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Immunization of West Nile recombinant envelope Domain III with equine CD40 ligand protein vaccine induced specific immune response in rabbits and horses

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IMMUNIZATION OF WEST NILE RECOMBINANT ENVELOPE DOMAIN III WITH EQUINE CD40 LIGAND PROTEIN VACCINE INDUCED SPECIFIC IMMUNE RESPONSE IN RABBITS AND HORSES

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ABSTRACT

West Nile virus (WNV) is one of several flaviruses known to infect mammalian species, including humans. There were 15,257 horse cases reported in 2002 and 1,086 in 2006 in United States. Recently, significant increases in equine and human cases have been reported in United States. Domain III of the WNV envelope protein binds to cellular receptors, and induces a significant portion of the neutralizing antibody response against the virus. CD40 Ligand (CD40L, CD154) enhances productive interactions between T cells and APC and has been shown to function as a potential adjuvant. In this study, we constructed and expressed a fusion protein consisting of the Domain III of WNV envelope protein fused in-frame with the soluble portion of the equine CD40L. Immunizations of rabbits revealed that the recombinant protein induced antibody that specifically reacted with the WNV and neutralized the virus. Similar experiments were performed with horses. Western immunoblots confirmed that vaccinated horses produced antibodies that specifically reacted with the recombinant WNV E DIII proteins. The recombinant DIII protein with TiterMax or CD40L or both as adjuvant(s) induced significantly higher anti-WNV E DIII antibody activities than control and DIII alone groups after first vaccination. The recombinant DIII-CD40L protein vaccine continually induced the anti-WNV E DIII antibody activities without the adjuvant TiterMax. Moreover, the groups immunized with DIII-CD40L+TiterMax and DIII-CD40L showed stronger neutralization activities from week 8 than the other groups, and they maintained the high titers for at least 10 weeks. The results showed that healthy horses vaccinated with recombinant WNV E DIII protein with equine CD40L demonstrated an antigen specific humoral immune response. The responses were enhanced by booster vaccination.

Vaccination with this recombinant WNV E DIII-CD40L protein induced a WNV specific immunity in healthy horses that might contribute to protection from WNV-associated disease. CD40L could be utilized as a non-toxic, alternative adjuvant to boost the immunogenicity of subunit vaccines in horses.

INTRODUCTION

Taxonomy and epidemiology of West Nile virus

West Nile virus (WNV) was first isolated from a febrile patient in the West Nile province of Uganda in 1937 (Smithburn, 1940). After that, it has spread to more temperate regions that include Africa, Europe and North America (Bertelsen et al., 2004; McDonald et al., 2007; Nash et al., 2001; Shirafuji et al., 2009). WNV outbreaks were reported in Egypt and Israel (1951 and 1957), France (1962-1965), South Africa (1974), India (1980-1981), Ukraine (1985). More recent history includes outbreaks in Algeria (1994), Romania (1996), Morocco (1996 and 2003), Tunisia (1997 and 2003), Italy (1998), Czech Republic (1997), Israel (1997, 2000 and 2003), Russia (1999-2001), France (2000, 2003-2004) (Bernkopf, Levine, and Nerson, 1953; Chowers et al., 2001; Dauphin et al., 2004; George et al., 1984; Gerhardt, 2006; Hayes, 2001; Hayes et al., 2005; Hubalek and Halouzka, 1999; Joubert et al., 1970; Martina et al., 2011; Murgue et al., 2001; Murgue, Zeller, and Deubel, 2002; Platonov et al., 2001; Schneeweiss et al., 2011; Schuffenecker et al., 2005; Tsai et al., 1998; Zeller and Schuffenecker, 2004). WNV is primarily transmitted in nature by Culex mosquitoes. Birds are the amplifiers of the WNV diseases, and equines play an import indicator role in WNV outbreaks but are dead-end hosts.

WNV spread to all parts of continental U.S. after its initial introduction in New York at 1999 (Asnis et al., 2000; Iyer et al., 2009; Novello, 2000). There were 738 equine cases reports in USA during 2001. However, the number of WNV cases number increased to 15,257 in 2002 (Fig. 1) according to CDC. In the 1999 outbreak 36% of the

infected horses either died or were euthanized (Ostlund et al., 2001). The common clinical symptoms include weakness, incoordination and ataxia (Trock et al., 2001). Other studies showed a 22-28% mortality rate in horses after WNV infection (Salazar et al., 2004; Schuler et al., 2004).

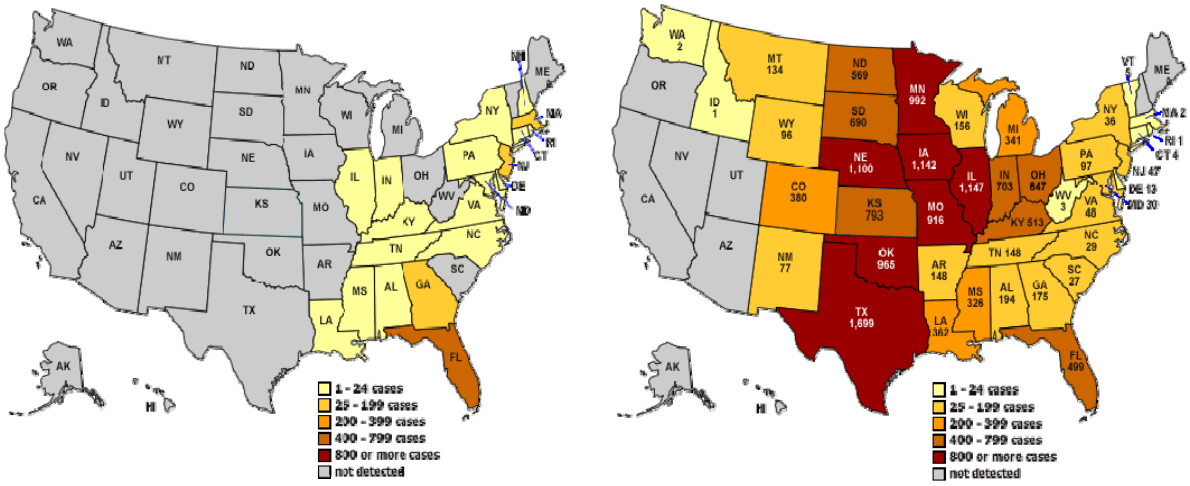

Figure 1. Equine cases of WNV infection in 2001 and 2002 (CDC, 2012).

Molecular characteristics of WNV

WNV belongs to flavivirus genus in the family Flaviviridae. It has an approximately 11kb single-stranded positive (+) sense RNA genome and a single open reading frame. The genome is translated as a single polyprotein (Fig. 2), which is subsequently cleaved by host as well as virally encoded proteases to give three structural proteins and seven non-structural proteins (Biedenbender et al., 2011; Chu and Ng, 2002; Lindenbach BD, 2007; Nowak, Farber, and Wengler, 1989). The structural proteins include a capsid (C) protein, a pre-membrane (prM) / membrane(M) and an envelope (E)

glycoprotein that mediates viral attachments, virus-induced membrane fusion and virion assembly (Mukhopadhyay et al., 2003; Mukhopadhyay, Kuhn, and Rossmann, 2005; Whiteman et al., 2010). The viral nonstructural proteins (NS1, NS2A, NS2B, NS3 NS4A, NS4B and NS5) regulate viral transcription/replication and attenuate host antiviral responses (Johansen et al., 2000; Lin et al., 2006) (Fig. 2). The icosahederal virion particles are about 500Å in diameter and the lipid-bilayer membrane of the nascent virus contains 180 molecules of the envelope (E) and premembrane (preM) proteins organized into 60 asymmetric trimeric spikes of preM-E heterodimers (Zhang et al., 2003). WNV envelope glycoprotein is the major antigenic determinant and is involved in virus binding and fusion (Bai et al., 2007; Iyer et al., 2009; Ledizet et al., 2007). The E protein exists as a dimer on the surface of mature virus particles and consists of a hydrophobic anchor responsible for the initiation of membrane fusion and 3 distinct domains (EI, EII, and EIII) essential for dimerization and receptor binding (Roehrig, 2003). EI and EII are formed from the folding of noncontiguous amino acid (AA) segments. EIII is formed by about 100 AAs and contains a single stretch and a fold that is immunoglobulin-like domain (Roehrig, 2003). More importantly, WNV Domain III region contains immune-dominant neutralizing epitopes (Chu, Chiang, and Ng, 2007a; Chu, Chiang, and Ng, 2007b; Kanai et al., 2006; Martina et al., 2011; Schneeweiss et al., 2011) (Fig. 3). This particular domain is an ideal vaccine target because it is the receptor-binding domain of envelope and it encodes the majority of the flavivirus type-specific (including WNV) epitopes, which is responsible for receptor specificity, vector preference, host range, and neutralization of WNV (Chu, Chiang, and Ng, 2007b; Martina et al., 2008; Martina et al., 2011; Schneeweiss et al., 2011).

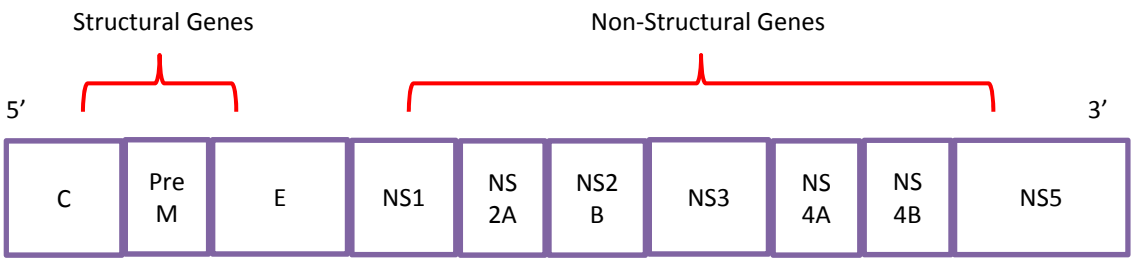

Figure 2. Organization of WNV genome. WNV has an approximately 11kb RNA genome which is transcribed as a single polyprotein. The polyprotein is cleaved by various viral and host proteases. The Capsid (C), pre-membrane (PreM) and envelope glycoproteins constitute the structural genes. WNV also encodes seven non-structural (NS) that are vital in transcription, translation and pathogenesis of the virus.

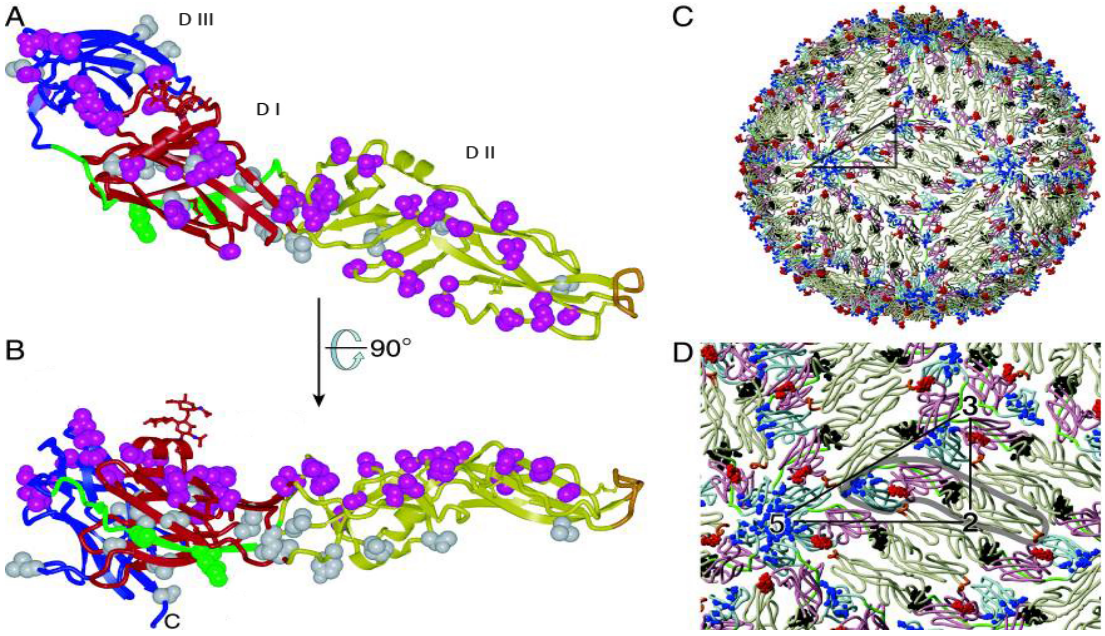

Figure 3. A and B: Structure of WNV envelope glycoprotein. The view in panel B is perpendicular to the viral surface. C: Atomic model of the WNV outer protein shell. D: Close-up of panel C. Modified from Kanai and Kar et al. (Kanai et al., 2006)

Immune responses

Humoral immune response plays a crucial role in protection against WNV infection (Diamond et al., 2003; Engle and Diamond, 2003). Immunological studies appeared in the literature in the 1950's (Hammon and Imam, 1957; Hammon and Sather, 1956; Imam and Hammon, 1957a; Imam and Hammon, 1957b). A majority of the

antibodies in flavivirus infections are directed against the E glycoprotein while a smaller population may be directed to preM/M (Colombage et al., 1998; Falconar, 1999; Pincus et al., 1992; Vazquez et al., 2002) and NS1 (Chung et al., 2006). The DII, containing the fusion loop, and DIII, containing the putative receptor binding domain of WNV E, are major targets for virus neutralization. Antibodies against the DII domain were found to be protective against WNV in mice (Gould et al., 2005). However, the most potent neutralizing antibodies are those that bind to the lateral surface of DIII domain and this forms the basis of many subunit vaccines (Alonso-Padilla et al., 2011; Beasley and Barrett, 2002; Choi et al., 2007; Chu, Chiang, and Ng, 2007a). Both $CD4^+$ (Burke, Wen, and King, 2004; Kulkarni, Mullbacher, and Blanden, 1991) and CD8 T^+ (Liu, Blanden, and Mullbacher, 1989) cells have been shown to respond in WNV infection. Wang *et al.* showed that mice infected with the Sarafend strain of WNV showed influx of $CD8^+$ but not $CD4^+$ T cells in the brain (Wang and Lobigs et al. 2003). Additionally, $CD8^+$ T cells in the brain and spleen also expressed the early activation marker CD69 along with CD25.

Diagnostic methods

Preliminary diagnosis of WNV infection is often based on the patient's clinical features, places and dates of travel, activities, and epidemiologic history of the location where infection occurred. Laboratory diagnosis of WNV infections is generally accomplished by testing of serum or cerebrospinal fluid (CSF) to detect virus-specific IgM and neutralizing antibodies. IgM capture EIAs can be used for diagnosis early in disease (Calisher et al., 1988; Calisher et al., 1985). The IgM response is relatively specific for each antigenic complex and is useful even at later times, because IgM persists

for at least 2 to 3 weeks after onset of disease (Calisher et al., 1986; Calisher et al., 1983). Real time RT-PCR can amplify the conserved region of WNV (Meyers, Rumenapf, and Thiel, 1989) and is ideal for rapid diagnosis on mass samples. Also, histopathology with immunohistochemistry and virus culture of autopsy tissues can be useful in fatal cases.

Current vaccines against WNV

Live attenuated vaccines: A live attenuated vaccine was developed by passaging the Israeli strain of WNV in mosquito cell line and selecting escape mutant using monoclonal antibodies (Lustig et al., 2000). The resulting virus WNV-25A was found to be genetically stable and had lost all neuroinvasiveness. This vaccine protected 100% of vaccinated mice and geese up on lethal challenge. Pletnev *et al.* generated two chimeric viruses (Pletnev et al., 2003). One of these had the WNV membrane precursor and envelope on a Dengue 4 (WNV/DEN4) background and the other had a 30 nucleotide deletion in the 3' non-coding region of DEN4 (WNV/DEN4-3'Δ30). Both these vaccines were attenuated in rhesus macaques, elicited high neutralizing antibody titers and prevented viremia in the monkeys against challenge. A follow-up study with the WNV/DEN4-3'Δ30 virus showed that it was unable to infect geese. The virus also showed reduced neurovirulence in intracranially-challenged suckling mice but had lost all neurovirulence in immunocompromised mice and was significantly attenuated monkeys (Pletnev et al., 2006). In a similar study, Huang *et al.* constructed two chimeric Dengue 2 viruses expressing WNV NY99 pre-M and E glycoprotein. These vaccines were shown to be attenuated and protected mice on challenge with the NY99 strain of WNV (Huang et

al., 2005). However, the risk of the attenuated virus undergoing recombination and changing of tropism from using is still questionable (Chu and Chiang et al. 2007).

Inactivated vaccines: A formalin-inactivated whole-virus veterinary vaccine developed by Fort Dodge Animal Health, IA and is sold under the brand name Innovator®. The vaccine was shown to be safe and efficacious (Ng et al., 2003) in horses and was granted full license by the USDA. This vaccine, however failed to elicit neutralizing antibodies in flamingos and red-tailed hawks (Nusbaum et al., 2003). Another experiment in golden hamsters vaccinated with the Fort Dodge vaccine showed that all vaccinated animals survived challenge with WNV. Eight out of nine animals (88.8%) showed hemagglutination Inhibition (HI) and complement fixing (CF) antibodies while 55.5% animals showed low levels of WNV neutralizing antibodies (Iyer et al., 2009; Tesh et al., 2002). Boehringer Ingelheim introduces five new Vetera® Vaccines including Vetera Gold + VEE, the only equine vaccine with 8-way disease protection in one convenient vaccine on 2010 (http://www.bi-vetmedica.com/news-and-announcements/equine_news/equine_press_releases/Boehringer-Ingelheim-Introduces-Five-New-Vetera-Vaccines.html). There are 9 killed/inactivated microorganisms including WNV in the vaccine.

Nucleic acid/DNA vaccines: A DNA vaccine expressing the WNV NY99 capsid gene was constructed and tested in mice (Yang et al., 2001). This vaccine was shown to elicit a strong Th1 immune response producing a robust peak in IL-2 and IFN-γ levels. Davis *et al.* engineered a DNA vaccine expressing WNV preM and E proteins (Davis et al., 2001). The vaccine protected 100% of the mice and generated robust neutralizing

antibody response in horses on challenge. In another study, a plasmid DNA encoding the infectious full length RNA genome of Kunjin virus was used to vaccinate mice. A single mutation in the NS1 gene of the Kunjin virus attenuated it in sucking mice. The vaccine was shown to protect against intracerebral and intraperitoneal challenge with both WNV NY99 and the Kunjin virus (Hall et al., 2003). Martin *et al.* carried out phase I clinical trials for a DNA vaccine expressing WNV NY99 preM and E genes In 2007 (Martin et al., 2007). The vaccine was safe and well tolerated, and elicited strong humoral and T cell responses. More recently, a capsid deleted Kunjin virus DNA vaccine was developed with the capsid being provided *in trans*. These viruses replicate only one time to generate Virus Like Particles (VLP) progeny that was found to be highly immunogenic in mice and horses (Chang et al., 2008)

Recombinant/subunit vaccines: Recently, several recombinant WNV vaccines, especially recombinant WNV E DIII proteins, have been developed for use against WNV infection in different animal species (Chang et al., 2007; Chu, Chiang, and Ng, 2007b; Demento et al., 2010; Fassbinder-Orth et al., 2009; H. El Garcha, 2008; Ishikawa et al., 2007; Martina et al., 2008; McDonald et al., 2007; Redig et al., 2011; Wang et al., 2001; Whiteman et al., 2010). Immunized mice generated high titer of WNV-neutralizing Abs after the immunization of recombinant WNV E DIII protein and the IgG isotypes generated were predominantly IgG2a in the murine sera against the recombinant protein (Chu, Chiang, and Ng, 2007b). Recombinant domain III (DIII) of WNV E protein in a mouse model induced high neutralizing antibody titers in the mouse model and protection against lethal WNV infection in C57BL/6 mice. This vaccine preparation also afforded partial protection against lethal JEV infection (Martina et al., 2008). A recombinant

influenza virus expressing DIII of the WNV glycoprotein E (Flu-NA-DIII) was evaluated as a WNV vaccine candidate in a mouse model. FLU-NA-DIII-vaccinated mice were protected from severe body weight loss and mortality caused by WNV infection, and subcutaneous immunization with 10^5 $TCID_{50}$ Flu-NA-DIII provided 100% protection against challenge. Adoptive transfer experiments demonstrated that protection was mediated by antibodies and CD4+T cells (Martina et al., 2011).

CD40 Ligand (CD40L, CD154) functions

CD40L is a trans-membrane protein expressed on activated T cells, granulocytes, macrophages, endothelial cells, vascular smooth muscle cells, and activated platelets (D'Aversa, Eugenin, and Berman, 2008; Hill et al., 2008; Schneider et al., 2007; Sitati et al., 2007). CD40L, one potential immune-enhancing molecule, is critical to productive interactions between T cells and APC and regulates equine inflammatory responses after exposure to flavivirus-infected cell (Klein and Diamond, 2008). Incubation of Human Brain Microvascular Endothelial Cells (HBMEC) with soluble CD40L resulted in increased expression of the adhesion molecules E-selectin, VCAM-1 and ICAM-1. Consequently, the adhesion of both resting and anti-CD3 activated CD4+ T lymphocytes to CD40L treated HBMEC was significantly increased compared to unstimulated EC, which suggests a potential mechanism by which activated CD40L expressing T cells could enhance adhesion and migration of inflammatory cells across the blood–brain barrier to sites of inflammation in the human central nervous system (Omari and Dorovini-Zis, 2003). Binding of CD40 by CD40L on activated $CD4^+$ T cells provides an important co-stimulatory signal for immunoglobulin class switching, antibody affinity

maturation, and priming of $CD8^+$ T cells (Fischer et al., 2007; Gares et al., 2006; Tripp et al., 2000). A requirement for CD40L in anti-viral immune responses was shown in studies that examined the anti-adenovirus response in CD40L-/- mice. Diminished CD4+ T cell priming and reduced IL-4, IL-10, and IFN-γ cytokine expression occurred in the absence of CD40L (Yang et al., 1996). The importance of CD40L expression for both the B and T cell immune responses was also shown in CD40L-/- mice challenged with lymphocytic choriomeningitis virus (Borrow et al., 1996; Oxenius et al., 1996). Soluble multimeric forms CD40L have been used as an adjuvant to enhance vaccine responses in ducks (Gares et al., 2006). Moreover, CD40-CD40L interactions are necessary to control acute WNV infection in mice, facilitate T cell migration across the blood-brain barrier to control WNV infection (Sitati et al., 2007), and equine CD40L regulates inflammatory responses (Klein and Diamond, 2008).

We hypothesize that the recombinant protein vaccine consisting of the WNV E DIII domain fused in-frame with the equine CD40L would produce specific humoral immune responses against WNV capable of neutralizing WNV infectivity.

MATERIALS AND METHODS

Cells and viruses

WNV (LSU-AR01), which was isolated in 2001 from a dead blue jay (*Cyanocitta cristata*) at Louisiana Animal Disease Diagnostic Laboratory (LADDL), LSU School of Veterinary Medicine (LSU SVM), and Vero cells in this study were provided by Dr. Alma Roy (LADDL). The virus was grown on Vero cells with Dulbecco's modified minimum essential media (DMEM) containing 5% fetal bovine sera and 1% antibiotics throughout this study.

Cloning and expression of recombinant WNV E DIII and DIII-CD40L proteins

WNV (LSU-AR01) viral RNA was extracted from WNV-infected Vero cells using TRI reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions. RT-PCR was used to amplify the gene encoding the WNV E DIII protein using the Superscript one-step RT-PCR system (Invitrogen Life Technologies).The gene sequence corresponding to aa 297-406 of the WNV E DIII domain protein was amplified by PCR from the viral cDNA using Fail Safe DNA Polymerase (Epicentre Biotechnologies, Madison, WI). A PCR product was obtained after PCR amplification under the following conditions: 35 cycles of denaturation at 95^0C for min, annealing at 56^0C for 1min, and extension at 72^0C for 1.5 min. Then the PCR product was purified, digested with the restriction enzymes *Eco*RI and *Xho*I, and followed by ligation into the corresponding restriction sites of the GenScript pGS-21a vecter. The nucleotide sequence was confirmed by DNA sequence analysis. Novagen

BL21 (DE3) *Escherichia coli* cells was transformed with the pGS-21a vector containing the inserted fragment. The recombinant bacteria were grown until OD_{600} of the culture reached 0.6-0.8, and protein expression was induced by adding isopropyl β-D-thiogalactoside (IPTG) to a final concentration of 0.5 mM at 37^0C for 3 hr. Cells were harvested by centrifugation at 4000×g for 20 min at room temperature. The recombinant WNV E DIII protein was expressed mainly in insoluble fractions. The pellets were washed twice with STE buffer (150 mM Nacl, 10 mMT Tris, imM EDTA pH 8.00) and treated using lysosome and benzonase nuclease. Bacteria cell lysates were centrifuged at 16,000×g for 30 min at 4^0C (Beckman Coulter) to obtain the supernatant for protein purification.

Similarly, the WNV E DIII (327bp) fused in-frame with the equine CD40L (711bp) gene segment (digested with the restriction enzymes *Eco*RI and *Xho*I) were ligated into GenScript pGS-21a vector. The vector was then transformed into BL21 (DE3) *E. coli* cells. Bacteria were cultured and treated as previous method to obtain the supernatant for protein purification.

Purification of recombinant WNV E DIII and DIII-CD40L proteins

The protein was purified by using Mini Profinity IMAC purification cartridge and desalting cartridge (Bio-RAD Profinia protein purification system, Cat#: 732-4610). About 11 ml supernatant of cell lysate was used in one purification process. The samples went through binding, washing, elution, and desalting procedures. Then the Profinia purification system automatically selected for purification the first highest protein-containing fraction that was well separated during the elution cycle. WNV E DIII protein

(40KDa) has high concentration yield (0.7 mg/ml). WNV E DIII-CD40L protein (65KDa) has low concentration yield (0.1 mg/ml). The protein sizes (40KDa / 65KDa) and concentration were verified by SDS-PAGE.

Immunization procedures

Rabbits. Two rabbits were obtained from the Division of Laboratory Medicine (DLAM), LSU SVM and maintained strictly according to the approved Institutional Animal Care and Use Committee protocols (IACUC). These rabbits had no serological evidence of exposure to WNV or other vaccines. Each rabbit was vaccinated using recombinant protein vaccine consisting of WNV E DIII protein fused in-frame with the equine CD40L on one occasion (2ml@0.1mg/ml), and received booster vaccinations two weeks later.

Horses. Fifteen horses (Gelding, age from 5 to 13 years old, from LSU Equine Health Studies Program) were selected on the basis of no-detectable WNV antibody against WNV via the plaque reduction neutralization test ($PRNT_{90}$). These horses were cared for according to standard operating procedures of DLAM and IACUC. These horses were randomly divided into five different groups and were immunized via the following protocols: The first group of three horses was vaccinated by intramuscular injection (im) (mid-cervical region) with purified recombinant WNV E DIII protein mixed with TiterMax (T) as adjuvant; second group of three horses was immunized with recombinant WNV E DIII-CD40L protein with TiterMax as adjuvant; third group was immunized with the recombinant WNV E DIII protein alone without any adjuvant; fourth group was immunized with the recombinant WNV E DIII-CD40L protein alone without

any adjuvant; fifth group was immunized with the commercial vaccine Vetera® Gold + VEE (Boehringer Ingelheim). Each animal in the tested groups was vaccinated with a recombinant protein vaccine (2ml; 0.1mg/ml), received a booster immunization with the same recombinant protein (2ml; 0.1mg/ml) at two weeks post immunization and an additional booster immunization (2ml; 0.1mg/ml) using recombinant protein only without TiterMax as adjuvant at Week 6. Horses in the control group received commercial vaccine only once at the beginning of experiment. TiterMax® adjuvants were bought commercially from CytRx Corporation. TiterMax® adjuvants contain block copolymer, CRL-8300, and form a stable water-in-oil emulsion.

Sera collection

Blood samples were collected from rabbits before immunization, 4 weeks after the first immunization, and 19 weeks after the first immunization. Equine venous blood was collected from each horse prior to vaccination, one week after initial vaccination, two weeks after initial vaccination and biweekly thereafter until 16 weeks. Blood samples were centrifuged at 1000 g for 10 minutes after collecting, and sera were separated and stored at -20 ^{0}C for further test. The specificity of the equine sera for WNV E DIII protein was determined using Western blot analysis, ELISA, and virus plaque reduction neutralization test.

Serologic analysis for Equine IgG

Immunoglobulin G subtypes (IgGa, IgGb, and IgG(T)) concentrations were measured in triplicate using commercially available equine-specific ELISA quantification

kits (IgGa: kit E70-124; IgGb: kit E70-127; and IgG(T): kit E70-105; Bethyl Laboratories Inc., Montgomery, TX) following the instructions of the manufacturer. Horse IgG was purified from the horse sera using Thermo Scientific NAbTM Spin Kits (89950) following the instructions for the specific anti-WNV E DIII antibody test.

Western blotting

Purified WNV DIII protein and DIII-CD40L protein were run under on an SDS-PAGE gradient (4-20%) gel. The protein was transferred to nitrocellulose, was blocked with 0.5% BSA in 0.05 M Tris and then incubated with purified horse IgG. Substrate (5-Bromo-4-chloro-3-indolyl Phosphate/Nitroblue Tetrazolium (BCIP/NBT)) was added after the membrane was incubated with Anti-Horse IgG (whole molecule)-Alkaline Phosphatase conjugate.

Plaque reduction neutralization test (PRNT)

Sera samples were inactivated by incubation at 56 ^{0}C for 30 min. Serial two-fold dilutions of the sera were incubated with equal volumes of 50 PFU of WNV-LSU-AR01 at 35 ^{0}C for 1 h. Sera virus mixtures were then added to Vero cell mono layers in 24-well plates in triplicates and the plates were incubated at 35 ^{0}C for another hour. Plates were then overlaid with DMEM containing 1% methyl cellulose and 2% fetal bovine sera. Plates were incubated at 35 ^{0}C for 72 h and then fixed with 10% formalin in phosphate buffered saline (PBS). Plates were washed three times with PBS and stained with 0.1% crystal violet. Plaques were counted and the highest dilution of sera resulting in reduction of 90% of the plaques was noted.

Statistical analysis

Data analysis was performed with GraphPad Prism software (GraphPad Software Inc., SanDiego, CA). Statistical significance was based on two tailed tests resulting in *p*-value <0.05.

RESULTS

Expression of the recombinant WNV E DIII and WNV E DIII-CD40L proteins

A gene segment encoding the WNV E DIII alone or WNV E DIII in-frame fused with the equine CD40L was cloned into the GenScript pGS-21a plasmid vector as described in Materials and Methods (Fig. 4). Then the plasmid vector was transformed into Novagen BL21 (DE3) *E. coli* cells for protein expression purposes.

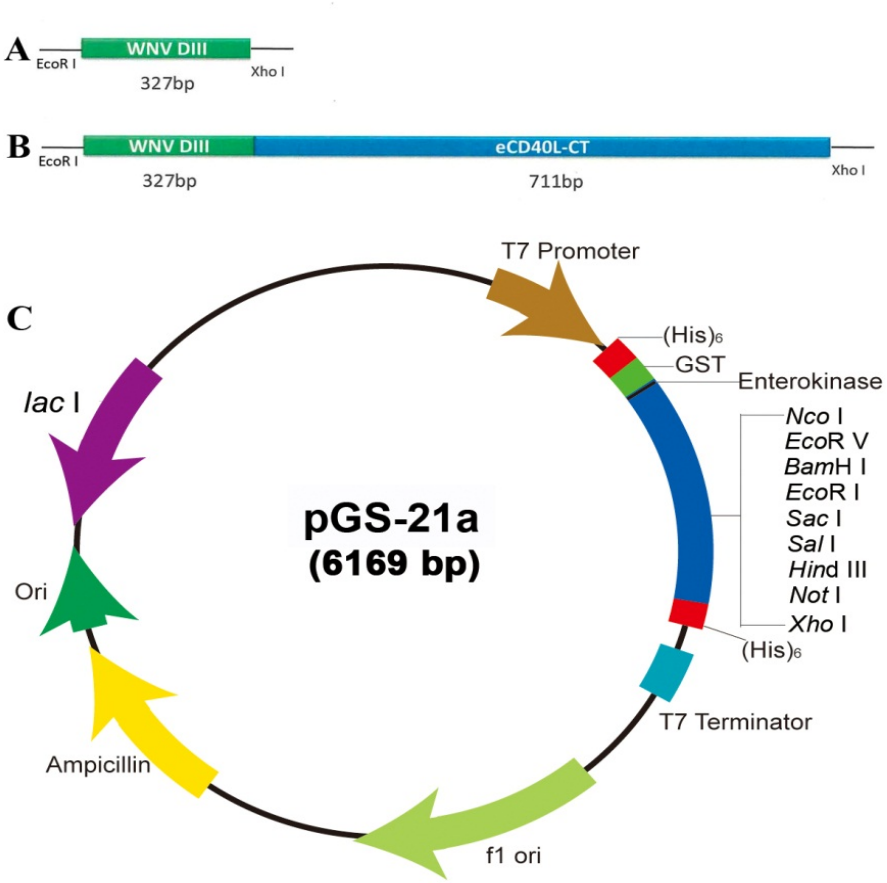

Figure 4. A: WNV E DIII sequence; B: WNV E DIII-CD40L sequence; C: GenScript pGS-21a vector sequence.

Recombinant protein expression and purification

Recombinant WNV E DIII / WNV E DIII-CD40L proteins were expressed in BL21 (DE3) *E. coli* cells transformed with the pGS21a expression vector after IPTG induction. Bacterial cell pellets and their supernatants were tested using SDS-PAGE after the harvested cells were centrifuged and washed with STE buffer (150 mM Nacl, 10 mMT Tris, imM EDTA pH 8.00). The expressed recombinant proteins were found only within cell pellets (Fig. 5). The recombinant WNV E DIII / WNV E DIII-CD40L proteins migrated with an apparent molecular mass of 40KDa and 65KDa, respectively (Fig. 6), in agreement with the predicted molecular masses. Higher amounts of soluble proteins after bacterial lysis were produced at lower temperature of 27^0C in comparison to 37^0C (Fig. 7). Substantially higher amounts of WNV DIII were expressed in *E. coli* in comparison to the DIII-CD40L protein (Fig. 6). The recombinant proteins within the cell pellets were purified using Mini Profinity IMAC purification cartridges and desalting cartridges (Bio-Rad Profinia Protein Purification System) after cell lysis using lysosome and benzonase nuclease treatments. Approximately 9-fold higher amount of total protein from *E. coli* lysates containing the WNV DIII domain were loaded on the purification columns in comparison to the WNV DIII-CD40L (Figs. 8, 9). The Bio-Rad Profinia purification system automatically selected for purification the first highest protein-containing fraction that was well separated during the elution cycle. The WNV DIII-CD40L purification resulted in proteins eluting after the collected fraction (Fig. 9), however, these fractions did not contain any specific protein bands relating to the desired WNV DIII-CD40L (not shown).

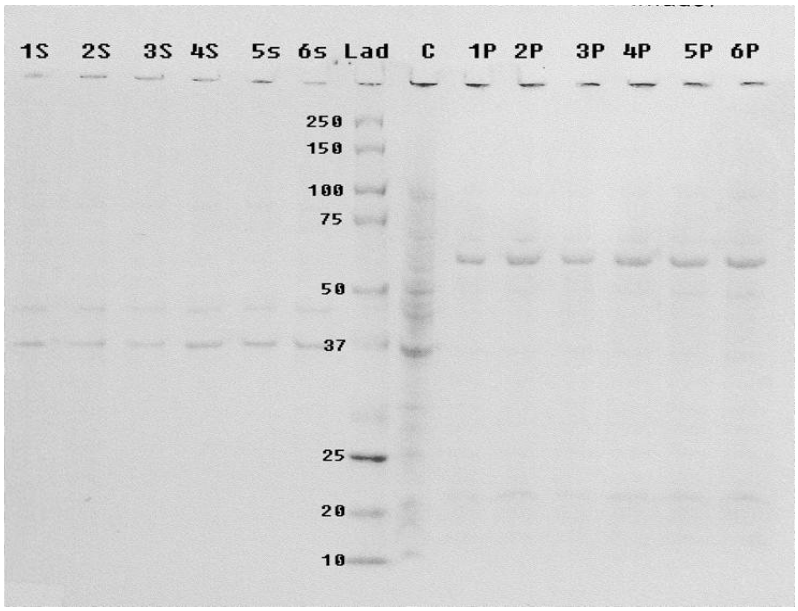

Figure 5. SDS-PAGE of WNV E DIII-CD40L proteins after IPTG induction and before bacterial lysis. 1S-6S: proteins from cell suspension; Lad: protein ladder; C: non-induced control; 1P-6P: proteins from cell pellets.

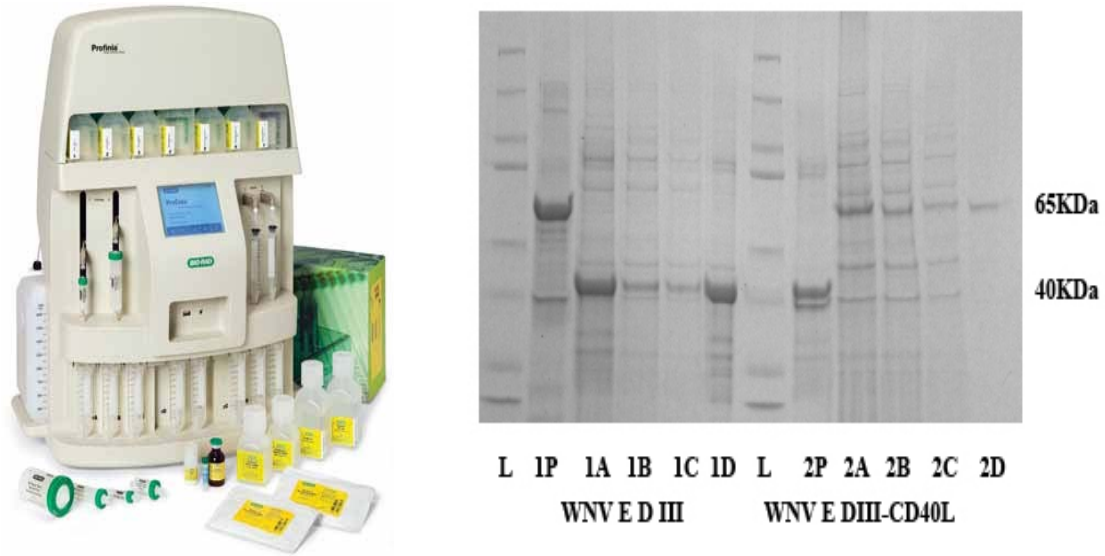

Figure 6. SDS-PAGE of recombinant WNV E DIII / DIII-CD40L (right) after purification using Bio-Rad Profinia protein purification system (left). L: ladders; 1P and 2P: cell pellet; 1A and 2A: soluble protein; 1B and 2B: flowthrough; 1C and 2C: wash 1; 1D and 2D: elution (purified proteins). The sizes of purified recombinant proteins were 40KDa and 65KDa, respectively.

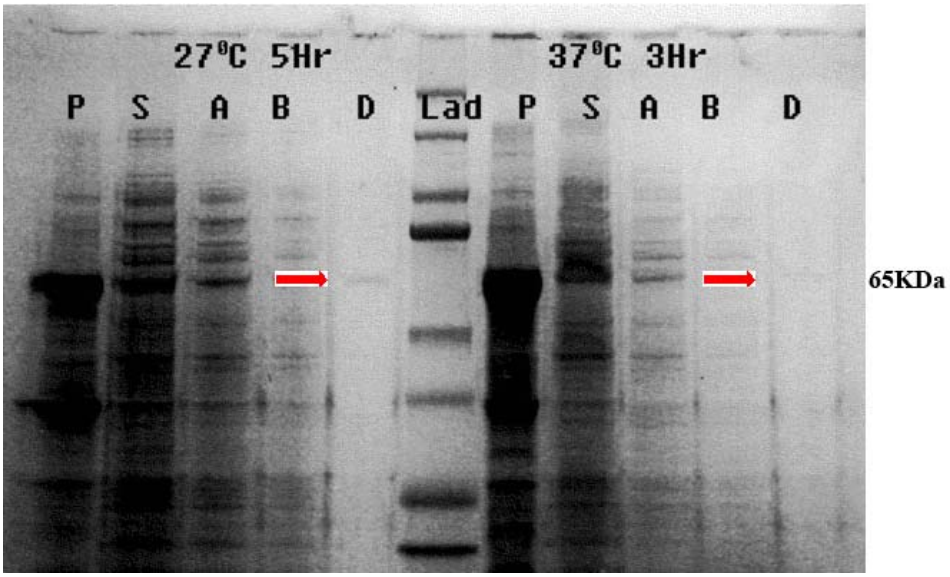

Figure 7. SDS-PAGE of recombinant WNV E DIII-CD40L purified proteins under different temperatures. L: ladder; P: cell pellet; S: soluble protein; A: flow through; B: wash; D: elution (purified proteins). Recombinant protein also can be induced at low temperature (27^0C).

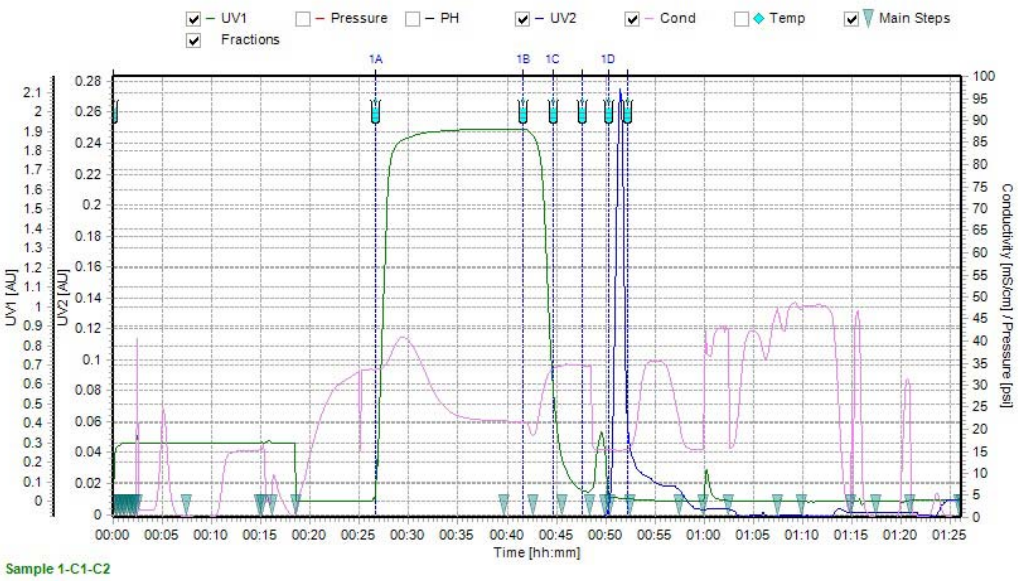

Figure 8. Purification of recombinant WNV E DIII protein through Mini Profinity IMAC purification cartridges and desalting cartridges (Bio-Rad Profinia Protein Purification System). 1A: flowthrough; 1B: wash 1; 1C: wash 2; 1D: elution (purified proteins).

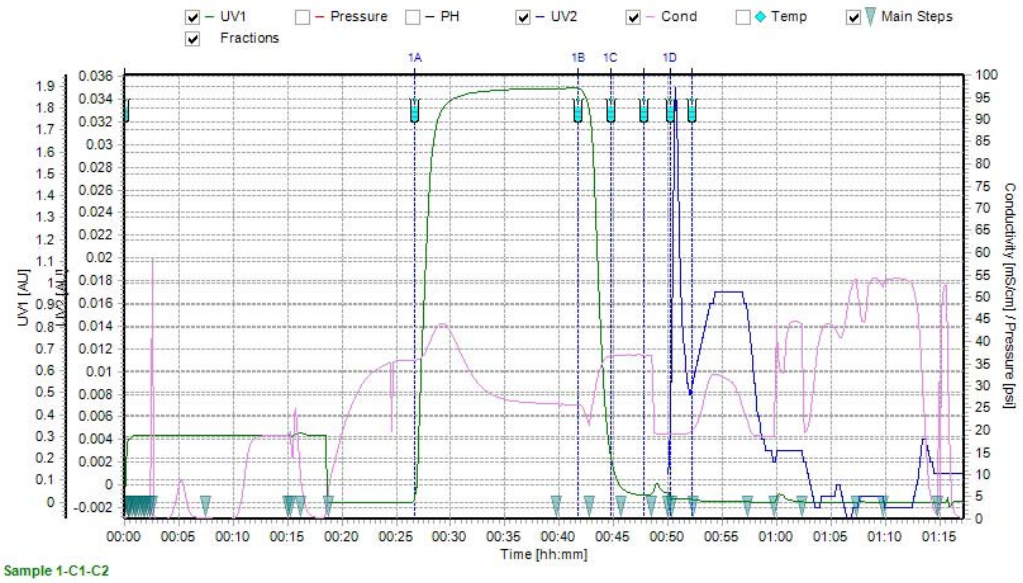

Figure 9. Purification of recombinant WNV E DIII-CD40L protein through Mini Profinity IMAC purification cartridges and desalting cartridges (Bio-Rad Profinia Protein Purification System). 1A: flowthrough; 1B: wash 1; 1C: wash 2; 1D: elution (purified proteins).

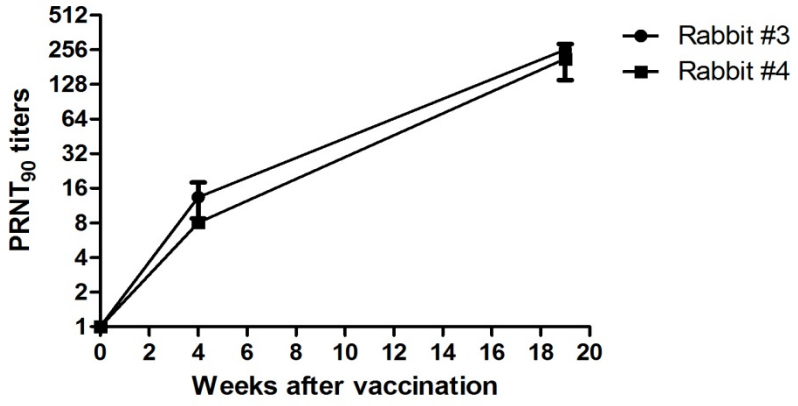

Figure 10. Sera neutralizing antibody titers ($PRNT_{90}$) from rabbits immunized with recombinant WNV E DIII-CD40L protein.

Rabbit immune responses to WNV E DIII-CD40L subunit protein vaccine

After the prime and boost immunization, the rabbits produced high antibody titers against WNV-LSU-AR01 strain. The $PRNT_{90}$ titers were between 1:128 to 1:256 at week

19 after vaccination (Fig. 10). The recombinant WNV E DIII-CD40L protein induced specific antibody reaction from rabbits after the immunization and maintained the titers for at least 19 weeks. Immunizations of rabbits revealed that the recombinant WNV E DIII-CD40L protein induced antibody that specifically reacted with the WNV envelope glycoprotein and neutralized the virus.

Clinical signs of immunized horses

Horses vaccinated with TiterMax as adjuvant exhibited inflammation at the injection site after the first booster vaccination. All other horses had no any symptoms. The injection site reaction for WNV E DIII resulted in plaques of edema ranging in size from 2 to 4 cm, while WNV E DIII-CD40L resulted in plaques of edema ranging in size of 4 to 7 cm. The injection sites of the horses vaccinated with WNV E DIII-CD40L+ TiterMax were warm and painful upon palpation lasting approximately 10 days. One injection site reaction included a purulent exudate. The edema at the reaction site of all the horses with reactions was resolved after four weeks post the first booster injection. Because of these apparent inflammatory reactions produced by TiterMax, additional booster vaccinations with recombinant proteins were applied by intramuscular injection on other side of neck without TiterMax.

Characterization of anti-WNV antibodies

Humoral immunity was measured following initial vaccination of horses for several immunoglobulin subtypes. The concentrations of serum IgGa in WNV E DIII-CD40L+T and WNV E DIII-CD40L groups were increased significantly after the second

booster vaccination and significantly higher than other groups (Fig. 11). The concentrations of serum IgGb and IgG(T) did not show significant changes during the experimental procedure among all five groups (Figs. 12, 13).

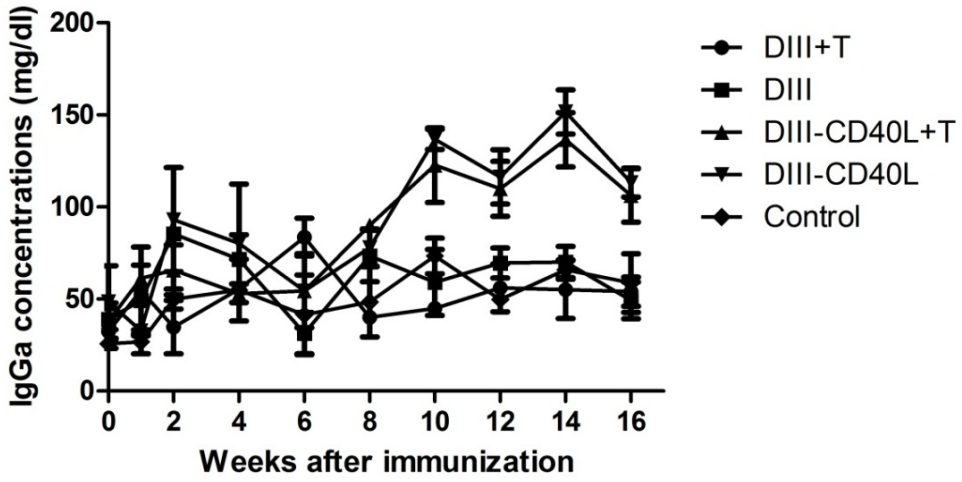

Figure 11. Time-dependent IgGa concentrations in sera obtained from vaccinated horses.

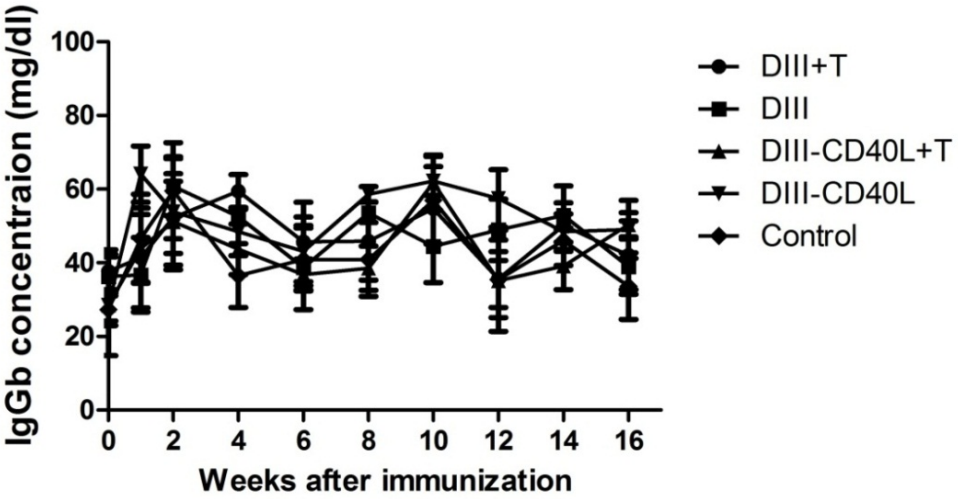

Figure 12. Time-dependent IgGb concentrations in sera obtained from vaccinated horses.

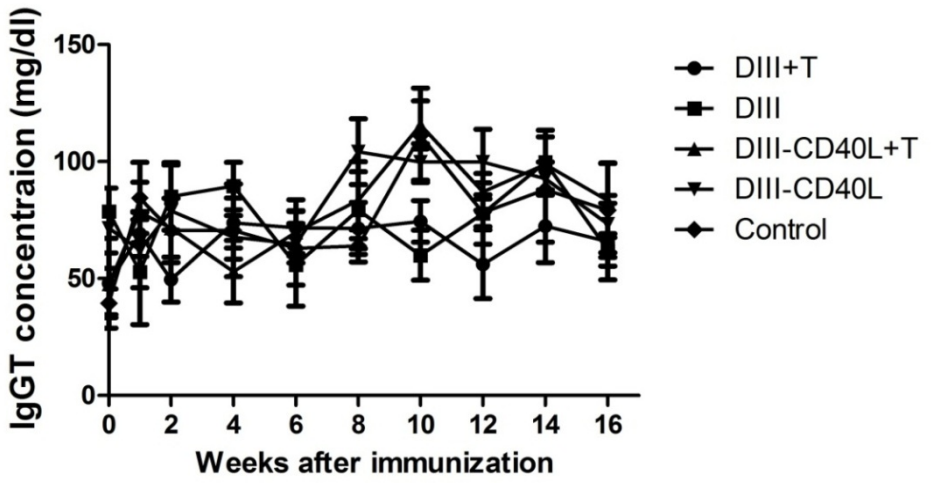

Figure 13. Time-dependent IgG(T) concentrations in sera obtained from vaccinated horses.

Horse IgG purification.

Horse sera were purified using Thermo Scientific NAb^{TM} Spin Kits (89950) to obtain pure IgGs, which eliminated the side-effect of other protein in sera for the ELISA results. Unpurified and purified IgG were run on SDS-PAGE (Fig. 14) and Western blot (Fig. 15). The purified recombinant WNV E DIII / DIII-CD40L proteins had very strong specific reaction with antibodies in sera from immunized horses (Fig. 15).

Specific reactions between recombinant WNV E DIII protein and antibodies in sera from the immunized horses

All horses immunized with the recombinant WNV E DIII / DIII-CD40L proteins developed specific anti-WNV E DIII antibody after the immunization. The group immunized with recombinant DIII only, showed similar antibody immune responses to recombinant WNV E DIII protein as the control group immunized with the commercial vaccine Vetera Gold + VEE (Boehringer Ingelheim). The groups immunized with

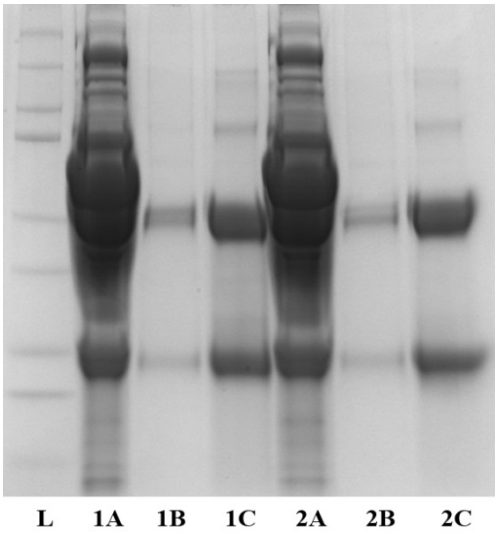

Figure 14. Antibody purification using Thermo Scientific NAb™ Spin Kits. L: ladder; 1A & 2A: horse serum before purification; 1B & 2B: purified IgG (first elution from the column); 1C & 2C: purified IgG (second elution from column).

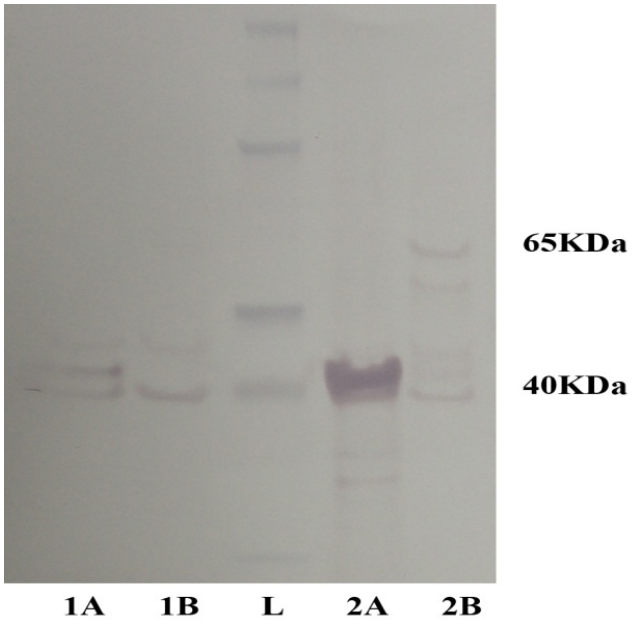

Figure 15. Detection of the recombinant WNV E DIII / DIII-CD40L proteins using Western immunoblot with sera from immunized horses. L: ladder; 1A: antibody from immunized horse sera slightly detected the WNV E DIII protein from un-induced *E. coli* cells lysis; 2A, antibody from immunized horse sera were able to detect the WNV E DIII protein; 1B: antibody from immunized horse sera were not able to detect the recombinant WNV E DIII-CD40L protein from un-induced *E. coli* cells lysis; 2B, antibody from immunized horse sera were able to detect the recombinant WNV E DIII-CD40L protein.

WNV E DIII+T / WNV E DIII-CD40L+T in the presence of TiterMax as adjuvant presented significantly strong anti-WNV E DIII antibody in the equine sera from Week 4 to Week 8 than other groups without TiterMax as adjuvant (Fig. 16). However, anti-WNV E DIII antibody activities in the group immunized with WNV E DIII-CD40L were significantly higher than other groups without the presence of CD40L after Week 14. The group immunized with DIII-CD40L+T showed the highest anti-WNV E DIII antibody activities during the period of Week 4 to the end of experiment.

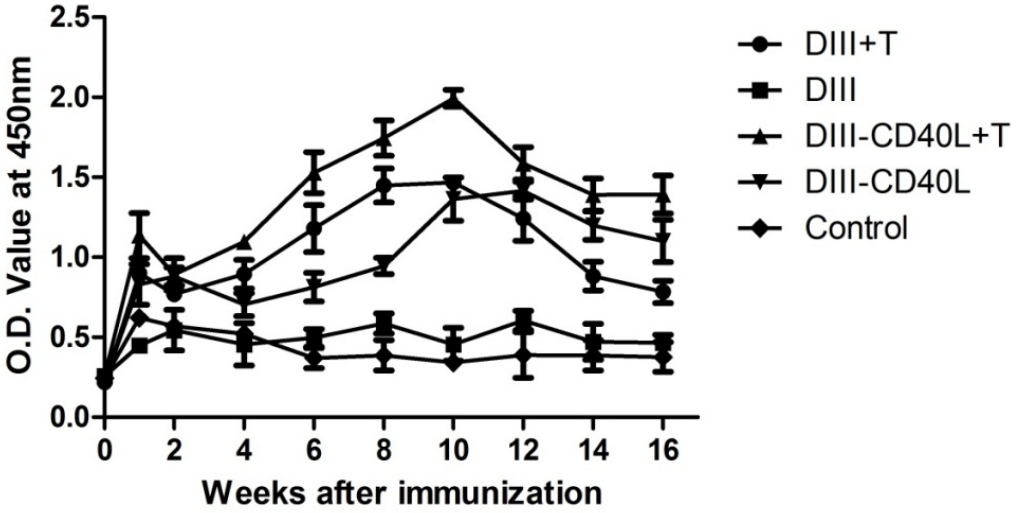

Figure 16. Time-dependent activities of specific antibody against WNV E DIII protein in sera obtained from vaccinated horses.

Sera from all five groups at week 12 after first immunization were tested using Western immunoblots assay. All the sera from the immunized horses presented specific anti-WNV E DIII antibody activities. Control group had the lowest anti-WNV E DIII antibody activities. The groups immunized with recombinant protein with CD40L (Fig. 17: lane 2 and 4) produced stronger specific anti-WNV E DIII antibodies than others groups (Fig. 17).

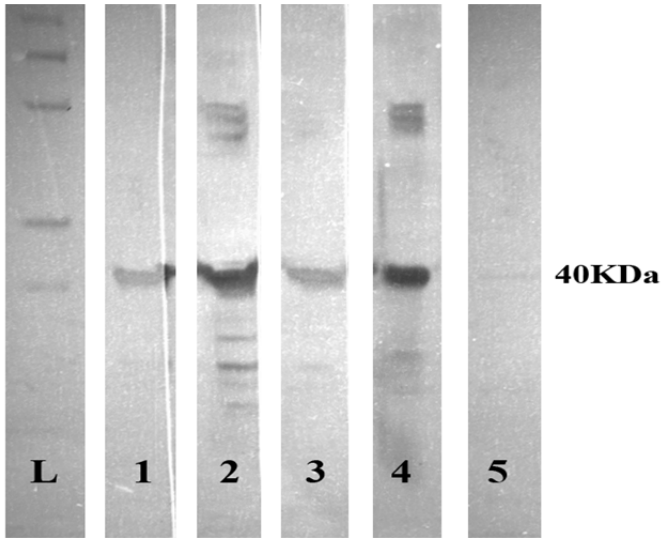

Figure 17. Testing of sera from immunized horses for ability to react with recombinant WNV E DIII protein in Western immunoblots. Sera from each group of horses at 12 weeks after vaccination were depicted in lanes numbered, 1, 2, 3, 4, and 5. Lane L: protein ladder; Lane 1: horse serum from group immunized with DIII+T; 2: horse serum from group immunized with DIII-CD40L+T; 3: horse serum from group immunized with DIII; 4: horse serum from group immunized with DIII+CD40L; 5: control group. Purified WNV E DIII proteins were used as antigen from lane 1 to 5.

Anti-WNV neutralization titers of horse antibodies

Plaque reduction neutralization tests were performed on the sera from immunized horses. The group immunized with WNV E DIII protein alone had the lowest $PRNT_{90}$ titer from the first vaccination to week 6, and was at the same level as control group from week 8 to week 16. The group immunized with recombinant WNV E DIII-CD40L protein showed stronger neutralization activity from week 6 in comparison to all other groups. Moreover, the group immunized with DIII-CD40L without the TiterMax as adjuvant also showed strong neutralization activities from week 8 in comparison with DIII+TiterMax and control groups and exhibited activity approaching the level of the group immunized with DIII-CD40L+T with TiterMax as adjuvant (Fig. 18). The group with DIII+TiterMax had significantly higher titers than DIII alone from week 2 to week

14, however, the titers decreased from week 12 and exhibited no significant difference compared to DIII alone at week 16.

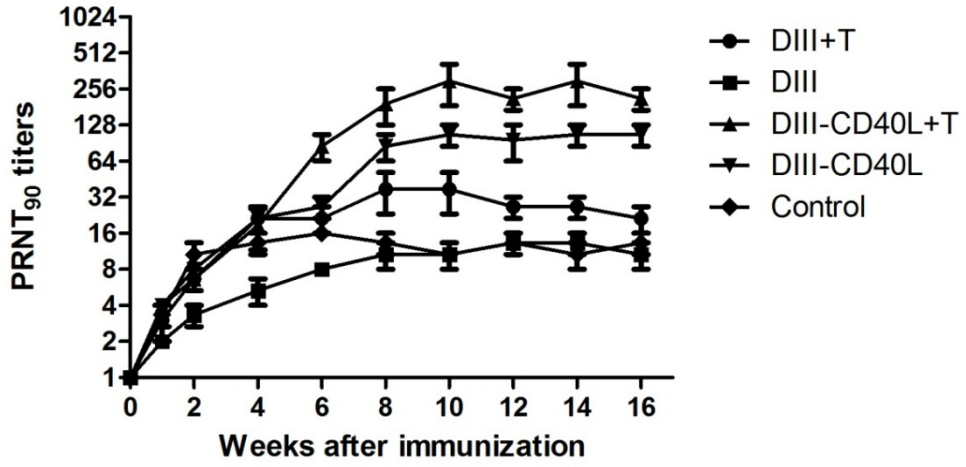

Figure 18. Sera neutralizing antibody titers ($PRNT_{90}$) from five groups of horses immunized with different vaccines.

DISCUSSION

As of November 6, 2012, 48 states have reported WNV infections in people, birds, or mosquitoes. A total of 5,054 cases of WNV disease in people, including 228 deaths, have been reported to CDC (Fig. 19). Of these, 2,559 (51%) were classified as neuroinvasive disease (such as meningitis or encephalitis) and 2,495 (49%) were classified as non-neuroinvasive disease.

The 5,054 cases reported thus far in 2012 is the highest number of WNV disease cases reported to CDC through the first week in November since 2003. Almost 80 percent of the cases have been reported from 12 states (Texas, California, Louisiana, Mississippi, Illinois, South Dakota, Michigan, Oklahoma, Nebraska, Colorado, Ohio, and Arizona) and over a third of all cases have been reported from Texas. (CDC, 2012).

With no specific treatments against WNV infection, developing an effective vaccine against WNV is an attractive proposition. A number of WNV vaccines had been evaluated in animal models and approved for usage on horses (Davis et al., 2001; El Garch et al., 2008; Kitai, Kondo, and Konishi, 2011; Kitai et al., 2011; Schneeweiss et al., 2011) . The current WNV vaccines on different species included inactivated / attenuated WNV horse vaccines (Monath et al., 2006; Orlinger et al., 2010; Samina et al., 2005) and recombinant vaccines using the canarypox virus, influenza A virus, vesicular stomatitis virus to express WNV antigens (El Garch et al., 2008; Iyer et al., 2009; Karaca et al., 2005; Martina et al., 2011; Minke et al., 2004).

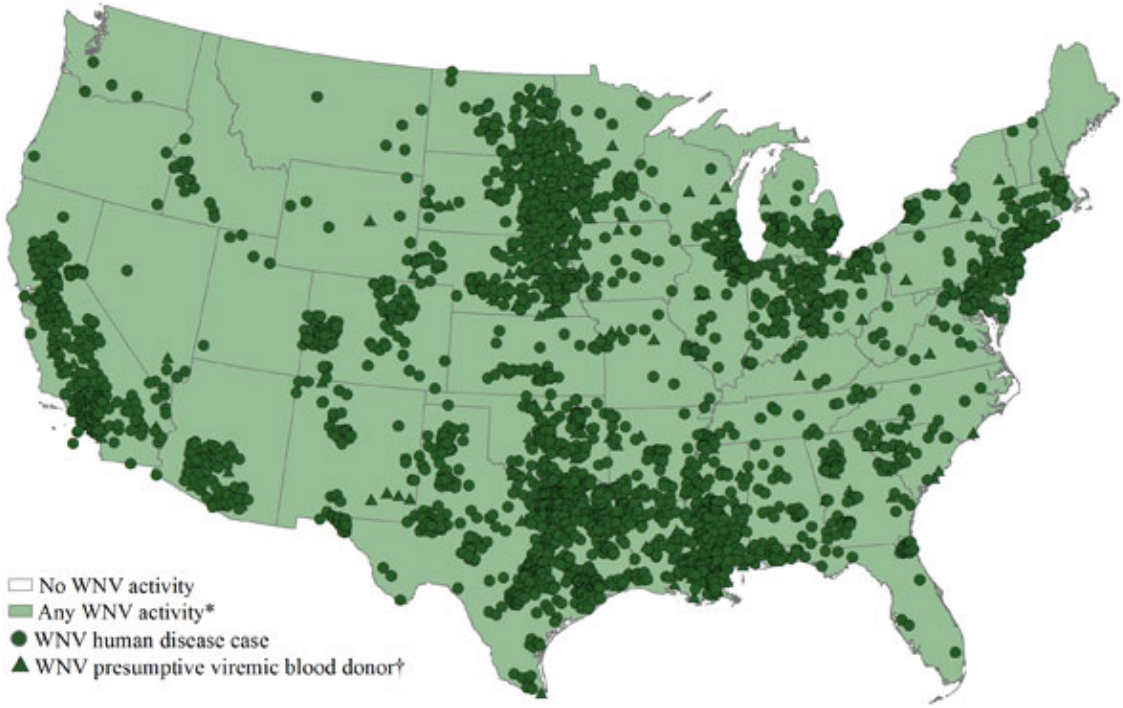

Figure 19. The map displays white areas that represent no WNV activity reported, light green areas that represent any WNV activity* , dark green circles that represent disease cases, and dark green triangles that represent presumptive viremic blood donors. * Includes WNV human disease cases, presumptive viremic blood donors, veterinary disease cases and infections in mosquitoes, birds, and sentinel animals. †Presumptive viremic blood donors have a positive screening test which has not necessarily been confirmed. Map shows the distribution of WNV activity* (shaded in light green), human infections (dark green circles), and presumptive viremic blood donors (dark green triangles) occurring during 2012 by state. If WNV infection is reported from any area of a state, that entire state is shaded. (CDC, 2012)

However, the risk of immune enhancement of heterologous flavivirus infection with inactivated virus and legitimate safety concerns with chimeric flavivirus are highly debatable (Chu, Chiang, and Ng, 2007b). Recently, researchers have focused on recombinant proteins as alternative vaccines on horses and other species (Alonso-Padilla et al., 2011; Bonafe et al., 2009; Chu, Chiang, and Ng, 2007b; Chu et al., 2005; Gershoni-Yahalom et al., 2010; Kitai, Kondo, and Konishi, 2011; Ledizet et al., 2005; Li et al., 2011; Martina et al., 2008; McDonald et al., 2007; Schneeweiss et al., 2011; Spohn et al., 2010; Wang et al., 2001; Whiteman et al., 2010). Moreover, many of the experiments

have investigated the WNV E DIII protein as safe and efficient vaccine (Alonso-Padilla et al., 2011; Chu, Chiang, and Ng, 2007b; Li et al., 2011; Martina et al., 2008; Martina et al., 2011; Schneeweiss et al., 2011). The WNV E DIII domain is highly conserved among several WNV strains. WNV E DIII functions as a receptor-binding domain, forming a continuous polypeptide segment that can fold independently, and therefore is an attractive candidate as a subunit vaccine.

Our results show that the recombinant WNV E DIII protein vaccine with the equine CD40L stimulates specific immune response in horses, and that equine CD40L enhances anti-WNV E DIII immune responses. Recombinant WNV E DIII was shown to be immunogenic and protective in mice challenged with virulent WNV and Japanese encephphalitis virus (JEV) (Chu, Chiang, and Ng, 2007b; Chu et al., 2005; Martina et al., 2008; Schneeweiss et al., 2011). In our study, the levels of anti-WNV E DIII antibodies increased in the sera from all horses after first immunization. Western immunoblots confirmed that vaccinated horses produced antibodies that specifically reacted with the recombinant WNV E DIII proteins (Fig. 16). The group immunized with WNV E DIII protein alone produced significantly lower anti-WNV E DIII antibody activity compared to other three test groups and also had lowest $PRNT_{90}$ titers before the second booster vaccination among all the groups (Fig. 16, 18). However, the recombinant DIII protein with TiterMax or CD40L or both as adjuvant(s) induced significantly higher anti-WNV E DIII antibody activities than control and DIII alone groups after first vaccination. These results suggest that both TiterMax and CD40L act as adjuvants to enhance humoral immune responses against the WNV DIII protein.

CD40L is a cell surface costimulatory molecule expressed mainly by activated T cells and CD40L is critically important for T-B cell and T cell-dendritic cell interactions. Previous studies suggested that CD40L might enhance both humoral and cellular immune responses and CD40L expression has been shown to enhance T cell and APC activation and signaling (Gurunathan et al., 1998; Lei et al., 1998). Constitutive retroviral immune responses of CD40L restored Ag-specific cytolytic and humoral immune responses in $CD40L^{-/-}$ mice (Brown et al., 1998). CD40-CD40L interactions are necessary to control acute WNV infection (Sitati et al., 2007).

CD40L, as adjuvant, stimulated the immune activity slower than TiterMax as adjuvant. The antibody activity in DIII-CD40L group was lower than DIII+TiterMax group from week 4 to week 8. But CD40L stimulated the immune activity continuously and the antibody activity in DIII-CD40L group was higher than DIII+TiterMax group after week 10. Also, the anti-DIII antibody activity induced by CD40L reached similar level in both DIII-CD40Land DIII-CD40L+TiterMax groups despite the existence of the adjuvant TiterMax (Fig. 16). Also, the groups immunized with DIII-CD40L and DIII+TiterMax had no significant difference on the $PRNT_{90}$ titers from week 0 to week 6. Moreover, the $PRNT_{90}$ titers in the group with DIII-CD40L dramatically increased from week 8 and significantly higher than the group with DIII+TiterMax from week 8 to week 16 (Fig. 18). These results suggest that CD40L could be applied as an efficient adjuvant to enhance humoral immune responses that could persist for longer times than those produced by synthetic adjuvants such as TiterMax.

The use of recombinant WNV E DIII protein as a WNV vaccine candidate has advantages over existing WNV immunization strategies. The recombinant WNV E DIII protein does not involve the use of attenuated or chimeric viruses, hence excluding the risk of the virus undergoing recombination and changing of tropism, which could result in increased pathogenicity (Chu, Chiang, and Ng, 2007b). And the safety issue using recombinant WNV E DIII protein for immunization had been addressed since no significant level of endotoxin contamination from bacteria was detected in the purified recombinant WNV E DIII protein used for immunization (Chu, Chiang, and Ng, 2007b). Also, further evaluation of this DIII-based vaccine in other mammalian species, including humans seems necessary.

Overall, our results showed that healthy horses vaccinated with recombinant WNV envelope Domain III protein with equine CD40L demonstrated the production of WNV- specific humoral immune responses. The responses were enhanced by booster vaccination. It could be concluded that vaccination with this recombinant WNV E DIII-CD40L protein induced a WNV specific immunity in healthy horses that could protect against WNV-associated disease. Overall, our study showed CD40L could be utilized as a non-toxic, alternative adjuvant to boost the immunogenicity of subunit vaccines in horses. Ultimately, vaccination-challenge experiments have to be performed in horses to assess whether this WNV DIII-CD40L recombinant protein vaccine can substantially reduce or eliminate WNV viremia and associated clinical disease symptoms in horses.

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