

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 titer, 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 its association with severe outbreaks and a high incidence of neurological disease and death. West Nile virus (WNV) is a 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 1 [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 virus specific antibody Immunoglobulin M, (IgM) and neutralizing antibodies by plaque reduction neutralization test (PRNT) assay [18,19]. The absence of effective treatment against WN 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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Yellow Fever, Japanese encephalitis and Tick-borne encephalitis viruses [20]. Several licensed veterinary vaccines are currently available. Two are inactivated whole WNV vaccines: the West Nile Innovator, whole formalin inactivated WNV-based vaccine, developed by Fort Dodge Animal Health, and commercialized by Pfizer [21], and Vetera vaccine (Boehringer Ingelheim). A West Nile-Innovator DNA vaccine (Fort Dodge Animal Health, commercialized by Pfizer,) approved in 2005 has now been discontinued. A recombinant canarypox vectored vaccine expressing the pre5 membrane (PrM) and the E protein of the NY99 strain 1 (Recombiteck Equine West Nile Virus Vaccine, Merial-Sanofi Aventis) are also commercialized [22,23]. All the vaccines currently commercialized require two to three injections and an annual immunization boost. Although there is still no human WNV vaccine approved today, several vaccine candidates are in development. A DNA vaccine encoding the pre-membrane and E glycoprotein's of the NY99 strain of WNV under the transcriptional control of the CMV/R promoter (cytomegalovirus enhancer/promoter with the human T-cell leukemia virus type 1 R region) was evaluated in phase I on 30 healthy adults, half aged 18–50 years and half aged 51–65 years. The major drawback of existing vaccines is that multiple doses required for protective immunity. However, subunit vaccines that employ only a portion of virus are safe, low cost, easy to scale up as compared to inactivated or virus like particles. This study focused on developing a recombinant subunit WNV vaccine

candidate using viral E and domain III proteins as the target antigens. Present study was aimed to assess the immunogenic potential and protective efficacy of recombinant whole envelope and domain III proteins of WNV in mice model were discussed.

Materials and Methods

Microorganism and cells

West Nile virus (Eg101 prototype strain GenBank accession number AF2609681) was included in this study. The virus was propagated in Vero cell line (African green monkey kidney cells). *Escherichia coli* host strain BL21 (DE3) and the plasmid vector pET 28a were obtained from Novagen (Madison, WI, USA). Luria Bertani (LB) for bacterial media preparation from Difco laboratories, Detroit, Michigan; Ni-NTA super flow resin, viral RNA mini kit, and anti-His antibody from Qiagen, Hilden, Germany. Access quick one-step RTPCR kit (Promega, Madison, WI, USA). Prestained protein ladder and restriction enzymes were from MBI Fermentas, Hanover, USA. Isopropyl mthiogalactoside (IPTG), diaminobenzidine (DAB), Tetra methyl benzidine (TMB), 30% Hydrogen peroxide (H₂O₂), Kanamycin and all secondary antibody enzyme conjugates (anti-mouse, anti-rabbit and anti-human horseradish peroxidase) were from Sigma Chemical, St. Louis, MO, USA.

Cloning and expression of whole envelope and domain III genes

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 [WN Env F5-GGATCCTTTAACTGCCTTGGAAT 3 (BamHI) and the reverse primer WN-Env R5 GTCGACAGCGTGCACGTTCA 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 by using the forward primer [WN DIII F5- GGATCCTTGAAGGGAACAACCTA-3'(BamHI) and the reverse primer WN DIII R5- GTCGACTTAGCTTCCAGACTTGT-3'(SalI)] which corresponds to 1855–2175 position of the genome of WN Eg101 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 an expression host. The positive clones were confirmed by PCR and restriction analysis followed by nucleotide sequencing. 1 The logarithmic phase cultures of recombinant whole envelope and DIII were induced with different conc. of IPTG (0.5, 1, 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 sonicating the induced cell pellet and the resultant cell lysate were centrifuged at 18,600×g for 30min 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, 1mol/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 mmol/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 1 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].

Mice immunization

The animal experiments were formed according to the protocol approved by the Institutional Animal Ethics Committee (IAEC) at DRDE, Gwalior. Four weeks old Balb/Cmice (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

of Freund's complete adjuvant at the first immunization. Booster injections with Freund's incomplete adjuvant formulation were given on 14, 28, 42 and 56 days after first immunization.

Determination of Humoral Immune Response

Antibody titre by enzyme linked immunosorbent assay

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/well of the purifier. Recombinant proteins rWNE_{env} and rWNDIII in 0.1M carbonate bicarbonate 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 mins intervals with PBS-T (0.05% T). After washing, goat anti-mouse IgG Horseradish peroxidase (dilution

1 : 5000 in 3 % BSA) was added to the wells and plate was incubated for 1 h at 37°C followed by washing with PBS-T. The reaction was developed at room temperature by adding 100 µl of tetramethyl benzidine (TMB) in all the wells. The reaction was stopped with 2N sulphuric acid. The absorbance was measured at 450 nm using an ELISA plate reader. Mice sera from each group were used for the determination of ELISA end point 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 mins 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 2×10^5 cells/well and incubated overnight at 37°C in a 5% CO₂ incubator. 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 a 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 Eg101 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 dispensed 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/ml (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

Six groups of four weeks old balb/c mice (n=10) were taken to determine the lethal dose of West Nile Virus. West Nile virus was administered 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

challenge, 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 denaturing 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 rWNV 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 rWNV 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 dose of 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 *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. As compared to unstimulated splenocyte culture supernatant, the sensitized splenocyte produced high level of IFN- Y, IL-10 and moderate levels of IL-2, IL-4,

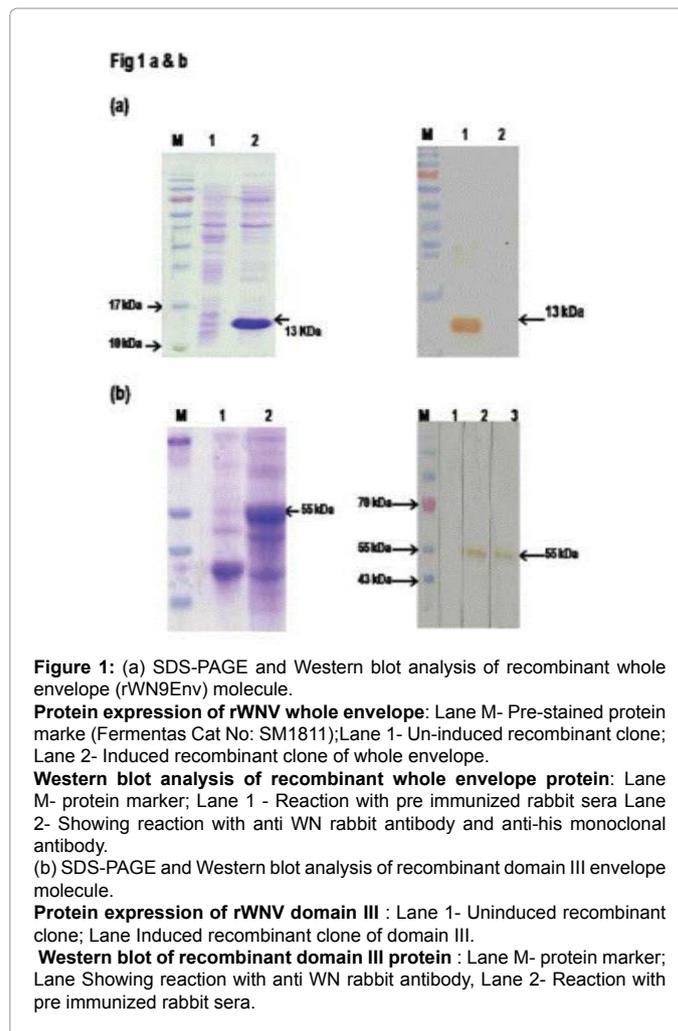


Figure 1: (a) SDS-PAGE and Western blot analysis of recombinant whole envelope (rWN9Env) molecule.

Protein expression of rWNV whole envelope: Lane M- Pre-stained protein marker (Fermentas Cat No: SM1811); Lane 1- Un-induced recombinant clone; Lane 2- Induced recombinant clone of whole envelope.

Western blot analysis of recombinant whole envelope protein: Lane M- protein marker; Lane 1 - Reaction with pre immunized rabbit sera Lane 2- Showing reaction with anti WN rabbit antibody and anti-his monoclonal antibody.

(b) SDS-PAGE and Western blot analysis of recombinant domain III envelope molecule.

Protein expression of rWNV domain III : Lane 1- Uninduced recombinant clone; Lane Induced recombinant clone of domain III.

Western blot of recombinant domain III protein : Lane M- protein marker; Lane Showing reaction with anti WN rabbit antibody, Lane 2- Reaction with pre immunized rabbit sera.

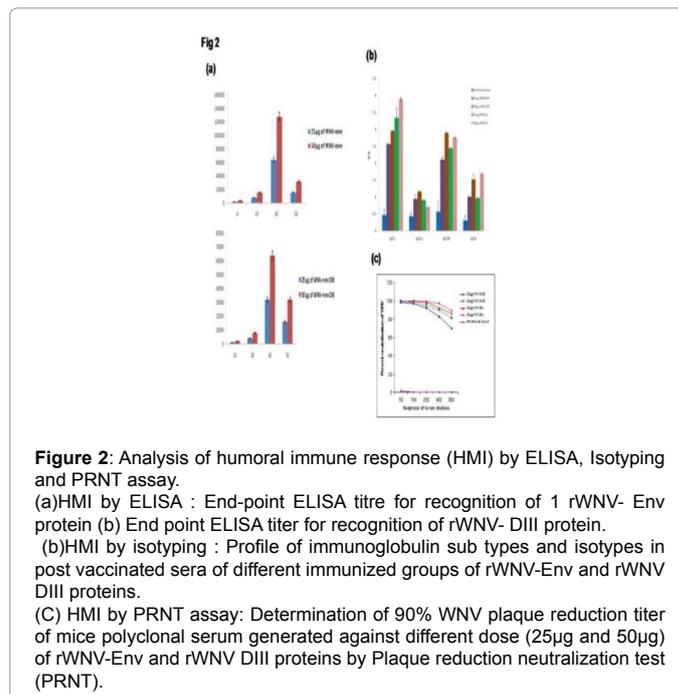


Figure 2: Analysis of humoral immune response (HMI) by ELISA, Isotyping and PRNT assay.

(a)HMI by ELISA : End-point ELISA titre for recognition of 1 rWNV- Env protein (b) End point ELISA titer for recognition of rWNV- DIII protein.

(b)HMI by isotyping : Profile of immunoglobulin sub types and isotypes in post vaccinated sera of different immunized groups of rWNV-Env and rWNV DIII proteins.

(C) HMI by PRNT assay: Determination of 90% WNV plaque reduction titer of mice polyclonal serum generated against different dose (25µg and 50µg) of rWNV-Env and rWNV DIII proteins by Plaque reduction neutralization test (PRNT).

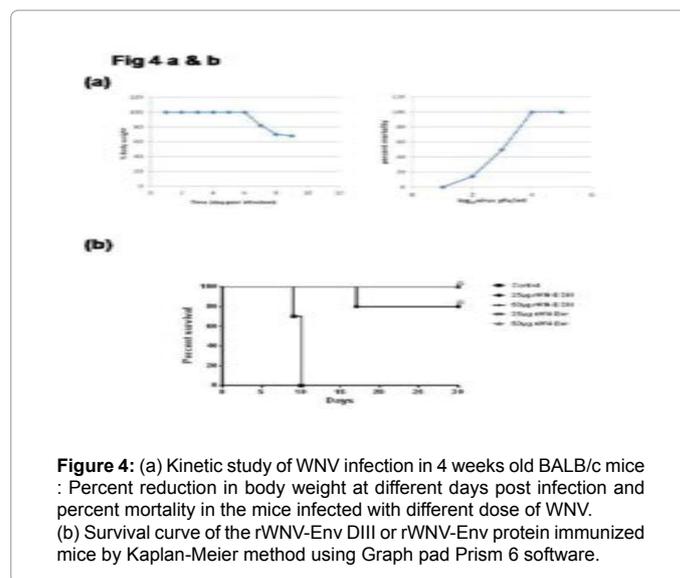
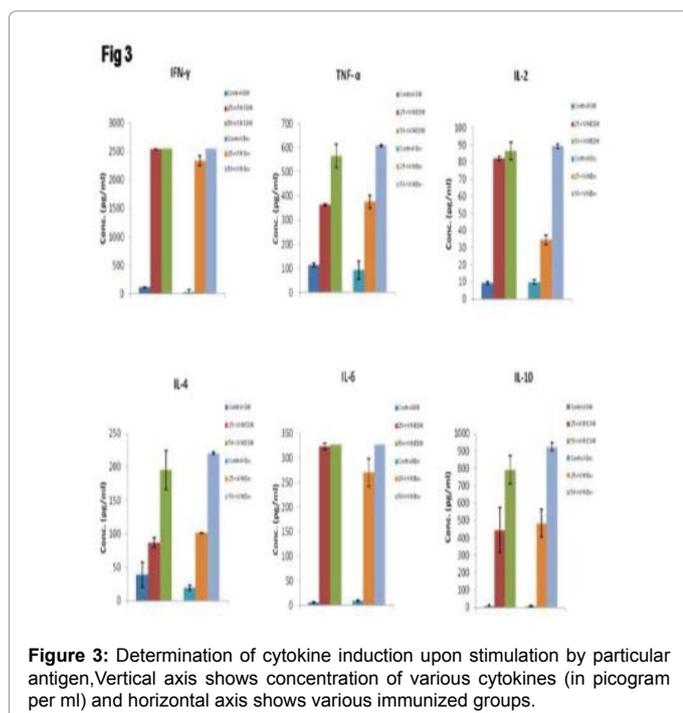
IL-6 and TNF alpha after 48 hrs stimulation (Figure 3).

Kinetics of protection induced by West Nile virus strain: To determine how rapidly protection developed after 1 vaccination with West Nile virus, susceptible mice were challenged with a high dose of West Nile virus at different time points after immunization. All unvaccinated mice and those challenged at 1 day postvaccination developed severe infection followed by hind limb paralysis. In contrast to unvaccinated controls, mice challenged as early as 1 week post vaccination were fully protected against West Nile virus infection. To determine whether this rapid protection was immunological in nature, mice were depleted of CD4+ and CD8+ T cells prior to vaccination. At 1 week after vaccination, the animals were challenged with West Nile virus strain. After challenge all the T cell depleted animals developed rapid and lethal disease, presenting a requirement for T cells in protection (Data not shown).

Protective potential of the subunit vaccine candidates: Before challenging the Balb/C mice, 50 % lethal dose of West Nile Eg101 virus was calculated by Reed and Munich method [28] which was found to be 103.185 pfu/ml of the WNV. Moreover, infected mice have shown hind limb paralysis as a prominent symptom on 9 dpi with approximately 30% decrease in body weight. WNV causes death in the mice at 10 day of post infection (Figure 4a). Both the recombinant proteins and dose administered, anti-WNV neutralizing antibodies elicited were able to protect all animals. Vaccinated mice along with controls were administered with 100LD50 dose of WNV and observed up to 30 days post infection. Mice immunized with 25 µg and 50 µg dose of recombinant whole envelope protein confers 100 % protection. Whereas Similarly recombinant DIII protein provide 83 % protection in Balb/C mice (Figure 4b).

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



WNV 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 DIIs 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

SDS-PAGE showed a high degree of purity (95%) that was achieved in a single step. Further Balb/ mice were immunized with whole envelope and domain III recombinant proteins at a conc. of 25 and 50 µg at a 15 days intervals. Two booster dose were given subsequently. The sera were collected on different time point and analyze for humoral immune response. Splenocytes from the immunized mice were cultured in-vitro to determine the recombinant protein induced cell mediated immunity. Protective efficacy of all these groups was calculated against lethal WNV challenge. Humoral immune response was assessed by ELISA titer, isotyping and PRNT assay. Both the recombinant proteins exhibited promising antibody titer eliciting a strong humoral response and neutralizing antibodies in mice. IgG isotyping revealed that both the protein elicited generation of all type of IgG antibody with 1 a predominance of IgG1 and IgG2b. This finding further proves in the stimulation of cytokine level. Since IgG1 and IgG2b type antibody generation leads to Th2 biased immune response and IgG3 favors Th1 biased immune response. In our cytokine profiling results both the pro inflammatory cytokines i.e. IL-2, IFN-γ, TNF-α and anti-inflammatory cytokines i.e. IL-4, IL-6, IL-10 were significantly increased. Level of IFN-γ was majorly enhanced irrespective of dose due to the presence of viral protein. Cell mediated immune response induced by recombinant proteins of WNV with balanced Th1/Th2 activation. Immunized mice were challenged with 100 LD50 and observed for a period of 4 weeks post challenge for mortality. All the unimmunized mice died with WNV. whereas 100% protection was observed in mice immunized with recombinant envelope proteins at a concentration of 25 and 50 µg and also recombinant envelope DIII protein at a concentration of 50 µg Whereas 25µg dose of rWN- E DIII confers 80% protection. In conclusion, in these studies we have established the bacterial expression system as a potential method to produce recombinant subunit envelope antigen for human vaccine development. The purified recombinant envelope antigen is stable, non17 toxic, immunogenic and efficacious in mouse model after WNV challenge.

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