Assessment of Immunogenic Potential and Protective Efficacy of Recombinant Whole Envelope and Domain III Subunit Vaccine Candidate against West Nile Virus

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Abstract
West Nile virus is an emerging arthropod borne flavivirus with increasing distribution worldwide that is responsible for severe and fatal encephalitis in humans. Currently, there is no licensed human vaccine or specific therapy to prevent WNV infection. Present study was aimed to assess the immunogenicity and protective efficacy of recombinant whole envelope (rEnv) and domain III (rDIII) in mice model. Briefly, the gene coding for the envelope and domain III protein of WNV was cloned and expressed in pET 28a expression vector followed by purification. Balb/C mice were immunized with the purified recombinant proteins of rEnv and rDIII with a conc. of 25 and 50μg with Freund’s adjuvant. Booster injections with same formulation were given on 14 and 28 days after first immunization. Two booster doses were given subsequently. Humoral and cell mediated immune responses were determined by ELISA titr, PRNT assay and cytokine ELISA. Further assessment of cell mediated immune response from immunized mice revealed higher levels of both pro- and anti-inflammatory cytokines indicating a balance of Th1/Th2 type of immune response. All the mice were challenged with 100LD50 dose of WNV after 30 days of last booster and observed for 1 month. Mice immunized with recombinant whole envelope protein showed 100 % protection whereas domain III only confers 83 % protection. These results indicate that WNV whole envelope protein can be better candidate molecule for vaccine studies.

Keywords: WNV infection; PET 28a expression vector; Th1/Th2 type; Culex pipiens; Meningo encephalitis

Introduction
The reemergence of West Nile virus (WNV) since 1990s is a serious public health concern due to it associate with severe outbreaks and a high incidence of neurological disease and death. West Nile virus (WNV) is a 1 mosquito-borne zoonotic arbovirus with a single-stranded positive-sense RNA genome. It has been serologically classified in the Japanese encephalitis (JE) sero complex, which includes closely related viruses that are responsible for severe encephalitis in humans [1]. This flavivirus is found in temperate and tropical regions of the world. It was first identified in the West Nile sub region in the East African nation of Uganda in 1937. WNV is endemic in parts of Africa, Europe, the Middle East, and Asia [2-4] and following its emergence in the United States in 1999 it has rapidly spread across North America, and has recently been reported in Mexico, South America, and the Caribbean [5-7]. Until the 1990s, WNV had caused sporadic outbreaks with rare reports of encephalitis in humans and it re-emerged with virulence in Romania, Israel and Russia [8]. The West Nile virus (WNV) is transmitted through female mosquitoes, which are the prime vectors of the virus. Only females feed on blood, and different species have evolved to take a blood meal on preferred types of vertebrate hosts. The infected mosquito species vary according to geographical area; in the US, Culex pipiens (Eastern US), Culex tarsalis (Midwest and West), and Culex quinquefasciatus (Southeast) are the main sources.

Infection with WNV remains asymptomatic in the majority of cases or results in West Nile fever (a mild flu-like illness) in approximately 20 to 30% of infected cases [9-11]. The incubation period for WNV in humans is 2-14 days [12]. Symptoms may include fever, headaches, fatigue, muscle pain or aches, malaise, nausea, anorexia, vomiting, myalgias and rash. Less than 1% of the cases is severe and result in neurological disease when the central nervous system is affected [13,14]. The virus has a positive three structural (capsid [C], pre membrane [prM] or membrane [M], and envelope [E]) proteins and seven nonstructural (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5) proteins [15]. After a mosquito bite WNV infects keratinocytes cells, which migrate to regional lymph nodes where initial replication occurs WNV then spreads systemically to visceral organs, such as the kidney and spleen, where a second round of replication takes place, presumably in macrophages. Depending on the level of viremia WNV can cross the blood-brain barrier (BBB) into the brain and cause meningo encephalitis [16,17]. Diagnosis of West Nile Virus infections is generally accomplished by isolation and serologic testing of blood serum or cerebrospinal fluid (CSF), which is obtained via a lumbar puncture. Definitive diagnosis of WNV is obtained through detection of virus11specific antibody Immunoglobulin M, (IgM) and neutralizing antibodies by plaque reduction neutralization test (PRNT) assay [18,19]. The absence of effective treatment against WNV infection encourages vaccine development to prevent the infection. Although veterinary vaccines are commercialized to protect horses no licensed vaccine is available to prevent WNV infection in humans. The correlate of protection for flavivirus vaccines is the induction of neutralizing antibodies, as demonstrated by the vaccines against...

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Materials and Methods

Position of primers corresponds to the 967–2469 position of the genome of strain WNEG101 (GenBank accession no. AF260968.1). Similarly, domain III gene was amplified by using the forward primer [WN DIII F5- GGATCCTTTAACTGCCTTGGAAT 3' (BamHI)] and the reverse primer WN DIII R5- GTCGACTTAGCTTCCAGACCTTGTT-3' (SalI)] which corresponds to 1855–2175 position of the genome of WN Env 101 strain. The PCR purified products of both the genes were cloned in pET-28a expression vector and transformed into E. coli DH5α cells as per standard protocol. The construct of PET22 recombinant whole envelope and domain III were transformed into E. coli BL21 (DE3) competent cells as a expression host. The positive clones were confirmed by PCR and restriction analysis followed by nucleotide sequencing.  

The logarithmic phase cultures of recombinant whole envelope and DIII were induced with different conc. of IPTG (0.5, 1.5 and 2 mM) in Terrific and LB broth and checked at hourly interval for a period of 5 h. The localization of recombinant whole envelope and domain III protein were determined by sonication the induced cell pellet and the resultant cell lysate were centrifuged at 18,600 × g for 30 min at 4°C. The clear supernatant and remaining pellet were collected separately and then analyzed on 10% SDS-PAGE [24]. 2.3. Purification of the recombinant whole envelope and DIII proteins Recombinant clone of whole envelope gene in BL21 (DE3) cells was induced at 37 °C for 4 h in terrific broth containing 50 μg/ml kanamycin when the OD600 of the culture attained approx. 0.6. The cell pellet was suspended in 50 ml of cell lysis buffer and then sonicated for 5 min on ice. Supernatant was discarded and the inclusion body (IB) pellet was applied for further use. The IB pellet was suspended in 100 ml of IB wash buffer (pH 6.0) containing 50 mmol/l PO4, 200 mmol/l NaCl, 5 mmol/l EDTA, 1 mol/l urea, and 1% Triton-X100 followed by incubation at room temperature (RT) for 10 min and then centrifuged at 9500 × g at 4°C for 20 min. The supernatant was discarded and the purified IB pellet was used for solubilization. Inclusion body solubilization buffer (pH 8.0) containing 10 mmol/l Tris–Cl, 100 mmol/l NaCl, 100 mmol/l NaH2PO4, 1 mol/l EDTA, and 8 mol/l urea was added to the purified IB pellet and stirred overnight at RT. The recombinant protein was purified by immobilized metal affinity chromatography using a Ni-NTA super flow chelating agarose column [25]. Initially, the column was equilibrated with 20 ml of IB solubilization buffer (pH 8.0). Ultimately, the protein bound to the column was eluted by passing 15 ml of elution buffer (pH 4.0) containing 10 mmol/l Tris, 100 mmol/l NaCl, 100 mmol/l NaH2PO4, and 8 mol/l urea. Each elute was collected in 1ml fractions and analyzed on 10% SDS-PAGE. Similarly, domain III expressed protein was purified under native condition. Briefly, native lysis buffer was added at 5 ml/gram dry weight in the induced cell pellet and sonicated on ice. The lysate was centrifuged at 10000xg for 20 min at 4°C and the supernatant was preceded for further purification by immobilized metal affinity chromatography using a Ni-NTA super flow chelating agarose column. The column was first equilibrated with 20 ml of native lysis buffer (pH 8.0) followed by binding of the cell lysate. Next, the column was washed with 30 ml of native wash buffer (pH 8.0) and finally the protein bound to the column was eluted in 1 ml fractions by passing 15 ml of native elution buffer (pH 8.0). Both the purified proteins were dialyzed in dialyzing buffer (pH 6.0) and concentrated using 10kDa centrifugal filter device. Concentration of the purified protein was estimated by the bicinchoninic acid method [26].

Microorganism and cells

West Nile virus (WNV) RNA was extracted from 140μl of infected culture supernatant using QIAamp viral RNA mini kit (Qiagen, USA) according to manufacturer instruction. Whole envelope and domain III gene of WNV were cloned and expressed in E. coli expression system followed by purification by Ni-NTA affinity chromatography. Briefly, whole envelope gene of WNV was amplified using the forward primer specific for whole envelope gene [WNEnv F5- GGATCCTTTAACTGCCTTGGAAT 3' (BamHI)] and the reverse primer WN-Env R5- GTCGACTTAGCTTCCAGACCTTGTT-3' (SalI)]. The position of primers corresponds to the 967–2469 position of the genome of strain WNEG101 (GenBank accession no. AF260968.1). Similarly, domain III gene was amplified 

Cloning and expression of whole envelope and domain III genes

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Mice immunization

The animal experiments were performed according to the protocol approved by the Institutional Animal Ethics Committee (IAEC) at DRDE, Gwalior. Four weeks old Balb/C mice (n=6) were immunized subcutaneously at 2 weeks intervals with 25μg or 50μg per mouse of purified rEnv or rDIII proteins or PBS alone mixed with equal volume
Determination of Humoral Immune Response

Plaque reduction neutralization test (PRNT)

Antibodies titer generated against the recombinant whole 1 envelope and rWNDIII proteins were determined by standard indirect ELISA using the respective antigens. Briefly, 96-well micro titer plates were coated with 300 ng/ml of the purifier. Recombinant proteins rWNEnv and rWNDIII in 0.1M carbonate bicitarate buffer, (pH 9.6) overnight at 4°C. The coated wells were washed twice with PBS and blocked overnight at 4°C with 3% BSA in PBS. Serially two fold diluted sera in PBS starting with 1:500 were incubated in duplicate wells (100μl/well) at 37°C for 1 h. Pre-immunized sera were kept as control. The wells were washed three times at 2 min intervals with PBS-T (0.05% T). After washing, goat anti-mouse IgG Horseradish peroxidase (1:5000 dilution of goat anti4 mouse HRP conjugate) were applied and incubated at 37°C for 30 min. Finally the plates were washed thrice with PBS-T as mentioned above and developed the assay using TMB substrate and absorbance was measured at 450 nm using an ELISA plate reader. Mice sera from each group were used for the determination of ELISA endpoint titres.

Isotyping of antibody

Isotyping of immunized mice sera against whole envelope and domain III collected at 42 days of immunization were determined by mouse antibody subtyping kit (Sigma, St. Louis, MO, USA) following the manufacturer’s protocol. Briefly, 96 well microtiter plates were coated with the respective antigen and then blocked using 3% BSA. Further, the plate was incubated with 1:1000 dilution of immunized mice sera and pre immunized mice sera as control. After washing three times at 2 min interval with 10 PBST, wells were incubated with 1:1000 dilution of goat anti-1 mouse IgG each subtype specific antibodies IgG1, IgG2a, IgG2b, (Sigma, St. Louis, MO, USA), separately and incubated at 37°C for 1 h. Subsequently after washing, 1:5000 dilution of goat anti4 mouse HRP conjugate were applied and incubated at 37°C for 30 min. Finally the plates were washed thrice with PBST as mentioned above and developed the assay using TMB as substrate and absorbance was measured at 450 nm using ELISA reader.

Plaque reduction neutralization test (PRNT)

Sera obtained from mice immunized with recombinant proteins of whole envelope and domain III were evaluated for their neutralizing ability using Plaque reduction neutralization test (PRNT90) according to standard protocol [27] with pre immune sera used as a control. Briefly, Vero cells were seeded in a 24-well tissue culture plate at a density of 2×10^4 cells/well and incubated overnight at 37°C in 5% CO2 incubator. The next day, anti rWN-Env and rWN-E-DIII antibodies were serially two fold diluted starting from 1:20 to 1:640 and mixed with 1000 plaque-forming units (PFU) of West Nile virus and incubated at 37°C for 60 min. This virus antibody mixture was then inoculated (200 μl/well) to Vero cell monolayer along with suitable virus and cell controls and was allowed to adsorb for 2 hrs at 37°C in 5% CO2 incubator. Following adsorption, cells were washed with PBS and 1ml of MEM containing 2% FBS, 1.25% methyl cellulose was added to each well. The plate was kept at 37°C in 5% CO2 for 5 days. After washing with PBS, cells were fixed with chilled methanol and stained with 0.8% crystal violet solution. The neutralizing antibody titer was expressed as a reciprocal of the dilution of antibody, which showed a 1 90% reduction of plaque formation compared to the plaque numbers in the virus control [PRNT90].

In-vivo neutralization test

In vivo neutralization experiment was performed in 4 week old mice. In this study, mice sera collected after 42 days post vaccination with 25 and 50 μg dose of rWN-Env and rWN-E-DIII recombinant vaccine preparation was used as the source of neutralizing antibody. Serial two fold dilutions (1:10, 1:20 and 1:30) of heat inactivated post vaccinated serum samples were incubated with 104 PFU/ml of West Nile virus strain Flg101 for 2 hr at 37°C and injected subcutaneously into a group of 4 week old mice. Suitable healthy and virus control groups were also kept alongside. Mice were then observed for hind limb paralysis and death if any.

Assessment of cell-mediated immune response by cytokine profiling

Cytokine profiling of splenocytes (from various immunized groups) stimulated with particular antigen was done by commercially available ELISA kit (BD OptEIA™) according to manufacturer's instructions. After 7 day of booster dose immunization schedule, three mice from each group were dissected and the spleen were harvested. Splenocytes suspension was washed with PBS and then erythrocytes were lysed at room temperature using lysis solution containing 10mM NaHCO3 and 155 mM NH4Cl. The cell pellet was washed three times with RPMI 1640 media (Sigma, USA) and cells were re-suspended in RPMI 1640 medium supplemented with 10% FBS, 0.05 mM mercaptoethanol, 100U/ of penicillin per milliliter. Cells were then dispersed into each well of a round bottom 96 well cell culture plate at a density of 2×10^5 per ml. The splenocytes were then sensitized with the respective recombinant antigens at a final concentration of 10 μg/mI (in quadruplicate), along with unstimulated cell suspension as a negative control, and Concanavalin A (1 μg/ml) as a positive control. The plates were then incubated for 3 days at 37°C with 5% CO2. Culture supernatants were harvested and the concentration of the cytokine was analyzed by standard graph that was produced by known concentration of particular cytokine.

Determination of Lethal Dose (LD50) in balb/c Mice

Five groups of 4 week old mice were used to determine the lethal dose of West Nile Virus. West Nile virus was administrated subcutaneously at different pfu/ml in each group viz 101 pfu/ml, 102 pfu/ml, 103 pfu/ml, 104 pfu/ml and 105 pfu/ml along with one group was kept as PBS control. Mice were observed daily for developing virus symptoms and the number of mice died was recorded. The presence of WNV in the mice brain was confirmed by real time RT-PCR.

Challenge Studies in Mice Model

All the immunized mice group along with PBS control group was challenged with 100 LD50 dose of live WNV given subcutaneously and monitored daily for sign of illness for one month. Those showing signs of West Nile disease were anesthetized. In all the cases blood samples were collected prior to immunization and after the last booster doses, surviving mice were bled again 14 days after challenge. On day 30 after
challenege, all surviving mice were anesthetized.

**Results**

**Recombinant protein expression and purification**

A 1500 and 320bp DNA fragment of whole envelope and domain III gene of WNV were amplified. The cloned construct pET 28a-WNV whole envelope and DIII were confirmed with restriction digestion by specific restriction 1 enzymes. Optimal expression of the 55 and 13 kDa recombinant whole envelope and DIII protein were observed with induction of 1 mM IPTG for 4 h in the induced positive clone. Further SDS-PAGE analysis revealed the presence of recombinant proteins in insoluble and soluble fraction so the proteins were purified under denaturating and native condition. It was found to be approx. 95% pure by gel analysis with an estimated yield of 20 mg/l of shake flask culture. Antigenicity of the proteins were confirmed by western blot analysis which showed positive reactivity with polyclonal WN specific rabbit antisera (Figure 1a and 1b).

**Assessment of humoral immune response**

**Determination of antibody titer and IgG Isotype:** All the animals were bled 2 days prior to immunization to obtain pre-immune sera that served as control to assess different parameters. Post immunized mice sera were assessed for the generated antibodies by indirect ELISA with the respective antigens. The harvested sera were serially diluted and checked for presence of rWN Env and DIII antibodies by ELISA. A peak antibody ELISA titer of 50 and 25µg of rWN Env was 1:52,000 and 1:25,600 respectively, at 42 days of immunization schedule. Similarly, the highest antibody titer of 50 and 25 µg of rWN DIII was 1:64,000 and 1:32000 respectively, at 42 days of immunization. (Figure 2a). Sera collected on 42 day of post immunization from all the mice groups were analyzed for isotype. Sera against recombinant WN-Env and rWN-DIII showed similar pattern by inducing higher levels of IgG1 and IgG2b response with 25 or 50 µg doses. Whereas only 50 µg dose for both the recombinant proteins also showed IgG3 subtype of antibody (Figure 2b).

**In-vitro neutralization test:** In vitro virus neutralizing capacity of the antibodies for West Nile virus was determined by plaque reduction neutralization test (PRNT90). The PRNT90 titer of 25 and 50 µg doseof rWN E-DIII antibodies were observed 1:250 and 1:400 respectively. Similarly for 25 and 50 µg dose of rWN-Env antibodies, the PRNT90 titer were 1:640 and 1:700 respectively (Figure 2c). Recombinant whole envelope antigen shows complete protection in immunized mice at 25 µg WN-Env antibody.

**In-vivo neutralization test:** In vivo neutralization test, mouse protection was observed up to 1: 80 dilution of post vaccinated mice sera. Groups of 4 week old mice inoculated with post vaccinated sera and virus mix remained healthy, whereas, mice inoculated with virus only showed hind limb paralysis followed by death on 8-10 days of post inoculation.

**Assessment of cell-mediated immune response:** Splenocytes culture supernatants were analyzed for the level of interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-10 (IL-10), interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α). The level of cytokines have shown a dosedependency for both the proteins as 50µg dose induce more pronounced expression of both the pro and anti-inflammatory cytokines than the 25 µg dose of antigens. Ascompared to unstimulated splenocyte culture supernatant, the sensitized splenocyte produced high level of IFN-γ, IL-10 and moderate levels of IL-2, IL-4, IL-6, IL-10, TNF-α, and IL-12. Whereas, only 50 µg dose for both the recombinant proteins also showed IgG3 subtype of antibody (Figure 2b).

![Image](image-url)
Discussion

After WNV introduction in the US in 1999 and an unprecedented 1 peak epidemic in 2003, the number of cases dropped between 2008 and 2011. It was thus believed at that time that the virus would remain present at low-level transmission. However, in 2012, the US faced a new large epidemic with a high rate of neurologic disease, indicating that the country may expect periodic WNV outbreaks in the next decades associated with human morbidity and mortality. Besides, the recent cases reported in Europe indicate that WNV appears to be expanding its geographical range. The development of an effective WNV vaccine is therefore more than ever urgent to stop WNV spread to new areas and to protect the populations at risk from neurologic complications. So far, no human vaccine is available to control new WNV outbreaks and to avoid worldwide spreading. In contrast to the lack of WN vaccines for humans, a number of experimental vaccines have been successfully developed and tested, and several vaccines have been licensed for veterinary use. A formalin inactivated vaccine originally developed by Fort Dodge Animal Health, Fort Dodge, USA was licensed in 2003. This vaccine was shown to be safe and efficacious in horses [29]. Envelope is the major surface protein arranged in dimeric form and the role of envelope and its DIII gene is well determined as a vaccine candidate. The WNV envelope gene derived from a mosquito isolate was used to express the E glycoprotein. Mice vaccinated with the purified protein were shown to be protected on challenge [30]. Envelope protein was expressed in S2 cells were used to vaccinate mice and horses. All vaccinated mice survived and developed high WNV antibodies in mice and horses [31]. Several studies indicate that recombinant E-DIII protein can induce immune responses that protect mice from WNV infection when adjuvant with CpG oligo deoxynucleotides or in fusion with bacterial flagellin, a toll-like receptor-5 ligand [32]. A novel 1 kind of tetravalent DENV vaccine was produced based on the mixture of two bivalent E DIIIs of DENV in E. coli. This tetravalent vaccine, named MixBiEDIII, was evidenced to evoke humoral immune responses and protective antibodies against all four serotypes DENV [33]. The soluble fraction of the JEV domain III fusion protein expressed in E. coli maintained a proper folding of the native structure to induce neutralizing antibodies and protective immunity in mice [34]. Present study was aimed to assess the immunogenic potential of recombinant whole envelope and domain III protein of WNV followed by protective efficacy in mice model. For this study, recombinant whole envelope and DIII proteins were expressed in E. coli bacterial expression system under the control of an IPTG T7 promoter. Expressed whole envelope recombinant protein was purified under denaturing conditions whereas DIII was localized in soluble form followed by purification under native condition using Ni-NTA affinity chromatography. Analysis by