GSA Electronics) probe. The daily rectal temperatures were recorded in degrees Fahrenheit (°F).

WHITE BLOOD CELL COUNTS

Venous blood from each of the 26 vaccinated and control horses was collected daily on the Day of Challenge and for 10 days post-challenge directly into a vacutainer Disodium EDTA tube for WBC counts.

NASAL EXUDATE EVALUATION

All nasal exudate observations were made prior to collection of nasopharyngeal swabs. On the Day of Challenge and for 10 days post challenge, the nasal passages and muzzle of each of the 26 vaccinated and control horses were examined and graded using the grading and scoring description listed below.

The scoring grades of 0 through 6 were assigned on the basis of the severity of the disease indicated by each of the following classification:

Table 5: Scoring Grades

Score	Description of symptoms	Score sheet designation
0	Essentially normal indicates the horse was clean and essentially free of nasal exudate	EN
1	Slight clear serous discharge that may be frequently observed in both diseased and normal horses	C-1
2	Moderate clear serous discharge is indicative of a definite increase in volume over that normally observed	C-2
3	Copious clear serous discharge that is generally observed only in diseased horses	C-3
1.5	Very slight mucopurulent discharge indicates that mucus was definitely present in small amounts in either one or both nostrils	VSM
2	Slightly mucopurulent is a discharge easily observed in one	SM

Score	Description of symptoms	Score sheet designation
	or both nostrils	
4	Moderately mucopurulent indicates that mucoid discharges were present in large quantities in both nostrils	MM
6	Heavy mucopurulent indicates that copious amounts of a mucoid discharge filled both nostrils	HM

COUGHING

Episodes of coughing on each observation day were counted for each horse during the entirety of the observation period, whether or not the individual animal was being examined by the investigator at that time. Observers other than the investigator recorded the number of episodes of coughing of each individual horse during the observation period. Scoring of coughing episodes was actual counts of coughing episodes per horse.

CONJUNCTIVITIS

Conjunctivitis was evaluated daily at the time of nasal exudate evaluation. Conjunctivitis scores were recorded as 0 = normal; 1= mild to moderate conjunctivitis and 2 = severe conjunctivitis.

NASOPHARYNGEAL VIRAL ISOLATION/ HEMAGGLUTINATION (HA) METHODS

On each observation test day each nasal passage of each vaccinated and control was sampled deeply by means of sterile swabs. On collection, each of two swabs was immediately placed in a single tube containing 4 mL of chilled transport medium (Dulbecco's Minimal Essential Medium (DMEM) supplemented with 2% FBS, 2X Pen/Strep, 2X Amphotericin B).

For isolation of virus, the tubes were mixed, the swabs aseptically removed, and the medium centrifuged at 1500 rpm for 10 to 15 minutes to remove particulates. Medium was filtered through a 0.2μ syringe filter prior to inoculation on tissue culture cells. After filtration, 4-6% of sterile 85% sucrose solution was added to each sample for freezing at -80°C in order for all samples to be tested concurrently.

All samples were tested in sterile flat bottom microtiter plates using five wells per dilution and a 4 well dilution series for each of the 5 test wells. Upon thawing, 22 μL of the clarified sample medium was used to inoculate one day old monolayer of MDCK-S cells from which the growth medium had been aseptically removed and replaced with 200 μl of influenza

growth medium (DMEM containing 5-10 units/mL of 10,000U stock solution Porcine Trypsin, 2mM L-glutamine, 1X Pen-Strep and 1X Amphotericin B). The plates were then incubated at 35° C in a CO_2 incubator for 5-7 days. After the 5-7 day incubation period, 50µl from all wells of the titration plates were transferred directly into a labeled 96 well vinyl HA plate. Chicken red blood cells were added to each well and allowed to settle for 30-90 minutes at room temperature. Wells were read for positive agglutination as evidence of presence of equine influenza virus.

HEMAGGLUTINATION INHIBITION (HI) TESTING PROCEDURE

Serum samples were prepared by dispensing 0.15 ml of each sample into a test tube and extracting with 0.3 mL of 0.01M Sodium Periodate Solution at room temperature for 15 minutes. Glycerol Solution 3% (0.125 mL) was added to each tube, mixed and incubated at room temperature for 15 minutes. All samples were then heat-inactivated at 56°C for 30 minutes.

A 0.5% solution of chicken red blood cells was prepared in PBS (SAFC catalog number 59321C) and standardized to an optical density of 0.5 at 550 nm.

Extracted serum samples were tested in duplicate in U bottom polystyrene plates using a 2-fold dilution scheme in PBS ranging from 1:4 to 1:256, 25ul per well. Influenza A/Equi2/Ohio03 stock virus (25µL) was added to serum sample dilution. Plates were gently tapped to mix, and incubated at room temperature for 30 minutes. After incubation, chicken red blood cells were added to each well and incubated undisturbed at room temperature for 1 to 1.5 hours. Results were read by observing plates for presence or absence of agglutinated red blood cells in each well. Antibody titer was determined as the highest dilution of serum at which agglutination did not occur.

Results and Conclusions

When pooled across all timepoints post-challenge, vaccinated animals had lower total clinical scores than the control animals. When the total daily scores were summed over the post-challenge period, horses in the vaccinated group had lower total scores than those in the control group ($P < 0.05$). The mitigated fraction was estimated to be 0.6485 (95% ASE CI: 0.3258, 0.9712).

TABLE 6: Total Clinical Score

Outcome variable	Vaccination group	Day	Group by day interaction
Total clinical score[1]	**< 0.0001**	<0.0001	0.1321

[1] The GLIMMIX procedure would not converge, thus an ANOVA approach was used to evaluate the effect of vaccination over time after challenge. Results were interpreted through the bolded values.

TABLE 7:Mitigated fraction – total cumulative clinical score

	Control	Vaccinate	P-value[1]	Mitigated fraction[2] (95% ASE CI)
Total cumulative clinical score[2]	18.36[3]	9.93	0.0055	0.6485 (0.3258, 0.9712)

[1] P-value from Wilcoxon's rank sum test

[2] Nasal discharge score, conjunctivitis score and coughing score were summed with day and across all time points for each animal then ranked for the estimation of the mitigated fraction.

[3] Mean rank

NASAL DISCHARGE

The main effect of vaccination was statistically significant and reduced nasal discharge due to the influenza challenge. When pooled across all time points post-challenge, vaccinated animals had lower nasal discharge scores than the control animals.

TABLE 8: Nasal Discharge Score

Outcome variable	Vaccination group	Day	Group by day interaction
Nasal discharge score[1]	**0.0012**	<0.0001	0.4627

[1] The GLIMMIX procedure would not converge, thus an ANOVA approach was used to evaluate the effect of vaccination over time after challenge. Results were interpreted through the bolded values.

CONJUNCTIVITIS

For conjunctivitis, the main effect of vaccination was statistically significant. When pooled across all time points post-challenge, vaccinated animals had reduced conjunctivitis due to influenza infection as demonstrated by lower conjunctivitis scores than the control animals.

TABLE 9: Conjunctivitis Score

Outcome variable	Vaccination group	Day	Group by day interaction
Conjunctivitis score[1]	**0.0187**	0.0001	0.2498

[1] The GLIMMIX procedure would not converge, thus an ANOVA approach was used to evaluate the effect of vaccination over time after challenge. Results were interpreted through the bolded values.

COUGHING

Vaccine also protected against the cough resulting from equine influenza infection. Vaccinated animals had lower scores ($P < 0.05$,) on Days 3, 5, 7, 8, and 9 post-challenge than control animals.

TABLE 10: Coughing Score

Outcome variable	Vaccination group	Day	Group by day interaction
Coughing score[1]	0.0004	0.0009	**0.0275**

[1] The GLIMMIX procedure would not converge, thus an ANOVA approach was used to evaluate the effect of vaccination over time after challenge. Results were interpreted through the bolded values.

VIRUS SHEDDING (NASAL SWABS)

The vaccination also reduced the percent of horses shedding virus ($P < 0.05$). The figure below represents that the percentage of vaccinated animals shedding virus was lower ($P < 0.05$) on Days 3, 4, and 5 post-challenge than control animals.

TABLE 11: Mitigated fraction – days virus shedding

	Control	Vaccinate	P-value[1]	Mitigated fraction[2] (95% ASE CI)
Days virus positive[2,3]	2[3]	0	0.0004	0.7939 (0.5343, 1.0000)

[1] P-value from Wilcoxon's rank sum test

[2] The number of days of viral shedding was calculated then ranked for the estimation of the mitigated fraction. Asymptotic standard errors (ASE) were used to estimate the 95% confidence intervals (CI).

[3] The median number of days positive results was obtained from the virus isolation assay.

HI TITERS

The vaccine was also effective in eliciting protective antibody titers to equine influenza virus. Statistically significant higher titers in the vaccinated horses were seen on Day 36 (relative to vaccination), Day 154 (the day of challenge), 159 and 164. Horses in the vaccinated group had higher titers on each of these days than those in the control group ($P < 0.05$).

WBC and LYMPHOCYTE COUNTS

The vaccination also protected horses from reduction in white blood cell counts seen following influenza virus challenge. ($P < 0.05$). Vaccination with the combination vaccine provided statistically significant protection that was seen on Days 2 and 7 for WBC counts, and Days 2, 6, 7, and 8 post-challenge. Horses in the vaccinated group had higher WBC and lymphocyte counts than those in the control group ($P < 0.05$). A four month Duration of Immunity challenge was performed to demonstrate efficacy of the influenza virus fractions of a multi-component vaccine that included West Nile Virus vaccine (Encephalomyelitis-Rhinopneumonitis-Influenza-West Nile Virus Vaccine, Eastern, Western & Venezuelan, Killed Virus, Tetanus Toxoid) containing 3 Equine influenza A/equi-2 virus strains, ATCC Accession Nos. PTA-9522, PTA-9523, and PTA-9524, each of which is currently relevant in the equine population of the Americas, Europe and Asia. Twenty-six horses (15 vaccinates and 11 controls) were vaccinated twice in 3 week intervals with a 1 mL dose of vaccine, or were mock vaccinated with adjuvanted media components of the vaccine without viral antigen. Four months post-booster vaccination, horses were challenged with a virulent live Equine Influenza A/equi-2/Ohio03 virus. This virulent virus is the current Equine Influenza A/equi-2 strain recommended for inclusion into vaccines by OIE and is currently recognized as the most pertinent strain involved in outbreaks in the United States.

Results from this 4-month DOI challenge study show significant protective effects from challenge by vaccination with the test vaccine, a combination West Nile Virus vaccine with flu

and other pertinent equine antigens. Importantly, vaccinated horses displayed statistically lower total clinical signs of influenza virus (nasal discharge, conjunctivitis, and coughing, P=0.0055) with a mitigated fraction estimated to be 0.6485 (95% ASE CI: 0.3258, 0.9712). Additionally, viral shedding was statistically lower in vaccinated horses than control horses (P =.0004) with a mitigated fraction estimated to be 0.7939 (95% ASE CI: 0.5343, 1.0000). Hemagglutination inhibition titers were significantly higher in vaccinated horses than control horses, and white blood cell and lymphocyte counts remained significantly higher in vaccinated horses on multiple days of the study over those of control horses. No differences in rectal temperature were determined between the two groups.

In conclusion, the data from this study demonstrate that administration of 2 x 1 mL intramuscular doses of this West Nile Virus combination vaccine administered at a 21 day interval to foals 4 to 5 months of age protected against virulent challenge with the Equine Influenza A/equi-2/Ohio03 virus and provided a duration of immunity of at least 4 months for this product.

Example 5

This example illustrates the efficacy of an immunogenic composition of the present invention when challenged with (Equine Herpes Virus Type 1) EHV-1.

Materials and Methods

The EHV-1 viral antigen used in the vaccine evaluated in this study was produced on Madin Darby Bovine Kidney (MDBK) cells. Following growth, viral fluids were filtered, BPL inactivated, and concentrated. The inactivated viral fluids were tested for residual live virus after inactivation. On completion of satisfactory residual live virus testing, the inactivated viral fluids were then used to formulate a vaccine which also contained inactivated Venezuelan Equine Encephalomyelitis, TC-83 strain (ATCC Accession No. PTA-9411) Eastern Equine Encephalomyelitis, NJO strain (ATCC Accession No.PTA-9412) and Western Equine Encephalomyelitis, Fleming strain (ATCC Accession No.PTA-9410) viruses, inactivated influenza A/equine-2/Kentucky/95 (ATCC Accession No. PTA-9523), influenza A/equine-2/NewMarket/2/93 (ATCC Accession No. PTA-9524) and influenza A/equine-2/Ohio/03 (ATCC Accession No. PTA-9522) viruses, inactivated West Nile Virus (ATCC Accession No. PTA-9409) and tetanus toxoid.

Vaccine was formulated to minimum specifications for all antigens included in the product. EHV-1 antigen was added to the vaccine at a pre-inactivation titer of $10^{7.0}$ $TCID_{50}$/mL.

The final formulated vaccine contains the following ingredients per 1 mL dose:

EHV-1	$10^{7.0-9.0}$ $TCID_{50}$/mL
Influenza A2/Ohio/03	$10^{6.7-9.5}$ $TCID_{50}$/mL
Influenza A2/KY/95	$10^{6.7-9.5}$ $TCID_{50}$/mL
Influenza A2/NewMarket/2/93	$10^{6.7-9.5}$ $TCID_{50}$/mL
Tetanus Toxoid	5-10 CPU
Eastern Equine Encephalomyelitis	$10^{6.7-9.2}$ $TCID_{50}$/mL
Western Equine Encephalomyelitis	$10^{6.7-9.2}$ PFU/mL
Venezuelan Equine Encephalomyelitis	$10^{6.7-9.2}$ $TCID_{50}$/mL
West Nile Virus	$10^{7.0--9.0}$ $TCID_{50}$/mL
Adjuvant (preferably mineral oil)	100-200 µl
Glycerol	100-200 µl
EDTA 240 mM solution	10-20 µl
Diluent – DMEM containing	q.s.
Gentamicin (30 µg/mL of diluent volume)	
Formaldehyde (0.1-0.2% of diluent volume)	

Forty (40) horses, 4-5 months of age, were used in this study. Horses were randomly assigned to either vaccinate or control groups and were micro-chipped and then vaccinated. Twenty horses served as vaccinates and twenty horses were mock-vaccinated control horses. All horses had negative to low (< 1:6) EHV-1 serum neutralization (SN) titers prior to initiation of the study, indicative of horses susceptible to infection.

Vaccine was administered intramuscularly in a 1 mL dose volume to each of 20 horses in the vaccinate group. Twenty horses in the control group received a 1 mL dose of adjuvanted DMEM (Lot 004) containing excipients used in the 9-way vaccine (Gentamycin and formaldehyde) but no antigens. Challenge inoculation of virulent EHV-1 A183 strain virus was performed 15 days post-booster vaccination.

Serum samples for serological evaluation were collected from the vaccinated and control horses prior to initial vaccination, at 21 days post first dose vaccination (day of booster vaccination), on the day of challenge, and at 7 and 14 days post challenge. Body temperature,

whole blood samples, and nasal swabs were obtained from each horse on the day of challenge, and daily throughout the 14 day post-challenge observation period for a total of 15 observation days. Clinical data was also recorded daily for each horse for the 15-day observation period.

CHALLENGE PROCEDURE

CHALLENGE VIRUS

The original challenge virus seed used in this challenge study was the first passage of the original seed virus on Equine Dermal (ED) cells. This challenge virus was harvested and frozen at a titer of $10^{6.2}$ $TCID_{50}$/mL.

INTRANASAL CHALLENGE METHOD

Sedivet® (romifidine hydrochloride), a sedative and analgesic, was administered intravenously to each horse prior to challenge at a dosage of 50μg/kg of body weight. Each horse was then challenged with approximately $10^{6.5}$ $TCID_{50}$ of EHV-1 strain. The challenge virus was administered intranasally as an aerosol produced by a nebulizer into an Equine AeroMask (Trudell Medical International, Ontario, Canada) by the following method:

A pressure hose was fitted from an air compressor to the inlet port of the nebulizer. The outlet tube was then inserted into the AeroMask attached to the head of the horse being challenged and approximately 10 psi of air pressure was applied to the inlet port for four minutes. During this time approximately two milliliters of a $10^{6.2}$ $TCID_{50}$/mL challenge virus fluid was aerosolized directly into the nostrils of the horse being challenged.

PRE AND POST CHALLENGE EVALUATION PARAMETERS

TEMPERATURE

Daily morning rectal temperatures were recorded for each of the 40 vaccinated and control horses on Day of Challenge and for 14 days post challenge by means of a calibrated, electronic thermometer (GSA Electronics) probe. The daily rectal temperatures were recorded in degrees Fahrenheit (°F).

WHITE BLOOD CELL COUNTS

Venous blood from each of the 40 vaccinated and control horses was collected daily on the Day of Challenge and for 14 days post-challenge directly into a vacutainer Disodium EDTA tube for WBC counts.

NASAL EXUDATE EVALUATION

All nasal exudate observations were made prior to collection of nasopharyngeal swabs. On the Day of Challenge and for 14 days post challenge, the nasal passages and muzzle of each of the 40 vaccinated and control horses were examined and graded using the grading and scoring description listed below.

The scoring grades of 0 through 6 were assigned on the basis of the severity of the disease indicated by each of the following classification:

(EN) Essentially normal indicates the horse was clean and essentially free of nasal exudate, score, 0;

(C-1) Slight clear serous discharge that may be frequently observed in both diseased and normal horses, score 1;

(C-2) Moderate clear serous discharge is indicative of a definite increase in volume over that normally observed, score 2;

(C-3) Copious clear serous discharge that is generally observed only in diseased horses, score 3;

(VSM) Very slight mucopurulent discharge indicates that mucus was definitely present in small amounts in either one or both nostrils, score 1.5;

(SM) Slightly mucopurulent is a discharge easily observed in one or both nostrils, score 2;

(MM) Moderately mucopurulent indicates that mucoid discharges were present in large quantities in both nostrils, score 4; and

(HM) Heavy mucopurulent indicates that copious amounts of a mucoid discharge filled both nostrils, score 6.

NASOPHARYNGEAL VIRAL ISOLATION METHODS

On each observation test day each nasal passage of each vaccinated and control was sampled deeply by means of a sterile swabs. On collection, each of two swabs were immediately placed in a single tube containing 4 mL of chilled transport medium (Dulbecco's Minimal Essential Medium (DMEM) supplemented with 2% FBS, 2X Pen/Strep, 2X Gentamicin, and 2X Amphotericin B).

For isolation of virus, the tubes were mixed, the swabs aseptically removed, and the medium centrifuged at 1500 rpm for 10 minutes to remove particulates. Medium was filtered through a 0.2μ syringe filter prior to inoculation on tissue culture cells. One mL of the clarified transport medium was used to inoculate a 2 cm^2 one day old monolayer of ED cells grown in a 24 well tissue culture plate from which the growth medium had been aseptically removed. Following inoculation, the inoculum was allowed to adsorb on the cell monolayer for one hour at 37° C in a humidified incubator containing a 5% CO_2 atmosphere. After the absorption period, an additional 1 mL of re-feed medium (DMEM containing 2-5% fetal bovine serum (FBS), 2mM L-glutamine and 3X Gentamicin and 2X Amphotericin B) was added to each well. Following addition of re-feed media the plates were then incubated at 37° C in a CO_2 incubator. Each test and control tissue culture well was examined microscopically for 7 days for signs of cytopathic effect (CPE) typical of the EHV-1 A183 challenge virus. Wells that were negative at the end of the 7 day observation period were subcultured onto fresh cells and observed for an additional 7 days.

WBC BUFFY COAT VIRUS ISOLATION

Venous blood from each of the 40 vaccinated and control horses was collected on the Day of Challenge and daily for 14 days post-challenge by vacutainer into a Disodium EDTA tube. After permitting gravity sedimentation of the erythrocytes in the tube of EDTA anti-coagulated blood, the plasma and white blood cells were pipetted off and placed in a sterile 5 mL snap-cap tube. The plasma and white blood cell mixture was centrifuged at 1500 RPM for 10-15 minutes to pellet the white blood cells. The pellet was washed twice with 3 mL of phosphate buffered saline (PBS) containing 2X Pen/Strep, 2X Gentamicin, and 2X Amphotericin B. Cells were then suspended in 4 mL of DMEM supplemented with 2% fetal bovine serum (FBS) and 2X Pen/Strep, 2X Gentamicin, and 2X Amphotericin B. One mL buffy coat suspension was used to inoculate a 2 cm^2 one day old monolayer of ED cells grown in a 24 well tissue culture plate from which the growth medium had been aseptically removed. Following inoculation, the inoculum was allowed to adsorb on the cell monolayer for one hour at 37° C in a humidified incubator containing a 5% CO_2 atmosphere. After the adsorption period, an additional 1 mL of re-feed medium (DMEM containing 5-7% fetal bovine serum (FBS), 2mM L-glutamine and 1X Gentamicin was added to each well. Following addition of re-feed media the plates were then

incubated at 37° C in a CO_2 incubator. Wells could not be observed microscopically due to large volume of white blood cells settled on the monolayer. Therefore, at the end of 7 days, all wells were subcultured onto fresh ED cells using 0.5 ml of the 1st passage as inoculum. The subculture was observed for 7 days for CPE typical of challenge virus infection.

SERUM NEUTRALIZATION TESTING PROCEDURE

A standard microtiter serum neutralization test was employed in this study. All sera were tested in sterile flat bottom microtiter plates using five wells per dilution and an 8 well dilution series for each of the 5 test wells. Each of the 5 test wells contained 25 µl of serum dilution mixed with 25 µl of the indicator virus and 150 µl of a freshly planted ED cell suspension containing approximately 5 X 10^4 cells. The test indicator virus used was EHV-1 subtype 1 strain A183. In all tests the indicator virus back titration titers ranged between 109 to 263 $TCID_{50}$/25 µl. Serum neutralizing antibody titers are expressed as Reed-Muench ID_{50} titers.

For performance of the test, two-fold dilutions of each test serum was made in a sterile flat bottom microtiter plate using five replicate wells per test serum and an 8 well dilution series. Dilutions were made with an adjustable volume single or multi-channel pipetting instrument using sterile microtiter tips. The volume of serum added each of 5 wells of the first row was 50 µl. All other wells contained 25 µl of DMEM (no FBS). Following serial dilution down the plate, 25 µl was discarded from the last row. 25 µl of a pre-determined dilution of the indicator virus was added to each test well. Plates were then mixed and incubated for one hour at 37°C in 5% CO_2. On conclusion of the incubation period, 150 µl of a suspension containing 5 x 10^4 ED cells was added to each test and cell control well. The plates were incubated at 37° C in a CO_2 incubator for 3 days, at which time plates were microscopically examined for CPE typical of EHV-1. Alternatively, any conventional or commercial available assay can be used or those of skill in the art would be able to follow the guidance herein.

Results and Conclusion

Nasal discharge scores, nasal shedding of EHV-1 and conjunctivitis scores were considered the primary outcome variables. All other outcomes were considered secondary.

TABLE 12: Summary of the statistical analysis (P-values)

Outcome variable	Vaccination group	Day	Group by day interaction
Nasal discharge score[1]	0.0001	<.0001	**<0.0001**
Virus shedding[1]	**0.0028**	<0.0001	0.0863
Conjunctivitis score[1]	0.0020	<0.0001	**0.0017**
SN Titers	< 0.0001	< 0.0001	**< 0.0001**
WBC	0.3064	< 0.0001	**< 0.0001**

[1] The GLIMMIX procedure would not converge, thus an ANOVA approach was used to evaluate the effect of vaccination over time after challenge. Results were interpreted through the bolded values.

NASAL EXUDATE EVALUATION

The vaccination group by day interaction was statistically significant for the nasal discharge scores ($P < 0.05$). Statistically significant group effects were seen on Days 4, 5 and on Days 7 – 11 post-challenge (lower nasal scores in the vaccinated group, $P < 0.05$,). When the daily scores were summed over the post-challenge period, horses in the vaccinated group had lower total scores than those in the control group ($P < 0.05$). The mitigated fraction was estimated to be 0.7250 (95% ASE CI: 0.4886, 0.9614).

TABLE 13: Mitigated fraction – nasal discharge and conjunctivitis scores, nasal virus shedding (mean ranks)

	Control	Vaccinate	P-value[1]	Mitigated fraction[2] (95% ASE CI)
Nasal discharge	27.75	13.25	< 0.0001	0.7250 (0.4886, 0.9614)
Days shedding virus[2]	24.43	15.78	0.0068	0.4925 (0.1896, 0.7954)
Conjunctivitis	25.80	15.20	0.0038	0.5300 (0.2463, 0.8137)

[1] P-value from Wilcoxon's rank sum test

[2] Nasal discharge and conjunctivitis scores were summed across all time points then ranked for the estimation of the mitigated fraction. The number of days of viral shedding was calculated then ranked for the estimation of the mitigated fraction. Asymptotic standard errors (ASE) were used to estimate the 95% confidence intervals (CI).

TABLE 14: Mean nasal discharge score (N = 20 horses per group)

Days post-challenge	Control	Vaccinated	P-value[1]
0	0.00	0.00	1.0000

Days post-challenge	Control	Vaccinated	P-value[1]
1	0.00	0.00	1.0000
2	0.15	0.00	0.6029
3	0.33	0.48	0.6029
4	1.08	0.23	**0.0033**
5	1.43	0.40	**0.0004**
6	1.05	0.55	0.0833
7	1.50	0.68	**0.0044**
8	1.68	0.63	**0.0003**
9	2.13	0.50	**<.0001**
10	1.58	0.80	**0.0074**
11	0.98	0.23	**0.0095**
12	0.90	0.35	0.0568
13	1.23	0.90	0.2599
14	0.93	0.43	0.0833

[1] The GLIMMIX procedure would not converge, thus an ANOVA approach was used to evaluate the effect of vaccination over time on the nasal discharge score.

CONJUNCTIVITIS

The vaccination group, by day interaction, was statistically significant for the conjunctivitis scores ($P < 0.05$). Statistically significant group effects were seen on Days 5 and 6, and on Days 9 – 14 post-challenge (lower scores in the vaccinated group, $P < 0.05$). When the daily scores were summed over the post-challenge period, horses in the vaccinated group had lower total scores than those in the control group ($P < 0.05$). The mitigated fraction was estimated to be 0.5300 (95% ASE CI: 0.2463, 0.8137).

TABLE 15: Mean conjunctivitis score (N = 20 horses per group)

Days post-challenge	Control	Vaccinated	P-value[1]
0	0.00	0.00	1.0000
1	0.00	0.00	1.0000
2	0.00	0.00	1.0000
3	0.05	0.00	0.7321
4	0.15	0.15	1.0000
5	0.70	0.25	**0.0022**
6	0.85	0.25	**<.0001**
7	0.75	0.65	0.4936
8	0.45	0.35	0.4936
9	0.50	0.15	**0.0168**
10	0.45	0.15	**0.0403**

Days post-challenge	Control	Vaccinated	P-value[1]
11	0.50	0.05	**0.0022**
12	0.45	0.00	**0.0022**
13	0.45	0.05	**0.0063**
14	0.35	0.05	**0.0403**

[1] The GLIMMIX procedure would not converge, thus an ANOVA approach was used to evaluate the effect of vaccination over time.

VIRUS ISOLATION FROM NASOPHARYNGEAL SWABS

The main effect of vaccination group was statistically significant (fewer animals shedding in the vaccinated group, $P < 0.05$). When the number of days shedding was evaluated, horses in the vaccinated group had fewer days of virus shedding than those in the control group ($P < 0.05$, Table 2). The mitigated fraction was estimated to be 0.4925 (95% ASE CI: 0.1896, 0.7954).

TABLE 16: Proportion virus shedding (nasal swab, N = 20 horses per group)

Days post-challenge	Control	Vaccinated
0	0.00	0.00
1	0.00	0.00
2	0.05	0.05
3	0.05	0.10
4	0.20	0.05
5	0.25	0.15
6	0.45	0.25
7	0.45	0.35
8	0.50	0.10
9	0.45	0.15
10	0.25	0.00
11	0.35	0.10
12	0.30	0.10
13	0.15	0.00
14	0.05	0.00

WHITE BLOOD CELL COUNTS

The vaccination group by day interaction was statistically significant for WBC counts ($P < 0.05$, Table 1). Statistically significant group effects were seen on Days 2 and 3 post-challenge. Horses in the vaccinated group had higher WBC counts than those in the control

group, indicating the vaccine prevented the horses from suffering the leucopenia caused by infection with EHV 1 ($P < 0.05$).

TABLE 17: Mean WBC counts (N = 20 horses per group)

Days post-challenge	Control	Vaccinated	P-value[1]
1	14.0413	14.4887	0.6081
2	10.7963	14.1287	**0.0001**
3	11.1263	14.0687	**0.0008**
4	11.5013	13.1037	0.0667
5	10.7413	11.1987	0.6001
6	9.1063	9.4187	0.7203
7	10.1563	9.9037	0.7721
8	10.7813	10.6037	0.8386
9	11.1813	12.0737	0.3065
10	11.9713	12.4187	0.6081
11	12.6713	13.2137	0.5341
12	13.2913	13.5637	0.7549
13	14.7063	14.1737	0.5415
14	15.8463	14.4587	0.1121

[1] P-values from the ANOVA

SEROLOGICAL STUDIES

Titers were log transformed prior to the statistical analysis. The vaccination group by day interaction was statistically significant for SN titers ($P < 0.05$). Statistically significant group effects were seen on Days 35 (the day of challenge) and 7 and 14 days post-challenge (study days 42 and 49). Horses in the vaccinated group had higher titers than those in the control group ($P < 0.05$).

TABLE 18: Geometric mean – serum neutralization titers (N = 20 horses per group)

Study day	Control	Vaccinated	P-value[1]
0	3.987	3.384	0.4005
21	3.190	2.624	0.3168
35 (day of challenge)	3.480	6.863	**0.0006**
42	3.519	19.252	**<0.0001**
49	33.153	187.417	**<0.0001**

[1] P-values from the ANOVA. Serum neutralization titers were log (natural) transformed prior to the statistical analysis.

Results and Discussion

Respiratory disease caused by equine herpesvirus type 1 is usually an epidemic disease of naïve weanling and yearling horses that occurs in the first year of life, usually in the fall and winter months. Signs of acute infection include fever up to 106°F, viremia and leucopenia and/or neutropenia. Nasal discharge is usually evident during febrile periods of this first exposure. Natural infection by EHV-1 does not result in permanent immunity of the respiratory tract. Indeed, horses may be re-infected naturally every 3 to 6 months throughout life. After the first experience with this virus, re-infection results in production of virus, but usually without clinical signs of disease, resulting in carrier animals that act as natural reservoirs of the virus.

The equine herpesvirus-1 multi-component vaccine described in this report has been shown to be efficacious in reducing the respiratory manifestations, clinical symptoms and virus shedding from nasal exudate of horses challenged with a virulent heterologous strain of Equine Herpesvirus type 1. Reduction in shedding of virus from the respiratory route is important epidemiologically due to this being the natural route of exposure to naive animals as well as for re-infection of herd mates from those experiencing a natural infection. It was also a safe vaccine with no adverse reactions, either systemic or at the site of vaccine administration, observed following vaccine use in the study horses.

In this study, vaccination group by day interaction showed statistical significance for the primary outcome variables nasal discharge scores and conjunctivitis. Statistically significant group effects were seen in the vaccinate group for nasal discharge on Days 4, 5 and on Days 7-11 post-challenge. Group effects for conjunctivitis were also statistically significant on Days 5 and 6 and 9-14 with lower scores in the vaccinate group ($P<0.05$). This is significant epidemiologically because the EHV-1 virus is delicate and does not survive in the environment readily. Close contact is important for transmission of disease through nasal secretions containing virulent EHV-1 virus (Campbell and Studdert, 1983).

Importantly, another primary outcome variable in this study, virus shedding in nasal exudates, showed a main effect of vaccination as statistically significant ($P<0.05$). Horses in the

vaccinated group also had statistically fewer days of virus shedding than those in the control group ($P<0.05$).

Serum neutralization titers were statistically significant after vaccination and throughout the challenge period in vaccinates versus control horses ($P<0.05$). Humoral immunity and mucosal antibodies may be important in determining whether an EHV-1 infection becomes a productive or limited infection event (Kidd, Smith, Hannant, et. al, 1994).

Example 6

This example illustrates the efficacy and 6 month duration of immunity of an immunogenic composition of the present invention when challenged with West Nile Virus.

Materials and Methods

The WNV viral antigen used in the vaccine evaluated in this study was produced on E vero cells as described in Example 1. A total of 15 horses were randomly divided into groups, one being a control group of 5 horses. The vaccinated group of 10 horses received 2 doses of vaccine at 21-day intervals cells. On completion of satisfactory residual live virus testing the inactivated viral fluids were then used to formulate a vaccine which also contained inactivated Venezuelan Equine Encephalomyelitis, TC-83 strain (ATCC Accession No. PTA-9411), Eastern Equine Encephalomyelitis, NJO strain (ATCC Accession No. PTA-9412), and Western Equine Encephalomyelitis, Fleming strain (ATCC Accession No.PTA-9410) viruses, inactivated influenza A/equine-2/Kentucky/95 (ATCC Accession No. PTA-9523), influenza A/equine-2/NewMarket/2/93 (ATCC Accession No. PTA-9524) and influenza A/equine-2/Ohio/03 (ATCC Accession No. PTA-9522) viruses, inactivated West Nile Virus (ATCC Accession No. 9409) and tetanus toxoid. Vaccine was formulated to minimum specifications for all antigens included in the product.

The final formulated vaccine contains the following ingredients per 1 mL dose:

EHV-1	$10^{7.0-9.0}$ $TCID_{50}$/mL
Influenza A2/Ohio/03	$10^{6.7-9.5}$ $TCID_{50}$/mL
Influenza A2/KY/95	$10^{6.7-9.5}$ $TCID_{50}$/mL
Influenza A2/NewMarket/2/93	$10^{6.7-9.5}$ $TCID_{50}$/mL
Tetanus Toxoid	5-10 CPU
Eastern Equine Encephalomyelitis	$10^{6.7-9.2}$ $TCID_{50}$/mL

Western Equine Encephalomyelitis	$10^{6.7-9.2}$ PFU/mL
Venezuelan Equine Encephalomyelitis	$10^{6.7-9.2}$ $TCID_{50}$/mL
West Nile Virus	$10^{7.0--9.0}$ $TCID_{50}$/mL
Adjuvant (preferably mineral oil)	100-200 µl
Glycerol	100-200 µl
EDTA 240 mM solution	10-20 µl
Diluent – DMEM containing	q.s.
Gentamicin (30 µg/mL of diluent volume)	
Formaldehyde (0.1-0.2% of diluent volume)	

Fifteen horses were used in this study. Horses were randomly assigned to either vaccinate or control groups and then vaccinated. Ten horses served as vaccinates and five horses were mock-vaccinated control horses.

The vaccine was administered intramuscularly in a 1 mL dose volume to each of the horses in the vaccinate group. Each control received a 1 mL dose of adjuvanted DMEM containing excipients used in the 9-way vaccine (gentamycin and formaldehyde) but no antigens.

All groups were challenged approximately 6 months following vaccination with intrathecal inoculation of 1 ml PBS containing approximately 10^5 pfu of a heterologous strain of WNV (NY99, 4132, crow isolate). The challenge was conducted under ketamine-xylazine anesthesia.

Horses were monitored for a maximum of 14 days.

Results and Discussion

Viremia after challenge was considered the primary outcome variable in this study. The horses that had been vaccinated were 90% protected from viremia after challenge in this study. In comparison, all of the 5 control horses demonstrated viremia for 3-5 days post-challenge.

In addition, serum neutralization titers of vaccinated horses were significantly higher than those of control horses after vaccination. All the vaccinated horses developed measurable serum neutralization titers following vaccination, whereas none of the controls displayed any titer to WNV. This study demonstrated that 2 doses of the experimental combination vaccine reliably and effectively stimulated protective serological serum neutralization titers.

Since viremia is a prerequisite before virus can cross the blood-brain barrier to cause WNV encephalitis, viremia is well justified as the primary parameter for evaluation of protection in an experimental study of this type.

The results demonstrated that an immunogenic reaction is induced in the animals that were administered the vaccine, and that the vaccine is effective at providing protection for at least 6 months following vaccination. The effectiveness of the vaccine was evidenced in this example by reduction in WNV viremia and by stimulation of high serum neutralization titers to WNV. Because this vaccine is comprised of unique constituents including a long lasting non-metabolizable adjuvant, is formulated in a low 1mL dose volume to provide a high degree of safety as a highly immunogenic low passage whole inactivated virus WNV isolate of recent origin and high epidemiological prevalence (a North American Dominant WNV strain), and a WNV isolated from the tissues of an infected horse, it provides more comprehensive safety and long lasting effectiveness of at least 6 months duration than other vaccines currently available. Additionally, it has the effect of providing a safe vaccine when administered to animals, and in particular to horses.

Example 7

This example illustrates the efficacy of one embodiment of the immunogenic composition of the present invention including encephalomyelitis antigens with tetanus toxoid antigen.

Materials and Methods

Host animal and laboratory animal immunization/serology were evaluated to demonstrate efficacy of encephalomyelitis antigens and the tetanus toxoid antigen fraction in an Encephalomyelitis-Rhinopneumonitis-Influenza-West Nile Virus Vaccine, including Eastern, Western, and Venezuelan Encephalomyelitis, Killed Virus, and Tetanus Toxoid. The efficacy and lack of interference on equine encephalitis virus vaccines and tetanus toxoid fractions can be unequivocally demonstrated by laboratory animal potency testing of the combination vaccine. Demonstration of serological response following vaccination of horses is also indicative of vaccine-toxoid efficacy. Hence, both lab animal potency and host animal serology were used in this study to confirm the efficacy of the experimental vaccine. The vaccine was also evaluated for safety in animals including horses.

Horses 4-5 months of age, from non-vaccinated mares, were vaccinated with an efficacy serial of WNV combination vaccine containing inactivated Venezuelan Equine Encephalomyelitis Virus, TC-83 strain (ATCC Accession No. PTA-9411) Eastern Equine Encephalomyelitis Virus, NJO strain (ATCC Accession No.PTA-9412) Western Equine Encephalomyelitis Virus, Fleming strain (ATCC Accession No. PTA-9410) West Nile Virus (WNV), Horse Origin 2005 (ATCC Accession No. PTA-9409) Equine Herpesvirus Type 1 (ATCC Accession No. PTA-9525) (EHV-1), Influenza A/equine-2/Ohio/03 (ATCC Accession No. PTA-9522), Influenza A/equine-2/Kentucky/95 (ATCC Accession No. PTA-9523), Influenza A/equine-2/NewMarket/2/93 (ATCC Accession No. PTA-9524) and Tetanus Toxoid. Horses were vaccinated on Day 0 and Day 21 of the study. Blood samples were collected at Day 0, Day 21 and Day 35. Day 0 and Day 35 serological results are reported herein.

In addition, the same WNV combination vaccine used to vaccinate horses was tested for potency in guinea pigs. Data presented in this report collectively and definitively establish the efficacy of each antigen tested (EEE, VEE, WEE, tetanus) in this study and also confirm the safety of a WNV combination vaccine.

Bulk lots of EEE, WEE, and VEE viruses and tetanus toxoid were produced. Following growth, viral fluids were filtered, formalin inactivated, and concentrated. The inactivated viral fluids were tested for residual live virus after inactivation.

Inactivated viral and toxoid fluids described above were used to formulate a vaccine that also contained inactivated Equine Herpesvirus Type 1, inactivated influenza A/equine-2/Kentucky/95, influenza A/equine-2/NewMarket/2/93 and influenza A/equine-2/Ohio/03 viruses.

The vaccine was formulated to specifications for all antigens included in the product.

The final formulated vaccine contained the following ingredients per 1 mL dose:

EHV-1	$10^{7.0-9.0}$ $TCID_{50}$/mL
Influenza A2/Ohio/03	$10^{6.7-9.5}$ $TCID_{50}$/mL
Influenza A2/KY/95	$10^{6.7-9.5}$ $TCID_{50}$/mL
Influenza A2/NewMarket/2/93	$10^{6.7-9.5}$ $TCID_{50}$/mL
Tetanus Toxoid	5-10 CPU
Eastern Equine Encephalomyelitis	$10^{6.7-9.2}$ $TCID_{50}$/mL
Western Equine Encephalomyelitis	$10^{6.7-9.2}$ PFU/mL

Venezuelan Equine Encephalomyelitis	$10^{6.7-9.2}$ $TCID_{50}$/mL
West Nile Virus	$10^{7.0--9.0}$ $TCID_{50}$/mL
Adjuvant (preferably mineral oil)	100-200 µl
Glycerol	100-200 µl
EDTA 240 mM solution	10-20 µl
Diluent – DMEM containing	q.s.
Gentamicin (30 µg/mL of diluent volume)	
Formaldehyde (0.1-0.2% of diluent volume)	

Forty horses, four to 5 months of age, were used in this study. Horses remained with their dams on pasture throughout the vaccination period and were weaned from their dams when the 2-week post-booster sera were collected. Horses were assigned to either one of the two treatment groups randomly as they were vaccinated intramuscularly (IM) with a 1.0 ml dose. The primary immunization was followed three weeks later by a 1.0 ml IM booster vaccination. Twenty horses received vaccine. Twenty horses received placebo.

Guinea pigs were also vaccinated with the same combination WNV vaccine.

Horses were vaccinated and serum samples collected using the following schedule:

TABLE 19: Vaccination and Serum Sampling Schedule

Day of Test	Activity
0	Collect pre-vaccination blood and give primary vaccination
21	Collect blood and give booster vaccination
35	Collect blood for final serology

Guinea pigs were vaccinated and serum collected using the schedule outlined by 9 CFR, 113.207(b) and 113.114(c.).

Sera from horses in this study were tested following general guidelines. The assay was modified to determine titers by testing at 1:2 and 1:10 dilutions for Day 0 samples and at 1:10 and 1:40 dilutions for the 2-week post-booster serum samples. Sera were tested for EEE, WEE and VEE antibody and were tested for Tetanus toxoid antibody.

Results and Discussion

Horse Serological Evaluation for EEE, WEE and VEE

At Day 0 of the Study, not all foals were sero-negative to encephalomyelitis viruses. Five of the vaccinated foals had significant (>1:10) residual maternal antibody to EEE virus. In addition, two of the vaccinated foals had residual maternal antibody (>1:10) to WEE virus. Despite existing and potentially interfering passively acquired, maternal antibody at the time of administration of the first dose of the WNV combination vaccine, titers for all three fractions increased substantially (>4 fold in 80% of horses tested for EEE, >4 fold in 90% of horses tested for WEE and > 4 fold in 100% of horses tested for VEE) following vaccination, yet remained negative or low for the non-vaccinated foals. Individual foal data are presented below.

EEE, WEE and VEE Equine Serological Titers

TABLE 20: Plaque Reduction Neutralization Titration

Horse ID	Test Article	Day 0 EEE	Day 35 EEE	Day 0 WEE	Day 35 WEE	Day 0 VEE	Day 35 VEE
1	V	>10	>40	2	>40	<2	>40
2	V	<2	>40	<2	10	<2	40
3	V	<2	>40	<2	<10	<2	>40
4	V	<2	>40	<2	10	<2	>40
5	V	>10	>40	>10	>40	<2	>40
6	V	>10	>40	<2	>40	NS	10
7	V	<2	>40	<2	>40	<2	>40
8	V	>10	>40	2	>40	<2	>40
9	V	<2	>40	<2	>40	<2	>40
10	V	<2	>40	<2	>40	<2	10
11	V	<2	>40	<2	>40	<2	>40
12	V	<2	>40	<2	>40	<2	40
13	V	<2	>40	<2	>40	<2	>40
14	V	<2	>40	<2	10	<2	>40
15	V	>10	>40	>10	>40	2	>40
16	V	2	>40	<2	10	<2	>40
17	V	<2	>40	<2	10	<2	>40
18	V	<2	>40	<2	>40	<2	40
19	V	<2	10	<2	>40	<2	>40
20	V	2	>40	<2	>40	<2	10
21	C	<2	<10	<2	<10	<2	10
22	C	<2	<10	<2	<10	<2	<10
23	C	<2	<10	<2	10	<2	<10

Horse ID	Test Article	Day 0 EEE	Day 35 EEE	Day 0 WEE	Day 35 WEE	Day 0 VEE	Day 35 VEE
24	C	<2	<10	NS	<10	<2	<10
25	C	<2	<10	<2	<10	<2	<10
26	C	<2	<10	<2	10	<2	<10
27	C	<2	<10	<2	<10	<2	<10
28	C	2	<10	<2	<10	<2	10
29	C	2	<10	<2	10	<2	<10
30	C	<2	<10	<2	<10	<2	<10
31	C	<2	<10	<2	<10	<2	<10
32	C	<2	<10	<2	<10	<2	<10
33	C	>10	>40	<2	10	<2	<10
34	C	<2	<10	<2	<10	<2	<10
35	C	>10	10	2	<10	<2	10
36	C	2	<10	<2	<10	<2	<10
37	C	<2	<10	<2	<10	<2	<10
38	C	<2	<10	2	<10	<2	<10
39	C	<2	10	<2	<10	<2	<10
40	C	<2	>40	<2	<10	<2	<10

Guinea Pig Serological Evaluation for EEE, WEE, VEE and Tetanus toxoid

Nine of ten guinea pigs vaccinated with the combination vaccine seroconverted satisfactorily at (≥1:40) to EEE virus. Ten of ten guinea pigs had satisfactory titers for VEE virus (≥1:4) and ten of ten guinea pigs seroconverted satisfactorily to WEE virus (≥1:40). Also a serum pool from 10 vaccinated guinea pigs was tested for tetanus antibody and was shown to be satisfactory with a value of 4.3 anti-toxin units/ml (AU/ml).

Guinea pig potency tests were completed and found to be satisfactory for all four antigens including tetanus toxoid, EEE, VEE, and WEE.

The vaccine was also administered to horses (20 vaccinates and 20 controls) via primary immunization followed by booster immunization 3 weeks later. Fourteen days post-booster vaccination, horses were bled and serum collected for all serological testing. Equine response to encephalomyelitis antigens was tested utilizing 2 dilutions (1:2 and 1:10 for Day 0 samples and 1:10 and 1:40 for Day 35 samples) in 24-well plates to determine antibody titers.

The satisfactory guinea pig potency testing conclusively establishes the efficacy of 4 antigens (VEE, EEE, WEE and tetanus toxoid) in the West Nile Virus combination vaccine as a 9- antigen-containing vaccine-toxoid. Furthermore, satisfactory potency results are substantiated

and confirmed by host animal horse serology data in which vaccinated horses demonstrated a substantial rise in titer to each encephalitis virus fraction following vaccination. Additionally, the absence of observation of any adverse reactions in any of the vaccinated horses or guinea pigs confirms the safety of the WNV combination vaccine in animals.

Example 8

This example illustrates that a vaccine or immunogenic composition in accordance with the present invention has a duration of immunity of at least one year.

Materials and Methods

Host animal vaccination and challenge at least 1 year post-booster vaccination was used to confirm duration of immunity for the West Nile Virus antigen fraction in an Encephalomyelitis- Rhinopneumonitis-Influenza-West Nile Virus Vaccine, Eastern, Western & Venezuelan, Killed Virus, Tetanus Toxoid prepared from a North American Dominant isolate of WNV designated North American Equine E159 (NAEE159).

TABLE 21: The final formulated vaccine contains the following ingredients per 1 mL dose:

Ingredients	1 mL Dose
Eastern Equine Encephalomyelitis	$10^{7.5\text{-}9.2}$ $TCID_{50}$/mL
Western Equine Encephalomyelitis	$10^{8.2\text{-}9.2}$ PFU/mL
Venezuelan Equine Encephalomyelitis	$10^{7.7\text{-}9.2}$ $TCID_{50}$/mL
West Nile Virus (North American Dominant prepared from NAEE159)	$10^{8.0\text{-}9.2}$ $TCID_{50}$/mL
EHV-1	$10^{7.0\text{-}9.0}$ $TCID_{50}$/mL
Equine Influenza A2/Ohio/2003	$10^{7.3\text{-}9.5}$ $TCID_{50}$/mL
Equine Influenza A2/Kentucky/95	$10^{7.3\text{-}9.5}$ $TCID_{50}$/mL
Equine Influenza A2/NewMarket/2/93	$10^{7.3\text{-}9.5}$ $TCID_{50}$/mL
Tetanus Toxoid	5-10 CPU
Non-metabolizable Oil Adjuvant	100-200 µL
Diluent – DMEM containing	q.s.
Gentamicin	30 µg/mL of diluent volume
Formaldehyde	0.1% of diluent volume

Thirty horses (20 vaccinates and 10 controls), 4-5 months of age were used in this study. Horses were randomly assigned to one of two treatments and vaccinated intramuscularly (IM) with a 1.0 mL dose of the assigned vaccine or control product. The primary immunization was followed three weeks later by a 1.0 mL IM booster vaccination.

Horses were vaccinated once and then again about 30 days later. Horses were randomly assigned to either vaccine or control groups. Twenty horses received the vaccine group receiving VEWT/WNV/EHV-1/Influenza vaccine. Ten horses received adjuvanted DMEM containing excipients used in the vaccine (Gentamicin and formaldehyde) but no antigens. The non-metabolizable oil adjuvant used for all administrations was preferably mineral oil.

Challenge inoculation of the virulent heterologous WNV NY99 strain virus was performed 380 days post-booster vaccination. The second cohort of horses were challenged 408 days post-booster inoculation in a similar manner.

Serum samples for serological evaluation were collected from the vaccinated and control horses prior to initial vaccination, at 21 days post first dose vaccination (day of booster vaccination), monthly post-booster, on the day of challenge, and at 7 and 14 days post-challenge. Body temperature and serum samples were obtained from each horse on the day of challenge, twice daily on Days 1 through 6 post-challenge, and daily on Days 7-10 and Day 14 post-challenge. Clinical data was also recorded during those same time periods for the 15-day observation period.

The heterologous challenge virus, designated WNV NY99, was originally isolated from the brain of an infected crow (CDC, Ft. Collins, CO). On the day of challenge, the stock virus was thawed on ice and virus was diluted to the desired concentration in phosphate-buffered saline immediately prior to inoculation of horses.

Rectal temperatures were recorded for each of the vaccinated and control horses on the day before challenge, day of challenge and twice daily on days 1-14, then daily on Days 14-21 post challenge by means of a calibrated, electronic thermometer (GSA Electronics) probe. The daily rectal temperatures were recorded in degrees Fahrenheit (°F).

Venous blood from each of the vaccinated and control horses was collected on the Day of Challenge, twice daily on Days 1-6, and daily on Days 7-10 and Day 14 days post-challenge by Vacutainer into an SST tube. After centrifugation, serum was aliquotted and frozen immediately.

Vero Cells were grown in 6-well plates to confluency. To perform the plaque assay, serial 10-fold dilutions of serum were prepared in 96-well plates in BA-1 medium (MEM salts containing 1% BSA, 250 mg/L sodium bicarbonate, 50µl gentamicin and 2.5 µg amphotericin B/mL in 50mM Tris, pH 7.6). Serum dilutions (0.1 mL) were inoculated into each well of the 6-well plate and incubated for 45-60 minutes with rocking every 15 minutes. After the incubation period, 2 mL of overlay (2X medium containing MEM without phenol red prepared at twice the normal concentration and supplemented with 4% FBS, 200 IU penicillin G/mL and 100µg streptomycin/mL – warmed to 45°C) was added to each well. Plates were incubated at 37°C.

Two days after inoculation, 2 mL of a second overlay containing 2X agarose prepared by mixing equal volumes of 2X medium and 2X agarose was added to each well. Plates were examined and plaque numbers recorded in each well on days 3, 4 and 5 following inoculation. The virus titer per mL of original material is calculated as the number of plaques in a well (or average of multiple wells inoculated with the same dilution) times the dilution for the well being counted multiplied by 10.

A standard microtiter serum neutralization test was employed in this study. All sera were tested in sterile flat bottom 96 well microtiter plates using five wells per dilution and an 8 well dilution series for each of the 5 test wells. Each of the 5 test wells contained 25 µL of serum dilution mixed with 25 µL of the indicator virus and 150 µL of a freshly planted Vero cell suspension containing approximately 4 X 10^4 cells. The test indicator virus used was WNV NY99. Serum neutralizing antibody titers are expressed as Reed-Muench ID_{50} titers.

For performance of the test, two-fold dilutions of each test serum were made in a sterile flat bottom microtiter plate using five replicate wells per test serum and an 8 well dilution series. Dilutions were made with an adjustable volume single or multi-channel pipetting instrument using sterile microtiter tips. The volume of serum added to each of 5 wells of the first row was 50 µL. All other wells contained 25 µL of DMEM (no FBS). Following serial dilution down the plate, 25 µL was discarded from the last row. 25 µL of a pre-determined dilution of the indicator virus was added to each test well. Plates were then mixed and incubated for one hour at 37°C in 5% CO_2. On conclusion of the incubation period, 150 µL of a suspension containing 4 x 104 Vero cells were added to each test and cell control well. The plates were incubated at 37°C in a CO_2 incubator for 5-7 days, at which time plates were microscopically examined for CPE typical of WNV.

Histopathology was evaluated by a Board Certified Veterinary Pathologist. The scoring system used to describe defects in the pons or medulla was as follows:

Score:

0 = no significant lesions in section
0.5 = rare, small, multifocal glial nodules scattered throughout the parenchyma
1 = mild, nonsuppurative encephalitis. This is characterized by mild multifocal perivascular cuffs with lymphocytes and plasma cells and a rare neutrophil and scattered multifocal glial nodules composed of glial cells with a few mononuclear inflammatory cells. Occasionally within this grade, there may be minimal perivascular cuffing and more moderate scattered glial nodules.
2 = moderate nonsuppurative encephalitis characterized by moderate lymphoplasmacytic perivascular cuffs around many vessels and multifocal accumulations of glial nodules scattered throughout the parenchyma
3 = severe nonsuppurative encephalitis characterized by severe and thick lymphoplasmacytic perivascular cuffing with multiple scattered glial nodules throughout the parenchyma

Results and Discussion

There were no adverse reactions to vaccine administration at either dosing time point. All 4 to 5 month old foals receiving the experimental vaccine were free of either systemic or injection site adverse reactions in the study. This confirms the excellent safety of the vaccine of the present invention against WNV containing North American Dominant WNV antigen prepared from isolate NAEE159.

HORSE CHALLENGE WITH HETEROLOGOUS WEST NILE VIRUS

VIREMIA

Each of the 10 control horses (100%) were viremic for at least 1 day post-challenge, while only 2 of 20 horses (10%) in the WNV vaccine group were viremic.

CLINICAL SIGNS

Seven of the 10 horses (70%) in the control group developed signs of encephalomyelitis consistent with West Nile Virus infection. Each of these animals was viremic for at least one day during the challenge period. In the WNV vaccine group, 1 of the 20 horses (5%) developed signs consistent with West Nile Virus infection. Notably clinical signs progressed to death or euthanasia in 70% of the controls and only 5% of the vaccinates. All control mortalities were viremic, confirming fatal encephalitis due to WNV, whereas only one of two vaccinated animals that died was viremic during the challenge period.

SERUM NEUTRALIZATION TITERS

All vaccinated horses responded favorably to the WNV vaccine by developing protective levels of serum neutralizing (SN) antibody following vaccination. Over one year following vaccination, 17 of 20 (85%) of vaccinated horses maintained protective SN titers. By contrast, none of the control horses developed rising SN titers prior to virulent WNV challenge. Also, all vaccinated horses displayed an anamnestic rise in SN titers following virulent WNV challenge

HISTOPAHTOLOGY

Severity scores were provided for both the medulla and pons. Also with regard to this efficacy parameter, WNV vaccine containing North American Dominant WNV antigen prepared from isolate NAEE159 proved highly effective. Among the control horses, 50% displayed severe lesions of WNV encephalitis whereas only 10% of vaccinates were similarly affected.

Discussion and Conclusions

The WNV vaccine was prepared from a viral isolate (North American Equine E159) obtained from a horse in 2005 during the North American pandemic when a specific dominant WNV genotype emerged. This genotype is characterized by a specific valine to alanine amino

acid change at the 159th amino acid in the envelope (E) protein of the virus (when compared to the publicly available sequence for the WNV-NY99 isolate having the ATCC Accession No. AF196835), which has made all such isolates more robust and prolific, thereby displacing other WNV isolates, and making this genotype dominant among disease-causing WNV isolates in North America. Because it was prepared from the dominant genotype, the vaccine used in this study is indicative of the unique safety and efficacy achievable with vaccine prepared from all such North American Dominant isolates with this E protein profile and resulting prolificacy. Notably, all previously tested WNV vaccines have been prepared from a less prolific isolate of differing genotype and E protein amino acid sequence, namely WNV NY99. Based on this difference in nucleic acid sequence, E protein amino acid sequence, viral prolificacy, and unique ability to cause a pandemic, the North American Dominant isolates are displacing or have displaced NY99 from the environment. The unique genotype and phenotype (prolificacy), and, most importantly, the overwhelming environmental presence of North American Dominant WNV isolates and the absence of WNV NY99 is compelling evidence for the superiority of the North American Dominant West Nile Virus vaccine. Such superiority is confirmed by the safety and efficacy of the vaccine as demonstrated in this challenge study using vaccine prepared from North American Dominant isolate North American Equine E159 (NAEE159) (ATCC Accession No PTA-9409).

In this study, 4 to 5 month old horses were safely and effectively vaccinated with a multi-component VEWT/WNV/EHV-1/Equine Influenza vaccine batched at an appropriate antigen amount with the WNV component being North American Dominant WNV antigen prepared from isolate NAEE159c(ATCC Accession No. PTA-9409).

Study horses were intrathecally challenged at least 380 days post-booster vaccination with 10^5 PFU of a virulent heterologous West Nile Virus strain. Horses were evaluated for 14 days post-challenge for clinical signs (including temperature and mortality), viremia, serum neutralization titers, and histopathology scores from sections of the pons and medulla taken after euthanasia and necropsy.

Viremia after challenge and serum neutralization titers were key outcome variables in this study that were highly indicative of vaccine efficacy. Horses that had been vaccinated more than one year earlier with VEWT/WNV/EHV-1/Influenza Lot 916 were 90% protected from viremia after challenge in this study. In comparison, 100% of control horses demonstrated viremia post-

challenge. Additionally, serum neutralization titers of vaccinated horses were significantly higher than those of control horses at 14 days post-challenge, and displayed an anamnestic response typical of an effective vaccine following heterologous, virulent WNV challenge.

In addition the vaccine containing North American Dominant WNV antigen prepared from isolate NAEE159 reduced clinical signs and mortality resulting from encephalomyelitis following heterologous challenge with virulent WNV. Vaccine efficacy at least one year following vaccination was also confirmed by reduction in lesions typical of WNV infection.

This study demonstrated for the first time that 2 doses of the experimental combination vaccine prepared at appropriate doses of antigen including North American Dominant WNV antigen prepared from isolate NAEE159 administered to foals 4 to 5 months of age safely, reliably and effectively stimulated protective serological serum neutralization titers that resulted in duration of immunity of at least one year with protection from viremia, clinical signs, mortality, and encephalitic lesions after virulent heterologous challenge with West Nile Virus.

Example 9

In this study, a combination vaccine was prepared using a North American Dominant isolate of WNV, North American Equine E159(NAEE159) (ATCC Accession No. PTA-9409). The 14-day post second-vaccination sera samples from the guinea pigs vaccinated with this Encephalomyelitis-Rhinopneumonitis-Influenza- West Nile Virus Vaccine, Eastern, Western & Venezuelan, Killed Virus, Tetanus Toxoid were collected and tested for West Nile Virus plaque reduction neutralization (PRN). The sera from the vaccinated guinea pigs were tested for neutralizing antibody to both a North American Dominant isolate of WNV and to WNV isolate NY99. Notably, the vaccine displayed superior activity in stimulating neutralizing antibodies to North American Dominant WNV, as opposed to NY99 WNV. These data support the conclusion of the superior efficacy of WNV vaccines prepared from North American Dominant WNV isolates as contrasted with earlier less effective vaccines prepared from or based on the NY99 WNV isolate.

Furthermore, a vaccine prepared from an additional North American Dominant isolate of WNV, North American Donkey E159 (NADE159) will similarly demonstrate, as described above, the superior efficacy of such vaccines over the former NY99 based vaccines. Hence, data from multiple North American Dominant isolates cultivated from different host species,

originating from unique North American locations, and obtained at different times in North America will confirm the unexpected but superior efficacy of North American Dominant isolates of WNV for vaccine preparation.

The data from the plaque reduction neutralization assay also established that a vaccine prepared from a North American Dominant isolate of WNV that stimulates a titer of 1:12 or higher in vaccinated guinea pigs that provides 50% viral plaque reduction in at least 90% of vaccinated guinea pigs, correlates to vaccine protection against WNV challenge in the horse and provides for a duration of immunity of at least one year. West Nile Virus vaccination/challenge data in the horse at an antigen inclusion level of $10^{7.6-9.0}$ $TCID_{50}$ or higher per dose correlated with these guinea pig PRN titer results and confirmed the WNV immunizing dose that provides 1 year or longer duration of immunity in the horse. The corresponding dose in guinea pigs also stimulates serum neutralizing antibodies to a titer of at least 1:12 against North American Dominant WNV in guinea pigs.

Data presented in this report collectively demonstrate the unexpected efficacy of vaccines prepared from North American Dominant isolates of WNV, define the correlation between vaccine efficacy in the horse and guinea pig serum levels of neutralizing antibody, confirm that a 1:12 titer or higher in guinea pigs identifies an effective equine vaccine providing at least one year duration of immunity, and quite notably, demonstrate the superior efficacy of vaccines prepared from North American Dominant WNV as contrasted with NY99 WNV.

Materials and Methods

In order to demonstrate efficacy of the West Nile Virus antigen, prepared using a North American Dominant isolate of WNV, North American Equine E159(NAEE159) (ATCC Accession No. PTA-9409), in an Encephalomyelitis-Rhinopneumonitis-Influenza-West Nile Virus Vaccine, Eastern, Western & Venezuelan, Killed Virus, Tetanus Toxoid and to establish an effective dose measurable in horses or guinea pigs, host animal vaccination/challenge studies were performed in conjunction with guinea pig vaccination/serology studies. In this study, 14-day post second-vaccination sera samples from guinea pigs vaccinated with Encephalomyelitis-Rhinopneumonitis-Influenza-West Nile Virus Vaccine, Eastern, Western & Venezuelan, Killed Virus, Tetanus Toxoid were collected and tested. Additionally, a plaque reduction neutralization

assay was developed to measure the titer correlated to protection against challenge in the host animal. This titer was determined to be 1:12 or higher in the guinea pig.

Vaccine Formulations

Experimental Serials (Protective Dose Vaccine)

Experimental Serials were formulated to confirm protective antigen specifications for all antigens in the vaccine.

TABLE 22: The final formulated vaccines contained the following ingredients per 1 mL dose:

Ingredients	1 mL Dose
Eastern Equine Encephalomyelitis	$10^{7.5\text{-}9.2}$ $TCID_{50}$/mL
Western Equine Encephalomyelitis	$10^{8.2\text{-}9.2}$ PFU/mL
Venezuelan Equine Encephalomyelitis	$10^{7.7\text{-}9.2}$ $TCID_{50}$/mL
West Nile Virus (North American Dominant prepared from NAEE159)	$10^{7.3\text{-}9.2}$ $TCID_{50}$/mL
EHV-1	$10^{7.0\text{-}9.0}$ $TCID_{50}$/mL
Equine Influenza A2/Ohio/2003	$10^{7.3\text{-}9.5}$ $TCID_{50}$/mL
Equine Influenza A2/Kentucky/95	$10^{7.3\text{-}9.5}$ $TCID_{50}$/mL
Equine Influenza A2/NewMarket/2/93	$10^{7.3\text{-}9.5}$ $TCID_{50}$/mL
Tetanus Toxoid	5-10 CPU
Non-metabolizable Oil Adjuvant	100-200 µL
Diluent – DMEM containing	q.s.
Gentamicin	30 µg/mL of diluent volume
Formaldehyde	0.1% of diluent volume

Experimental serial 916 was formulated for host animal vaccination studies. Experimental serial 916 is a multi-component vaccine containing VEWT-WNV-EHV-1 and 3 strains of equine influenza type A2 virus. Experimental serial 916 is batched at $10^{7.6\text{-}9.2}$ $TCID_{50}$/mL of West Nile Virus antigen North American Equine E159(NAEE159). It is a 1mL dose vaccine in the horse.

This vaccine was also tested in guinea pigs at the time of host animal vaccinations to confirm the WNV efficacy and laboratory animal potency. Four replicate guinea pig sera

dilution experiments were performed for experimental serial 916 to validate a guinea pig assay criterion for this one-year duration of immunity (DOI) vaccine.

Experimental Serial 507 (Comparative Efficacy Serial)

Data from Experimental Serial 507 is included in this report to demonstrate that serials formulated with a North American Dominant isolate of WNV antigen show superior efficacy, measured as guinea pig titers, of the relevant North American Dominant isolates of WNV as compared to the earlier NY99 isolate.

Guinea Pig Serological Evaluation

Sera were tested for WNV antibody as follows:

1) West Nile Virus Indicator Strain: North American Equine E159(NAEE159) (ATCC Accession No. PTA-9409) & North American Donkey E159 (NADE159)
2) Growth medium for Vero Cells is DMEM + 5% FBS, 2 mM L-glutamine and 30 μg/mL gentamycin
3) Diluent for Test Serum is DMEM plus 30 μg/mL gentamycin
4) Diluent for indicator virus working solution is DMEM + 10% normal guinea pig serum (specific to WNV assay)
5) Guinea pig test sera is diluted 1:12
7) Use 4 mL overlay instead of 3 mL (specific to WNV assay)
8) Titers are calculated using 50% plaque reduction
9) Nine of ten vaccinated guinea pigs must have an antibody titer of ≥ 1:12 to demonstrate efficacy, and negative guinea pigs must be <1:4 (same criteria as VEE assay in SAM).

Results and Discussion

Serological Evaluation for West Nile Virus

Experimental Serial 916 Replicate I West Nile Guinea Pig

Plaque Reduction Neutralization

Results Using North American Equine E159(NAEE159)as Indicator Virus

The study was initiated and the guinea pigs were bled 25 days later.

TABLE 23: Number of Plaques/Sera Dilution

916 (I)	**2**	**3**	**4**	**6**	**8**	**12**	**16**	**32**	**64**
GP1	0	2	2	4	5.5	2.5	17	14	11
GP2	6.5	9.5	11	14	9	11.5	13	20	22.5
GP3	7	5	6	9.5	10	9.5	11	10	16.5
GP4	7	5	4	4	1	6	15.5	9.5	12.5
GP5	6	4.5	9	7	17.5	13.5	12	16	17
GP6	1	1	2.5	7.5	3	8	19.5	19.5	20.5
GP7	9.5	8	8	15.5	17.5	21.5	36	17.5	20.5
GP8	5	3.5	7.5	7.5	14.5	11	26.5	26.5	27.5
GP9	0	2	7	6	7	12	9	10.5	11.5
GP10	4.5	4.5	5.5	11	22	8.5	23	29	30.5
# Passed	**10/10**	**10/10**	**10/10**	**10/10**	**10/10**	**10/10**	**10/10**	**10/10**	**10/10**
Neg. Control 1	68.5	64.5	72.5	93.5	68	82	89.5	78.5	71
Neg. Control 2	72	61	87	69.5	70.5	85.5	77.5	69.5	88

Virus Control Values: 99. 70, 68, 88, 77, 64
Virus Control Average Plaques: 79
Virus Control 50% Reduction: 39.5

Table 24: Experimental Serial 916 Replicate II West Nile Guinea Pig

Plaque Reduction Neutralization

Results Using North American Equine E159(NAEE159)as Indicator Virus

Guinea Pigs were bled 35 days after the initiation of the study

Number of Plaques/Sera Dilution

916 (II)	2	3	4	6	8	12	16	32	64
GP1	1	3	6	6	14.5	6.5	13.5	18	17.5
GP2	1.5	3.5	6.5	6.5	8.5	9.5	10.5	12.5	19
GP3	12	5.5	22	21	41.5	34	41	41	43.5
GP4	4	8.5	17.5	16.5	21	36.5	28	41.5	42.5
GP5	3.5	4.5	9.5	15	26.5	25.5	13.5	23	40
GP6	1	3	7	13.5	17.5	14	24.5	26	23
GP7	8	5.5	14.5	11.5	21.5	15.5	34	26.5	28
GP8	1	1.5	2.5	3.5	4	11	9	20	17.5
GP9	13.5	18	25.5	29.5	29.5	35.5	28	37.5	43
GP10	9	8	10.5	21.5	21	20.5	27	31.5	27.5
# Passed	**10/10**	**10/10**	**10/10**	**10/10**	**9/10**	**10/10**	**9/10**	**8/10**	**6/10**

Neg. Control 1	69.5	67	71.5	69.5	72.5	73	83	68	78
Neg. Control 2	68	70.5	69	72.5	82.5	66	75	76	74

Virus Control Values: 99. 70, 68, 88, 77, 64
Virus Control Average Plaques: 79
Virus Control 50% Reduction: 39.5

Table 25: Experimental Serial 916 Replicate III West Nile Guinea Pig

Plaque Reduction Neutralization

Results Using North American Equine E159(NAEE159)as Indicator Virus

Guinea Pigs were bled 35 days after initiation

Number of Plaques/Sera Dilution

916 (III)	2	3	4	6	8	12	16	32	64
GP1	0.5	1.5	0	0	0	1	4	6	3.5
GP2	0	0	1	0.5	0	0	0	0	0
GP3	7.5	9	9	16	17	13.5	12	18.5	23
GP4	2.5	0	0	3	2	2	3.5	1	2.5
GP5	13.5	15.5	18	18	19.5	24	16.5	21.5	33.5
GP6	6.5	15.5	31.5	10	29.5	26.5	28.5	31.5	32
GP7	14.5	12	17.5	20.5	19.5	29.5	21.5	16	22.5
GP8	21	24.5	36	28	34.5	30.5	27.5	29	26
GP9	0.5	0.5	3	4	6.5	5	10	9.5	18
GP10	2.5	7.5	11	8	12.5	9	20	10.5	17
# Passed	**10/10**	**10/10**	**8/10**	**10/10**	**9/10**	**10/10**	**10/10**	**9/10**	**8/10**

Neg. Control 1	73	61.5	79.5	53.5	58.5	78	57	70	63
Neg. Control 2	55	54.5	64	52.5	58	68.5	66	67.5	79

Virus Control Values: 51, 58, 61, 66, 78
Virus Control Average Plaques: 62
Virus Control 50% Reduction: 31

TABLE 26: Experimental Serial 916 Replicate IV West Nile Guinea Pig
Plaque Reduction Neutralization
Results Using North American Equine E159(NAEE159)as Indicator Virus
Guinea Pigs were bled 30 days after the initiation of the study
Number of Plaques/Sera Dilution

916 (IV)	2	3	4	6	8	12	16	32	64
GP1	11.5	7	5.5	20.5	12	17.5	22.5	20	25
GP2	31	21.5	20.5	36.5	32	30	27	15	28.5
GP3	16	20.5	18	23.5	21.5	16	35.5	16.5	15
GP4	1	2	6.5	7	11	15	19	14	18.5
GP5	4	1	9.5	12	20	17	24.5	19.5	23.5
GP6	0	0	0	1.5	2.5	2.5	0.5	3	3
GP7	5.5	6.5	5.5	10	7	13	5.5	9.5	13
GP8	1	4.5	0.5	3	7.5	4.5	13.5	17.5	11.5
# Passed	**8/8**	**8/8**	**8/8**	**7/8**	**8/8**	**8/8**	**7/8**	**8/8**	**8/8**

Neg. Control 1	48	39	46.5	39	48	54	39.5	54	67.5
Neg. Control 2	40.5	53	44	44.5	54	48.5	59.5	61.5	45.5

Virus Control Values: 93, 53, 56, 92, 67, 44
Virus Control Average Plaques: 67.5
Virus Control 50% Reduction: 33.8

EXPERIMENTAL SERIAL 507(Demonstrating Superior Efficacy of North American Equine E159(NAEE159) Vaccines, and that other North American Dominant Vaccines, such as North American Donkey E159(NADE159 will provide superior efficacy to NY99 vaccines)

Guinea Pig Serological Evaluation for West Nile Virus

TABLE 27: Experimental Serial 507 West Nile Guinea Pig Plaque Reduction Neutralization Results using WNV NY1999 Isolate as Indicator Virus
Number of Plaques/Sera Dilution

Guinea Pig Number	**4**	**8**	**16**	**32**	**64**
GP1	≥10	≥15	≥15	≥14.5	≥10.5
GP2	4.5	5	7.5	9.5	13
GP3	1	3.5	3.5	6	6.5
GP4	8	11.5	≥13.5	≥14.5	≥14.5
GP5	6	7.5	7.5	8.5	9
GP6	8.5	9.5	9.5	12	14
GP7	7	8.5	9	≥10.5	≥14.5
GP8	5	10.5	10.5	14	14
GP9	5.5	7	7.5	10	10
GP10	5.5	6.5	8.5	≥12.5	14
# Passed	**9/10**	**6/10**	**5/10**	**2/10**	**1/10**
Neg. Control 1	≥13.5	≥13.5	≥13	≥13	≥14.5
Neg. Control 2	≥15.5	≥14.5	≥16	≥17	≥16.5

Virus Control Values: ≥24, ≥15, ≥14, ≥16, ≥18, ≥20
Virus Control Average Plaques: ≥17.8
Virus Control 50% Reduction: ≥8.9

TABLE 28: Experimental Serial 507 West Nile Guinea Pig Plaque Reduction Neutralization Results Using North American Equine E159(NAEE159)as Indicator Virus

Number of Plaques/Sera Dilution

Guinea Pig Number	2	4	8	16	32	64
GP1	17	14	18.5	14.5	19.5	26
GP2	2.5	3.5	2	6	17	15
GP3	5.5	10	4.5	9	5	11.5
GP4	11.5	10	15	15.5	19	19.5
GP5	13	23	23.5	19.5	32.5	13
GP6	5.5	8.5	12	11	13	14
GP7	8.5	12	14	15	16	12
GP8	14.5	14	18	19.5	25.5	27.5
GP9	1.5	3	3	8.5	15.5	7
GP10	12.5	12	7	18.5	14	13
# Passed	**9/10**	**9/10**	**7/10**	**7/10**	**5/10**	**7/10**
Neg. Control 1	24.5	21	22	23.5	22	25
Neg. Control 2	24	33	29	29	24.5	34.5

Virus Control Values: 40, 38, 34, 35, 28, 23
Virus Control Average: 33
Virus Control 50% Reduction: 16.5

Discussion and Conclusions

Guinea pigs were vaccinated and sera tested for West Nile Virus antibody. This assay established that a titer of 1:12 in vaccinated guinea pigs correlates to protection in a horse vaccination/challenge study that provides at least one year duration of immunity for WNV

vaccine prepared using a North American Dominant isolate of WNV, such as North American Equine E159(NAEE159).

Concurrently with guinea pig vaccinations, WNV vaccine prepared using a North American Dominant isolate of WNV, North American Equine E159(NAEE159), was also administered to horses (20 vaccinates and 10 controls) via primary immunization followed by booster immunization 3 weeks later. More than one year post-booster vaccination, horses were subjected to virulent West Nile Virus challenge, and were protected when compared to non-vaccinated controls. Vaccinated horses were protected from viremia, clinical signs, mortality, and encephalitic lesions after virulent heterologous challenge with West Nile Virus.

In addition the data substantiate the superior efficacy of WNV vaccines prepared using North American Dominant WNV, as opposed to previously developed vaccines derived from WNV NY99. The sera from the vaccinated guinea pigs were tested for neutralizing antibody to both a North American Dominant isolate of WNV and to WNV isolate NY99. Titers to the isolate frequently occurring in North America, namely North American Dominant (NAEE159), were consistently higher in vaccinated guinea pigs as compared to titers to the isolate that is no longer reported to be present in nor causing disease in North America, WNV NY99. Hence, the vaccine displayed superior activity in stimulating neutralizing antibodies to North American Dominant WNV, as opposed to NY99 WNV. These data support the conclusion of the superior efficacy of WNV vaccines prepared from North American Dominant WNV isolates as contrasted with earlier less effective vaccines prepared from or based on the NY99 WNV isolate.

Example 10

This Example illustrates the genetic differences between North American WNV strains and North American Dominant WNV strains, as used in the present invention.

Materials and Methods

Relevant areas of the genome of WNV NY99 and the North American Dominant WNV isolates suitable for preparation of a novel, superior vaccine were sequenced and compared to confirm the key genetic differences. Examples of North American Dominant isolates used in vaccine preparation include North American Equine E159(NAEE159) (ATCC Accession No. PTA-9409) and North American Donkey E159(NADE159).

Results and Conclusions

The critical Envelope (E) protein and Non-Structural 5 (NS5) protein was sequenced in these WNV isolates using standard laboratory techniques to determine genetic differences in nucleotide sequence as contrasted with WNV NY99. Notably, the North American Dominant isolates, of which specific examples are North American Equine E159(NAEE159) and North American Donkey E159(NADE159), displayed the changes which characterize North American Dominant WNV isolates and distinguish them from NY99 WNV, namely, the U to C mutation and C to U mutation at positions 1442 and 2466, respectively, of the nucleotide sequence encoding the E protein and the C to U mutation at position 9352 in the sequence encoding the NS5 protein. Figs. 10-17 show the sequence alignments of various regions of isolates. The alignments in the E region are relative to publically available reference sequences for a NY 99 isolate (deposited in GenBank as AY590210) and a North American Dominant isolate (WN 02 isolate) deposited in GenBank as AY590223. The alignments in the NS5 region are also relative to publically available reference sequences for a NY 99 isolate (deposited in GenBank as AY369442) and a North American Dominant isolate (WN 02 isolate) deposited in GenBank as AY369440). As shown by these alignments, North American Dominant WNV isolates have the same sequence changes relative to the NY 99 isolate as those defined in the definition for a North American Dominant WNV isolate. These sequences are provided herein as SEQ ID NOS. 1-22, the full length genome of a WN99 isolate is provided as

SEQ ID NO. 23, and the protein encoded by the full length genome of SEQ ID NO. 23 is provided as SEQ ID NO. 24.

Claims

1. An immunogenic composition comprising one or more strains of killed or inactivated West Nile Virus or any antigen thereof, wherein said immunogenic composition further comprises at least one antigen selected from the group consisting of Eastern Equine Encephalomyelitis Virus, Western Equine Encephalomyelitis Virus and Venezuelan Equine Encephalomyelitis Virus, Tetanus Toxoid, and combinations thereof, and wherein said West Nile Virus is one of the strains selected from the group consisting of Horse Origin 2005, deposited with the ATCC under accession number PTA-9409; NAEE159, deposit at the United States Department of Agriculture Isolate under accession number 405330; NY2002Nassau; NY2002Clinton; NY2002Queens; GA20021; GA20022; TX20021; TX20022; IN2002; NY2003Albany; NY2003Suffolk; NY2003Chatauqua; CO20031; CO20032; TX2003; TX2003Harris4; TX2003Harris6; TX2003Harris7; TX2003Harris10; AZ2004; TX2004Harris4; and combination thereof.

2. The immunogenic composition according to claim 1, wherein said immunogenic composition comprises at least one additional strain selected from the group consisting of Eastern Equine Encephalomyelitis Virus, Western Equine Encephalomyelitis Virus and Venezuelan Equine Encephalomyelitis Virus, Tetanus Toxoid, and combinations thereof.

3. The immunogenic composition according to any one of claims 1 to 2, wherein said immunogenic composition comprises one or more strains of Eastern Equine Encephalomyelitis, Western Equine Encephalomyelitis, Venezuelan Equine Encephalomyelitis Virus and Tetanus Toxoid.

4. The immunogenic composition according to any one of claims 1 to 3, wherein said immunogenic composition comprises at least one antigen or one additional strain of Equine Herpes Virus.

5. The immunogenic composition according to any one of claims 1 to 4, wherein said immunogenic composition comprises at least one additional strain of Equine Herpes Virus.

6. The immunogenic composition according to any one of claims 1 to 5, wherein said immunogenic composition comprises at least one antigen or one additional strain of Equine Influenza Virus.

7. The immunogenic composition according to any one of claims 1 to 6, wherein said immunogenic composition comprises at least one additional strain of Equine Influenza Virus.

8. The immunogenic composition according to any one of claims 1 to 7, wherein said immunogenic composition comprises one or more strains of Eastern Equine Encephalomyelitis Virus, Western Equine Encephalomyelitis Virus, Venezuelan Equine Encephalomyelitis Virus, Equine Influenza Virus, Equine Herpes Virus and Tetanus Toxoid.

9. The immunogenic composition according to any one of claims 1 to 8, wherein said West Nile Virus is a North American Isolate.

10. The immunogenic composition according to any one of claims 1 to 9, wherein said West Nile Virus is a North American Dominant Isolate.

11. The immunogenic composition according to any one of claims 1 to 10, wherein said Western Equine Encephalomyelitis Virus is the strain deposited with the ATCC under accession number PTA-9410.

12. The immunogenic composition according to any one of claims 1 to 11, wherein said Venezuelan Equine Encephalomyelitis Virus is the strain deposited with the ATCC under accession number PTA-9411.

13. The immunogenic composition according to any one of claims 1 to 12, wherein said Eastern Equine Encephalomyelitis Virus is the strain deposited with the ATCC under accession number PTA-9412.

14. The immunogenic composition according to any one of claims 5 to 13, wherein said Equine Herpes Virus is selected from the group consisting of the strains deposited with the ATCC under accession Nos. PTA-9525 or PTA-9526, and combinations thereof.

15. The immunogenic composition according to any one of claims 6 to 14, wherein said Equine Influenza Virus is selected from the group consisting of Influenza/Equine – 2/Ohio/03, Influenza/Equine-2/KY/95, Influenza/Equine-2/New Market/2/93, and combinations thereof.

16. The immunogenic composition according to any one of claims 6 to 15, wherein said Equine Influenza Virus is Influenza/Equine –2/Ohio/03, Influenza/Equine-2/KY/95, and Influenza/Equine-2/New Market/2/93.

17. The immunogenic composition according to any one of claims 6 to 16, wherein said Equine Influenza Virus is selected from the group consisting of the strains deposited with the ATCC under accession Nos. PTA-9522, PTA-9523 or PTA-9524 and combinations thereof.

18. The immunogenic composition according to any one of claims 1 to 17, wherein any of the strains are present in an amount from about $10^{2.0}TCID_{50}/mL - 10^{10.0}TCID_{50}/mL$ per dose.

19. The immunogenic composition according to any one of claims 1 to 18, wherein said immunogenic composition further comprises suitable excipients or pharmaceutical carrier.

20. The immunogenic composition according to claim 19, wherein said suitable excipients or pharmaceutical carrier is/are selected from the group consisting of a diluent, adjuvant, antimicrobial agent, inactivating agent, and combinations thereof.

21. The immunogenic composition according to claim 19 or 20, wherein said adjuvant is selected from the group consisting of, a polymer of acrylic or methacrylic acid, non-metabolized oil, and combinations thereof.

22. The immunogenic composition according to any one of claims 1 to 21, wherein said composition is to be administered to an equine.

23. The immunogenic composition according to any one of claims 1 to 22, wherein said immunogenic composition is to be administered in one or more doses.

24. The immunogenic composition according to any one of claims 1 to 23, wherein one dose of said immunogenic composition is formulated in 0.5ml to 2.5ml.

25. The immunogenic composition according to any one of claims 1 to 24, wherein said immunogenic composition provides duration of immunity of at least 12 months after the administration of one dose.

26. The immunogenic composition according to any one of claims 1 to 24, wherein said immunogenic composition is safe for use in foals or horses 4 months of age or older.

27. Use of an immunogenic composition according to any one of claims 1 to 26 in the manufacture of a medicament for reducing the incidence or lessening the severity of clinical symptoms associated with or caused by West Nile Virus, wherein said medicament is to be administered to an animal or a herd of animals in need thereof.

28. Use of an immunogenic composition according to any one of claims 1 to 26 in the manufacture of a medicament for reducing the incidence or lessening the severity of clinical symptoms associated with or caused by one or more of the pathogens selected from the group consisting of West Nile Virus, Eastern Equine Encephalomyelitis Virus, Western Equine Encephalomyelitis Virus, Venezuelan Equine Encephalomyelitis Virus and Clostridium tetani, wherein said medicament is to be administered to an animal or a herd of animals in need thereof.

29. Use of an immunogenic composition according to any one of claims 4 to 26 in the manufacture of a medicament for reducing the incidence or lessening the severity of clinical symptoms associated with or caused by one or more of the pathogens selected from the group consisting of: Eastern Equine Encephalomyelitis Virus, Western Equine Encephalomyelitis Virus, Venezuelan Equine Encephalomyelitis Virus, Equine Herpes Virus and Clostridium tetani, wherein said medicament is to be administered to an animal or a herd of animals in need thereof.

30. Use of an immunogenic composition according to any one of claims 6 to 26 in the manufacture of a medicament for reducing the incidence or lessening the severity of clinical symptoms associated with or caused by one or more of the pathogens selected from the group consisting of: West Nile Virus, Eastern Equine Encephalomyelitis Virus, Western Equine Encephalomyelitis Virus, Venezuelan Equine Encephalomyelitis Virus, Equine Herpes Virus, Equine Influenza Virus and Clostridium tetani, wherein said medicament is to be administered to in an animal or a herd of animals in need thereof.

31. The use according to any one of claims 27 to 30, wherein the incidence of clinical symptoms caused by one or more of said pathogens in a herd of animals is reduced from about 10% - 50% as compared to a herd not receiving said medicament.

32. The use according to any one of claims 27 to 31, wherein the administration of at least one dose of said medicament provides a duration of immunity of at least 12 months against one or more of said pathogens.

33. The use according to any one of claims 27 to 32, wherein said animal is a horse.

SEQUENCE LISTING

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caagttgtca cctacgccct aaacactttc accaacctgg ccgtccagct ggtgaggatg 540

atggaagggg aaggagtgat tggcccagat gatgtggaga aactcacaaa agggaaagga 600

cccaaagt 608

<210> 2
<211> 608
<212> DNA
<213> West Nile virus

<400> 2
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ctggagttcg aggctctggg ttttctcaat gaagaccact ggcttggaag aaagaactca 180

ggaggaggtg tcgagggctt gggcctccaa aaactgggtt acatcctgcg tgaagttggc 240

acccggcctg ggggcaagat ctatgctgat gacacagctg gctgggacac ccgcatcacg 300

agtgctgact tggaaaatga agctaaggtg cttgagttgc ttgatgggga acatcggcgt 360

cttgccaggg ccatcattga gctcacctat cgtcacaaag ttgtgaaagt gatgcgcccg 420

gctgctgatg gaagaaccgt catggatgtt atctccagag aagatcagag ggggagtgga 480

caagttgtca cctacgccct aaacactttc accaacctgg ccgtccagct ggtgaggatg 540

atggaagggg aaggagtgat tggcccagat gatgtggaga aactcacaaa agggaaagga 600

cccaaagt 608

<210> 3
<211> 909
<212> DNA
<213> West Nile virus

<220>
<221> misc_feature
<222> (57)..(57)
<223> n is a, c, g, or t

<400> 3
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gggaatgtca cacttgcatt tacaacatga tgggaaagag agagaaaaaa cccggagagt 180

tcggaaaggc taagggaagc agagccattt ggttcatgtg gctcggagct cgctttctgg 240

agttcgaggc tctgggtttt ctcaatgaag accactggct tggaagaaag aactcaggag 300

gaggtgtcga gggcttgggc ctccaaaaac tgggttacat cctgcgtgaa gttggcaccc 360

gacctggggg caagatctat gctgatgaca cagccggctg ggacacccgc atcacgagag 420

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ccagggccat cattgagctc acctatcgtc acaaagttgt gaaagtgatg cgcccggctg 540

ctgatggaag aaccgtcatg gatgttatct ccagagaaga tcagaggggg agtggacaag 600

ttgtcaccta cgccctaaac actttcacca acctggccgt ccagctggtg aggatgatgg 660

aaggggaagg agtgattggc ccagatgatg tggagaaact cacaaaaggg aaaggaccca 720

aagtcaggac ctggctgttt gagaatgggg aagaaagact cagccgcatg gctgtcagtg 780

gagatgactg tgtggtaaag cccctggacg atcgctttgc cacctcgctc cacttcctca 840

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attggcagc 909

<210> 4
<211> 975
<212> DNA
<213> West Nile virus

<220>
<221> misc_feature
<222> (3)..(3)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (12)..(12)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (813)..(813)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (881)..(882)
<223> n is a, c, g, or t

<400> 4
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cccaatacga ggaagatgta aacttgggaa gtggaaccag ggcggtggga aaacccctgc 180

tcaactcaga caccagtaaa atcaagaaca ggattgaacg actcaggcgt gagtacagtt 240

cgacgtggca ccacgatgag aaccacccat atagaacctg gaactatcac ggcagttatg 300

atgtgaagcc cacaggctcc gccagttcgc tggttaatgg agtggtcagg ctcctctcaa 360

aaccatggga caccatcacg aatgttacca ccatggccat gactgacact actcccttcg 420

ggcagcagcg agtgttcaaa gagaaggtgg acacgaaagc tcctgaaccg ccagaaggag 480

tgaagtacgt gctcaacgag accaccaact ggttgtgggc gtttttggcc agagaaaaac 540

gtcccagaat gtgctctcga gaggaattca taagaaaggt caacagcaat gcagctttgg 600

gtgccatgtt tgaagagcag aatcaatgga ggagcgccag agaagcagtt gaagatccaa 660

aattttggga gatggtggat gaggagcgcg aggcacatct gcgggggggaa tgtcacactt 720

gcatttacaa catgatggga aagagagaga aaaaacccgg agagttcgga aaggctaagg 780

gaagcagagc catttggttc atgtggctcg ganctcgctt tctggagttc gaggctctgg 840

gttttctcaa tgaagaccac tggcttggaa gaaagaactc nngaggaggt gtcgagggct 900

tgggcctcca aaaactgggt tacatcctgc gtgaagttgg cacccgacct gggggcaaga 960

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<210> 5
<211> 957
<212> DNA
<213> West Nile virus

<220>
<221> misc_feature
<222> (8)..(8)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (31)..(31)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (51)..(51)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (83)..(83)
<223> n is a, c, g, or t

<220>

<221> misc_feature
<222> (130)..(130)
<223> n is a, c, g, or t

<400> 5
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cgaaagctcn tgaaccgcca gaaggagtga agtacgtgct caacgagacc accaactggt 180

tgtgggcgtt tttggccaga gaaaaacgtc ccagaatgtg ctctcgagag gaattcataa 240

gaaaggtcaa cagcaatgca gctttgggtg ccatgtttga agagcagaat caatggagga 300

gcgccagaga agcagttgaa gatccaaaat tttgggagat ggtggatgag gagcgcgagg 360

cacatctgcg gggggaatgt cacacttgca tttacaacat gatgggaaag agagagaaaa 420

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<210> 6
<211> 961
<212> DNA
<213> West Nile virus

<220>
<221> misc_feature
<222> (30)..(30)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (41)..(41)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (62)..(63)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (107)..(107)
<223> n is a, c, g, or t

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cnngctcaac tcagacacca gtaaaatcaa gaacaggatt gaacgantca ggcgtgagta 120

cagttcgacg tggcaccacg atgagaacca cccatataga acctggaact atcacggcag 180
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tttgggtgcc atgtttgaag agcagaatca atggaggagc gccagagaag cagttgaaga 540
tccaaaattt tgggagatgg tggatgagga gcgcgaggca catctgcggg gggaatgtca 600
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aaatgaagct aaggtgcttg agttgcttga tggggaacat cggcgtcttg ccagggccat 960
c 961

<210> 7
<211> 1503
<212> DNA
<213> West Nile virus

<400> 7
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atcgatgtga agatgatgaa tatggaggcg gccaacctgg cagaggtccg cagttattgc 180
tatttggcta ccgtcagcga tctctccacc aaagctgcgt gcccgaccat gggagaagct 240
cacaatgaca aacgtgctga cccagctttt gtgtgcagac aaggagtggt ggacaggggc 300
tggggcaacg gctgcggact atttggcaaa ggaagcattg acacatgcgc caaatttgcc 360
tgctctacca aggcaatagg aagaaccatc ttgaaagaga atatcaagta cgaagtggcc 420
atttttgtcc atggaccaac tactgtggag tcgcacggaa actactccac acaggttgga 480
gccactcagg cagggagatt cagcatcact cctgcggcgc cttcacacac actaaagctt 540
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ggaagcagca ttggcaaagc ctttacaacc accctcaaag gagcgcagag actagccgct 1260

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acgcaaggat tgctgggggc tctcctgttg tggatgggca tcaatgctcg tgataggtcc 1440

atagctctca cgtttctcgc agttggagga gttctgctct tcctctccgt gaacgtgcac 1500

gct 1503

<210> 8
<211> 1503
<212> DNA
<213> West Nile virus

<400> 8
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gtggatttgg ttctcgaagg cgacagctgc gtgactatca tgtctaagga caagcctacc 120

atcgatgtga agatgatgaa tatggaggcg gccaacctgg cagaggtccg cagttattgc 180

tatttggcta ccgtcagcga tctctccacc aaagctgcgt gcccgaccat gggagaagct 240

cacaatgaca aacgtgctga cccagctttt gtgtgcagac aaggagtggt ggacaggggc 300

tggggcaacg gctgcggact atttggcaaa ggaagcattg acacatgcgc caaatttgcc 360

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atttttgtcc atggaccaac tactgtggag tcgcacggaa actattccac acaggctgga 480

gccactcagg cagggagatt cagcatcact cctgcggcgc cttcatacac actaaagctt 540

ggagaatatg gagaggtgac agtggactgt gaaccacggt cagggattga caccaatgca 600

tactacgtga tgactgttgg aacaaagacg ttcttggtcc atcgtgagtg gttcatggac 660

ctcaacctcc cttggagcag tgctggaagt actgtgtgga ggaacagaga gacgttaatg 720

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acctatggcg tctgttcaaa ggctttcaag tttcttggga ctcccgcaga cacaggtcac 960

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gct 1503

<210> 9
<211> 1376
<212> DNA
<213> West Nile virus

<220>
<221> misc_feature
<222> (1358)..(1358)
<223> n is a, c, g, or t

<400> 9
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ctaccgtcag cgatctctcc accaaagctg cgtgcccgac catgggagaa gctcacaatg 120

acaaacgtgc tgacccagct tttgtgtgca gacaaggagt ggtggacagg ggctggggca 180

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tccatggacc aactactgtg gagtcgcacg gaaactactc cacacaggtt ggagccactc 360

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cgggtcattt gaagtgtaga gtgaagatgg aaaaattgca gttgaaggga acaacctatg 780

gcgtctgttc aaaggctttc aagtttcttg ggactcccgc agacacaggt cacggcactg 840

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tggcttcatt gaacgaccta acgccagtgg gcagattggt cactgtcaac ccttttgttt 960

cagtggccac ggccaacgct aaggtcctga ttgaattgga accacccttt ggagactcat 1020

acatagtggt gggcagagga gaacaacaga tcaatcacca ttggcacaag tctggaagca 1080

gcattggcaa agcctttaca accaccctca aaggagcgca gagactagcc gctctaggag 1140

acacagcttg ggactttgga tcagttggag gggtgttcac ctcagttggg aaggctgtcc 1200

atcaagtgtt cggaggagca ttccgctcac tgttcggagg catgtcctgg ataacgcaag 1260

gattgctggg ggctctcctg ttgtggatgg gcatcaatgc tcgtgatagg tccatagctc 1320

tcacgtttct cgcagttgga ggagttctgc tcttcctntc cgtgaacgtg cacgct 1376

<210> 10
<211> 1376
<212> DNA
<213> West Nile virus

<400> 10
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ctaccgtcag cgatctctcc accaaagctg cgtgcccgac catgggagaa gctcacaatg 120

acaaacgtgc tgacccagct tttgtgtgca gacaaggagt ggtggacagg ggctggggca 180

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tccatggacc aactactgtg gagtcgcacg gaaactactc cacacaggct ggagccactc 360

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aggaaccaca cgccacgaag cagtctgtga tagcattggg ctcacaagag ggagctctgc 660

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cgggtcattt gaagtgtaga gtgaagatgg aaaaattgca gttgaaggga acaacctatg 780

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gcattggcaa agcctttaca accaccctca aaggagcgca gagactagcc gctctaggag 1140

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tcacgtttct cgcagttgga ggagttctgc tcttcctctc cgtgaacgtg catgct 1376

<210> 11
<211> 1376
<212> DNA
<213> West Nile virus

<400> 11
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aggcagggag attcagcatc actcctgcgg cgccttcata cacactaaag cttggagaat 420

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aggaaccaca cgccacgaag cagtctgtga tagcattggg ctcacaagag ggagctctgc 660

atcaagcttt ggctggagcc attcctgtgg aattttcaag caacactgtc aagttgacgt 720

cgggtcattt gaagtgtaga gtgaagatgg aaaaattgca gttgaaggga acaacctatg 780

gcgtctgttc aaaggctttc aagtttcttg ggactcccgc agacacaggt cacggcactg 840

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acatagtggt gggcagagga gaacaacaga tcaatcacca ttggcacaag tctggaagca 1080

gcattggcaa agcctttaca accaccctca aaggagcgca gagactagcc gctctaggag 1140

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atcaagtgtt cggaggagca ttccgcacac tgttcggagg catgtcctgg ataacgcaag 1260

gattgctggg ggctctcctg ttgtggatgg gcatcaatgc tcgtgatagg tccatagctc 1320

tcacgtttct cgcagttgga ggagttctgc tcttcctctc cgtgaacgtg catgct 1376

<210> 12
<211> 608
<212> DNA
<213> West Nile virus

<400> 12
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gctgctgatg gaagaaccgt catggatgtt atctccagag aagatcagag ggggagtgga 480

caagttgtca cctacgccct aaacactttc accaacctgg ccgtccagct ggtgaggatg 540

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cccaaagt 608

<210> 13
<211> 608
<212> DNA
<213> West Nile virus

<400> 13
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ctggagttcg aggctctggg ttttctcaat gaagaccact ggcttggaag aaagaactca 180

ggaggaggtg tcgagggctt gggcctccaa aaactgggtt acatcctgcg tgaagttggc 240

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agtgctgact tggaaaatga agctaaggtg cttgagttgc ttgatgggga acatcggcgt 360

cttgccaggg ccatcattga gctcacctat cgtcacaaag ttgtgaaagt gatgcgcccg 420

gctgctgatg gaagaaccgt catggatgtt atctccagag aagatcagag ggggagtgga 480

caagttgtca cctacgccct aaacactttc accaacctgg ccgtccagct ggtgaggatg 540

atggaagggg aaggagtgat tggcccagat gatgtggaga aactcacaaa agggaaagga 600

cccaaagt 608

<210> 14
<211> 981
<212> DNA
<213> West Nile virus

<400> 14
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gaaaaaaccc ggagagttcg gaaaggccaa gggaagcaga gccatttggt tcatgtggct 480

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agtgatgcgc ccggctgctg atggaagaac cgtcatggat gttatctcca gagaagatca 840

gaggggagt ggacaagttg tcacctacgc cctaaacact ttcaccaacc tggccgtcca 900

gctggtgagg atgatggaag gggaaggagt gattggccca gatgatgtgg agaaactcac 960

aaaagggaaa ggacccaaag t 981

<210> 15
<211> 974
<212> DNA
<213> West Nile virus

<400> 15
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cacgaaagct gaaccgccag aaggagtgaa gtacgtgctc aacgagacca ccaactggtt 180

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cggcgtcttg ccagggccat cattgagctc acctatcgtc acaaagttgt gaaagtgatg 780

cgcccggctg ctgatggaag aaccgtcatg gatgttatct ccagagaaga tcagaggggg 840

agtggacaag ttgtcaccta cgccctaaac actttcacca acctggccgt ccagctggtg 900

aggatgatgg aaggggaagg agtgattggc ccagatgatg tggagaaact cacaaaaggg 960

aaaggaccca aagt 974

<210> 16
<211> 970
<212> DNA
<213> West Nile virus

<400> 16
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gctctgaacc gccagaagga gtgaagtacg tgctcaacga gaccaccaac tggttgtggg 180

cgtttttggc cagagaaaaa cgtcccagaa tgtgctctcg agaggaattc ataagaaagg 240

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gagaagcagt tgaagatcca aaattttggg agatggtgga tgaggagcgc gaggcacatc 360

tgcggggga atgtcacact tgcatttaca acatgatggg aaagagagag aaaaaacccg 420

gagagttcgg aaaggctaag ggaagcagag ccatttggtt catgtggctc ggagctcgct 480

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tgatggaagg ggaaggagtg attggcccag atgatgtgga gaaactcaca aaagggaaag 960

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<210> 17
<211> 1503
<212> DNA
<213> West Nile virus

<400> 17
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cacaatgaca aacgtgctga cccagctttt gtgtgcagac aaggagtggt ggacaggggc 300

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atttttgtcc atggaccaac tactgtggag tcgcacggaa actactccac acaggttgga 480

gccactcagg cagggagatt cagcatcact cctgcggcgc cttcacacac actaaagctt 540

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gct 1503

<210> 18
<211> 1503
<212> DNA
<213> West Nile virus

<400> 18
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atcgatgtga agatgatgaa tatggaggcg gccaacctgg cagaggtccg cagttattgc 180

tatttggcta ccgtcagcga tctctccacc aaagctgcgt gcccgaccat gggagaagct 240

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tgctctacca aggcaatagg aagaaccatc ttgaaagaga atatcaagta cgaagtggcc 420

atttttgtcc atggaccaac tactgtggag tcgcacggaa actattccac acaggctgga 480

gccactcagg cagggagatt cagcatcact cctgcggcgc cttcatacac actaaagctt 540

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tactacgtga tgactgttgg aacaaagacg ttcttggtcc atcgtgagtg gttcatggac 660

ctcaacctcc cttggagcag tgctggaagt actgtgtgga ggaacagaga gacgttaatg 720

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gct 1503

<210> 19
<211> 968
<212> DNA
<213> West Nile virus

<220>
<221> misc_feature
<222> (7)..(7)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (10)..(10)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (13)..(13)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (941)..(942)
<223> n is a, c, g, or t

<400> 19
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atgacaaacg tgctgaccca gcttttgtgt gcagacaagg agtggtggac aggggctggg 180

gcaacggctg cggactattt ggcaaaggaa gcattgacac atgcgccaaa tttgcctgct 240

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ctcaggcagg gagattcagc atcactcctg cggcgccttc atacacacta aagcttggag 420

aatatggaga ggtgacagtg gactgtgaac cacggtcagg gattgacacc aatgcatact 480

acgtgatgac tgttggaaca aagacgttct tggtccatcg tgagtggttc atggacctca 540

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cgtcgggtca tttgaagtgt agagtgaaga tggaaaaatt gcagttgaag ggaacaacct 780

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cagtggcttc attgaacgat ctaacgccag tgggcagatt nntcactgtc aacccttttg 960

tttcagtg 968

<210> 20
<211> 979
<212> DNA
<213> West Nile virus

<220>
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<222> (4)..(4)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (6)..(6)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (10)..(10)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (974)..(974)
<223> n is a, c, g, or t

<400> 20
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gcacggaaac tactccacac aggctggagc cactcaggca gggagattca gcatcactcc 120

tgcggcgcct tcatacacac taaagcttgg agaatatgga gaggtgacag tggactgtga 180

accacggtca gggattgaca ccaatgcata ctacgtgatg actgttggaa caaagacgtt 240

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gatggaaaaa ttgcagttga agggaacaac ctatggcgtc tgttcaaagg ctttcaagtt 540

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cacactgttc ggangcatg 979

<210> 21
<211> 1004
<212> DNA
<213> West Nile virus

<220>
<221> misc_feature
<222> (4)..(4)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (12)..(12)
<223> n is a, c, g, or t

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<221> misc_feature
<222> (14)..(15)
<223> n is a, c, g, or t

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<221> misc_feature
<222> (17)..(17)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (32)..(32)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (35)..(35)
<223> n is a, c, g, or t

<400> 21
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<210> 22
<211> 1006
<212> DNA
<213> West Nile virus

<220>
<221> misc_feature
<222> (28)..(28)
<223> n is a, c, g, or t

<220>
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<222> (996)..(996)
<223> n is a, c, g, or t

<220>
<221> misc_feature
<222> (1004)..(1004)
<223> n is a, c, g, or t

<400> 22
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<210> 23
<211> 11029
<212> DNA
<213> West Nile virus

<400> 23
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Ala Leu Leu Ala Phe Phe Arg Phe Thr Ala Ile Ala Pro Thr Arg Ala
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Arg Arg Ser Ser Lys Gln Lys Lys Arg Gly Gly Lys Thr Gly Ile Ala
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Phe Gln Gly Lys Val Met Met Thr Val Asn Ala Thr Asp Val Thr Asp
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Val Ile Thr Ile Pro Thr Ala Ala Gly Lys Asn Leu Cys Ile Val Arg
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Ala Met Asp Val Gly Tyr Met Cys Asp Asp Thr Ile Thr Tyr Glu Cys
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Met Gln Arg Val Val Phe Val Val Leu Leu Leu Leu Val Ala Pro Ala
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Tyr Asn  Ala Asp Met Ile Asp  Pro Phe Gln Leu Gly  Leu Leu Val
    1145                1150                1155

Val Phe  Leu Ala Thr Gln Glu  Val Leu Arg Lys Arg  Trp Thr Ala
    1160                1165                1170

Lys Ile  Ser Met Pro Ala Ile  Leu Ile Ala Leu Leu  Val Leu Val
    1175                1180                1185

Phe Gly  Gly Ile Thr Tyr Thr  Asp Val Leu Arg Tyr  Val Ile Leu
    1190                1195                1200

Val Gly  Ala Ala Phe Ala Glu  Ser Asn Ser Gly Gly  Asp Val Val
    1205                1210                1215

His Leu  Ala Leu Met Ala Thr  Phe Lys Ile Gln Pro  Val Phe Met
    1220                1225                1230

Val Ala  Ser Phe Leu Lys Ala  Arg Trp Thr Asn Gln  Glu Asn Ile
    1235                1240                1245
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Leu Leu  Met Leu Ala Ala Val  Phe Phe Gln Met Ala  Tyr His Asp
    1250                 1255                 1260

Ala Arg  Gln Ile Leu Leu Trp  Glu Ile Pro Asp Val  Leu Asn Ser
    1265                 1270                 1275

Leu Ala  Val Ala Trp Met Ile  Leu Arg Ala Ile Thr  Phe Thr Thr
    1280                 1285                 1290

Thr Ser  Asn Val Val Val Pro  Leu Leu Ala Leu Leu  Thr Pro Gly
    1295                 1300                 1305

Leu Arg  Cys Leu Asn Leu Asp  Val Tyr Arg Ile Leu  Leu Leu Met
    1310                 1315                 1320

Val Gly  Ile Gly Ser Leu Ile  Arg Glu Lys Arg Ser  Ala Ala Ala
    1325                 1330                 1335

Lys Lys  Lys Gly Ala Ser Leu  Leu Cys Leu Ala Leu  Ala Ser Thr
    1340                 1345                 1350

Gly Leu  Phe Asn Pro Met Ile  Leu Ala Ala Gly Leu  Ile Ala Cys
    1355                 1360                 1365

Asp Pro  Asn Arg Lys Arg Gly  Trp Pro Ala Thr Glu  Val Met Thr
    1370                 1375                 1380

Ala Val  Gly Leu Met Phe Ala  Ile Val Gly Gly Leu  Ala Glu Leu
    1385                 1390                 1395

Asp Ile  Asp Ser Met Ala Ile  Pro Met Thr Ile Ala  Gly Leu Met
    1400                 1405                 1410

Phe Ala  Ala Phe Val Ile Ser  Gly Lys Ser Thr Asp  Met Trp Ile
    1415                 1420                 1425

Glu Arg  Thr Ala Asp Ile Ser  Trp Glu Ser Asp Ala  Glu Ile Thr
    1430                 1435                 1440

Gly Ser  Ser Glu Arg Val Asp  Val Arg Leu Asp Asp  Asp Gly Asn
    1445                 1450                 1455

Phe Gln  Leu Met Asn Asp Pro  Gly Ala Pro Trp Lys  Ile Trp Met
    1460                 1465                 1470

Leu Arg  Met Val Cys Leu Ala  Ile Ser Ala Tyr Thr  Pro Trp Ala
    1475                 1480                 1485

Ile Leu  Pro Ser Val Val Gly  Phe Trp Ile Thr Leu  Gln Tyr Thr
    1490                 1495                 1500
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Lys Arg Gly Gly Val Leu Trp Asp Thr Pro Ser Pro Lys Glu Tyr
1505 1510 1515

Lys Lys Gly Asp Thr Thr Thr Gly Val Tyr Arg Ile Met Thr Arg
1520 1525 1530

Gly Leu Leu Gly Ser Tyr Gln Ala Gly Ala Gly Val Met Val Glu
1535 1540 1545

Gly Val Phe His Thr Leu Trp His Thr Thr Lys Gly Ala Ala Leu
1550 1555 1560

Met Ser Gly Glu Gly Arg Leu Asp Pro Tyr Trp Gly Ser Val Lys
1565 1570 1575

Glu Asp Arg Leu Cys Tyr Gly Gly Pro Trp Lys Leu Gln His Lys
1580 1585 1590

Trp Asn Gly Gln Asp Glu Val Gln Met Ile Val Val Glu Pro Gly
1595 1600 1605

Lys Asn Val Lys Asn Val Gln Thr Lys Pro Gly Val Phe Lys Thr
1610 1615 1620

Pro Glu Gly Glu Ile Gly Ala Val Thr Leu Asp Phe Pro Thr Gly
1625 1630 1635

Thr Ser Gly Ser Pro Ile Val Asp Lys Asn Gly Asp Val Ile Gly
1640 1645 1650

Leu Tyr Gly Asn Gly Val Ile Met Pro Asn Gly Ser Tyr Ile Ser
1655 1660 1665

Ala Ile Val Gln Gly Glu Arg Met Asp Glu Pro Ile Pro Ala Gly
1670 1675 1680

Phe Glu Pro Glu Met Leu Arg Lys Lys Gln Ile Thr Val Leu Asp
1685 1690 1695

Leu His Pro Gly Ala Gly Lys Thr Arg Arg Ile Leu Pro Gln Ile
1700 1705 1710

Ile Lys Glu Ala Ile Asn Arg Arg Leu Arg Thr Ala Val Leu Ala
1715 1720 1725

Pro Thr Arg Val Val Ala Ala Glu Met Ala Glu Ala Leu Arg Gly
1730 1735 1740

Leu Pro Ile Arg Tyr Gln Thr Ser Ala Val Pro Arg Glu His Asn
1745 1750 1755

Gly Asn Glu Ile Val Asp Val Met Cys His Ala Thr Leu Thr His
1760 1765 1770

Arg Leu Met Ser Pro His Arg Val Pro Asn Tyr Asn Leu Phe Val
1775 1780 1785

Met Asp Glu Ala His Phe Thr Asp Pro Ala Ser Ile Ala Ala Arg
1790 1795 1800

Gly Tyr Ile Ser Thr Lys Val Glu Leu Gly Glu Ala Ala Ala Ile
1805 1810 1815

Phe Met Thr Ala Thr Pro Pro Gly Thr Ser Asp Pro Phe Pro Glu
1820 1825 1830

Ser Asn Ser Pro Ile Ser Asp Leu Gln Thr Glu Ile Pro Asp Arg
1835 1840 1845

Ala Trp Asn Ser Gly Tyr Glu Trp Ile Thr Glu Tyr Thr Gly Lys
1850 1855 1860

Thr Val Trp Phe Val Pro Ser Val Lys Met Gly Asn Glu Ile Ala
1865 1870 1875

Leu Cys Leu Gln Arg Ala Gly Lys Lys Val Val Gln Leu Asn Arg
1880 1885 1890

Lys Ser Tyr Glu Thr Glu Tyr Pro Lys Cys Lys Asn Asp Asp Trp
1895 1900 1905

Asp Phe Val Ile Thr Thr Asp Ile Ser Glu Met Gly Ala Asn Phe
1910 1915 1920

Lys Ala Ser Arg Val Ile Asp Ser Arg Lys Ser Val Lys Pro Thr
1925 1930 1935

Ile Ile Thr Glu Gly Glu Gly Arg Val Ile Leu Gly Glu Pro Ser
1940 1945 1950

Ala Val Thr Ala Ala Ser Ala Ala Gln Arg Arg Gly Arg Ile Gly
1955 1960 1965

Arg Asn Pro Ser Gln Val Gly Asp Glu Tyr Cys Tyr Gly Gly His
1970 1975 1980

Thr Asn Glu Asp Asp Ser Asn Phe Ala His Trp Thr Glu Ala Arg
1985 1990 1995

Ile Met Leu Asp Asn Ile Asn Met Pro Asn Gly Leu Ile Ala Gln
2000 2005 2010

Phe Tyr Gln Pro Glu Arg Glu Lys Val Tyr Thr Met Asp Gly Glu
2015 2020 2025

Tyr Arg Leu Arg Gly Glu Glu Arg Lys Asn Phe Leu Glu Leu Leu
2030 2035 2040

Arg Thr Ala Asp Leu Pro Val Trp Leu Ala Tyr Lys Val Ala Ala
2045 2050 2055

Ala Gly Val Ser Tyr His Asp Arg Arg Trp Cys Phe Asp Gly Pro
2060 2065 2070

Arg Thr Asn Thr Ile Leu Glu Asp Asn Asn Glu Val Glu Val Ile
2075 2080 2085

Thr Lys Leu Gly Glu Arg Lys Ile Leu Arg Pro Arg Trp Ile Asp
2090 2095 2100

Ala Arg Val Tyr Ser Asp His Gln Ala Leu Lys Ala Phe Lys Asp
2105 2110 2115

Phe Ala Ser Gly Lys Arg Ser Gln Ile Gly Leu Ile Glu Val Leu
2120 2125 2130

Gly Lys Met Pro Glu His Phe Met Gly Lys Thr Trp Glu Ala Leu
2135 2140 2145

Asp Thr Met Tyr Val Val Ala Thr Ala Glu Lys Gly Gly Arg Ala
2150 2155 2160

His Arg Met Ala Leu Glu Glu Leu Pro Asp Ala Leu Gln Thr Ile
2165 2170 2175

Ala Leu Ile Ala Leu Leu Ser Val Met Thr Met Gly Val Phe Phe
2180 2185 2190

Leu Leu Met Gln Arg Lys Gly Ile Gly Lys Ile Gly Leu Gly Gly
2195 2200 2205

Ala Val Leu Gly Val Ala Thr Phe Phe Cys Trp Met Ala Glu Val
2210 2215 2220

Pro Gly Thr Lys Ile Ala Gly Met Leu Leu Leu Ser Leu Leu Leu
2225 2230 2235

Met Ile Val Leu Ile Pro Glu Pro Glu Lys Gln Arg Ser Gln Thr
2240 2245 2250

Asp Asn Gln Leu Ala Val Phe Leu Ile Cys Val Met Thr Leu Val
2255 2260 2265

Ser Ala Val Ala Ala Asn Glu Met Gly Trp Leu Asp Lys Thr Lys
2270 2275 2280

Ser Asp Ile Ser Ser Leu Phe Gly Gln Arg Ile Glu Val Lys Glu
2285 2290 2295

Asn Phe Ser Met Gly Glu Phe Leu Leu Asp Leu Arg Pro Ala Thr
2300 2305 2310

Ala Trp Ser Leu Tyr Ala Val Thr Thr Ala Val Leu Thr Pro Leu
2315 2320 2325

Leu Lys His Leu Ile Thr Ser Asp Tyr Ile Asn Thr Ser Leu Thr
2330 2335 2340

Ser Ile Asn Val Gln Ala Ser Ala Leu Phe Thr Leu Ala Arg Gly
2345 2350 2355

Phe Pro Phe Val Asp Val Gly Val Ser Ala Leu Leu Leu Ala Ala
2360 2365 2370

Gly Cys Trp Gly Gln Val Thr Leu Thr Val Thr Val Thr Ala Ala
2375 2380 2385

Thr Leu Leu Phe Cys His Tyr Ala Tyr Met Val Pro Gly Trp Gln
2390 2395 2400

Ala Glu Ala Met Arg Ser Ala Gln Arg Arg Thr Ala Ala Gly Ile
2405 2410 2415

Met Lys Asn Ala Val Val Asp Gly Ile Val Ala Thr Asp Val Pro
2420 2425 2430

Glu Leu Glu Arg Thr Thr Pro Ile Met Gln Lys Lys Val Gly Gln
2435 2440 2445

Ile Met Leu Ile Leu Val Ser Leu Ala Ala Val Val Val Asn Pro
2450 2455 2460

Ser Val Lys Thr Val Arg Glu Ala Gly Ile Leu Ile Thr Ala Ala
2465 2470 2475

Ala Val Thr Leu Trp Glu Asn Gly Ala Ser Ser Val Trp Asn Ala
2480 2485 2490

Thr Thr Ala Ile Gly Leu Cys His Ile Met Arg Gly Gly Trp Leu
2495 2500 2505

Ser Cys Leu Ser Ile Thr Trp Thr Leu Ile Lys Asn Met Glu Lys
2510 2515 2520

Pro Gly Leu Lys Arg Gly Gly Ala Lys Gly Arg Thr Leu Gly Glu
2525 2530 2535

Val Trp Lys Glu Arg Leu Asn Gln Met Thr Lys Glu Glu Phe Thr
2540 2545 2550

Arg Tyr Arg Lys Glu Ala Ile Ile Glu Val Asp Arg Ser Ala Ala
2555 2560 2565

Lys His Ala Arg Lys Glu Gly Asn Val Thr Gly Gly His Pro Val
2570 2575 2580

Ser Arg Gly Thr Ala Lys Leu Arg Trp Leu Val Glu Arg Arg Phe
2585 2590 2595

Leu Glu Pro Val Gly Lys Val Ile Asp Leu Gly Cys Gly Arg Gly
2600 2605 2610

Gly Trp Cys Tyr Tyr Met Ala Thr Gln Lys Arg Val Gln Glu Val
2615 2620 2625

Arg Gly Tyr Thr Lys Gly Gly Pro Gly His Glu Glu Pro Gln Leu
2630 2635 2640

Val Gln Ser Tyr Gly Trp Asn Ile Val Thr Met Lys Ser Gly Val
2645 2650 2655

Asp Val Phe Tyr Arg Pro Ser Glu Cys Cys Asp Thr Leu Leu Cys
2660 2665 2670

Asp Ile Gly Glu Ser Ser Ser Ser Ala Glu Val Glu Glu His Arg
2675 2680 2685

Thr Ile Arg Val Leu Glu Met Val Glu Asp Trp Leu His Arg Gly
2690 2695 2700

Pro Arg Glu Phe Cys Val Lys Val Leu Cys Pro Tyr Met Pro Lys
2705 2710 2715

Val Ile Glu Lys Met Glu Leu Leu Gln Arg Arg Tyr Gly Gly Gly
2720 2725 2730

Leu Val Arg Asn Pro Leu Ser Arg Asn Ser Thr His Glu Met Tyr
2735 2740 2745

Trp Val Ser Arg Ala Ser Gly Asn Val Val His Ser Val Asn Met
2750 2755 2760

Thr Ser Gln Val Leu Leu Gly Arg Met Glu Lys Arg Thr Trp Lys
2765 2770 2775

Gly Pro Gln Tyr Glu Glu Asp Val Asn Leu Gly Ser Gly Thr Arg
2780 2785 2790

Ala Val Gly Lys Pro Leu Leu Asn Ser Asp Thr Ser Lys Ile Lys
2795 2800 2805

Asn Arg Ile Glu Arg Leu Arg Arg Glu Tyr Ser Ser Thr Trp His
2810 2815 2820

His Asp Glu Asn His Pro Tyr Arg Thr Trp Asn Tyr His Gly Ser
2825 2830 2835

Tyr Asp Val Lys Pro Thr Gly Ser Ala Ser Ser Leu Val Asn Gly
2840 2845 2850

Val Val Arg Leu Leu Ser Lys Pro Trp Asp Thr Ile Thr Asn Val
2855 2860 2865

Thr Thr Met Ala Met Thr Asp Thr Thr Pro Phe Gly Gln Gln Arg
2870 2875 2880

Val Phe Lys Glu Lys Val Asp Thr Lys Ala Pro Glu Pro Pro Glu
2885 2890 2895

Gly Val Lys Tyr Val Leu Asn Glu Thr Thr Asn Trp Leu Trp Ala
2900 2905 2910

Phe Leu Ala Arg Glu Lys Arg Pro Arg Met Cys Ser Arg Glu Glu
2915 2920 2925

Phe Ile Arg Lys Val Asn Ser Asn Ala Ala Leu Gly Ala Met Phe
2930 2935 2940

Glu Glu Gln Asn Gln Trp Arg Ser Ala Arg Glu Ala Val Glu Asp
2945 2950 2955

Pro Lys Phe Trp Glu Met Val Asp Glu Glu Arg Glu Ala His Leu
2960 2965 2970

Arg Gly Glu Cys His Thr Cys Ile Tyr Asn Met Met Gly Lys Arg
2975 2980 2985

Glu Lys Lys Pro Gly Glu Phe Gly Lys Ala Lys Gly Ser Arg Ala
2990 2995 3000

Ile Trp Phe Met Trp Leu Gly Ala Arg Phe Leu Glu Phe Glu Ala
3005 3010 3015

Leu Gly Phe Leu Asn Glu Asp His Trp Leu Gly Arg Lys Asn Ser
3020 3025 3030

Gly Gly Gly Val Glu Gly Leu Gly Leu Gln Lys Leu Gly Tyr Ile
3035 3040 3045

Leu Arg Glu Val Gly Thr Arg Pro Gly Gly Lys Ile Tyr Ala Asp
3050 3055 3060

Asp Thr Ala Gly Trp Asp Thr Arg Ile Thr Arg Ala Asp Leu Glu
3065 3070 3075

Asn Glu Ala Lys Val Leu Glu Leu Leu Asp Gly Glu His Arg Arg
3080 3085 3090

Leu Ala Arg Ala Ile Ile Glu Leu Thr Tyr Arg His Lys Val Val
3095 3100 3105

Lys Val Met Arg Pro Ala Ala Asp Gly Arg Thr Val Met Asp Val
3110 3115 3120

Ile Ser Arg Glu Asp Gln Arg Gly Ser Gly Gln Val Val Thr Tyr
3125 3130 3135

Ala Leu Asn Thr Phe Thr Asn Leu Ala Val Gln Leu Val Arg Met
3140 3145 3150

Met Glu Gly Glu Gly Val Ile Gly Pro Asp Asp Val Glu Lys Leu
3155 3160 3165

Thr Lys Gly Lys Gly Pro Lys Val Arg Thr Trp Leu Phe Glu Asn
3170 3175 3180

Gly Glu Glu Arg Leu Ser Arg Met Ala Val Ser Gly Asp Asp Cys
3185 3190 3195

Val Val Lys Pro Leu Asp Asp Arg Phe Ala Thr Ser Leu His Phe
3200 3205 3210

Leu Asn Ala Met Ser Lys Val Arg Lys Asp Ile Gln Glu Trp Lys
3215 3220 3225

Pro Ser Thr Gly Trp Tyr Asp Trp Gln Gln Val Pro Phe Cys Ser
3230 3235 3240

Asn His Phe Thr Glu Leu Ile Met Lys Asp Gly Arg Thr Leu Val
3245 3250 3255

Val Pro Cys Arg Gly Gln Asp Glu Leu Val Gly Arg Ala Arg Ile
3260 3265 3270

Ser Pro Gly Ala Gly Trp Asn Val Arg Asp Thr Ala Cys Leu Ala
3275 3280 3285

Lys Ser Tyr Ala Gln Met Trp Leu Leu Leu Tyr Phe His Arg Arg
3290 3295 3300

Asp Leu Arg Leu Met Ala Asn Ala Ile Cys Ser Ala Val Pro Val
3305 3310 3315

Asn Trp Val Pro Thr Gly Arg Thr Thr Trp Ser Ile His Ala Gly
3320 3325 3330

Gly Glu Trp Met Thr Thr Glu Asp Met Leu Glu Val Trp Asn Arg
3335 3340 3345

Val Trp Ile Glu Glu Asn Glu Trp Met Glu Asp Lys Thr Pro Val
3350 3355 3360

Glu Lys Trp Ser Asp Val Pro Tyr Ser Gly Lys Arg Glu Asp Ile
3365 3370 3375

Trp Cys Gly Ser Leu Ile Gly Thr Arg Ala Arg Ala Thr Trp Ala
3380 3385 3390

Glu Asn Ile Gln Val Ala Ile Asn Gln Val Arg Ala Ile Ile Gly
3395 3400 3405

Asp Glu Lys Tyr Val Asp Tyr Met Ser Ser Leu Lys Arg Tyr Glu
3410 3415 3420

Asp Thr Thr Leu Val Glu Asp Thr Val Leu
3425 3430