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Cloning and Expression of the E Protein from West Nile Virus

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Abstract: ***West Nile virus (WNV), genus Flavivirus, family Flaviviridae, is an "old world" arbovirus, transmitted mainly by infected mosquitoes. It was described as serious disease with a strong influence on public health. In this study, the cDNA template of E gene was amplified by PCR and cloned into pMD18-T simple vector. After double digestion with restriction enzyme (BamH1 and SAL1) and verified by sequencing. The gene was then inserted into the plasmid pET-32a (+), transformed into Escherichia coli BL21 (DE3) and expressed after induction by IPTG at the final concentration of 1.0 mmol.L-1, at 28 ℃ for 4 hours. Then the expression product was identified by SDS-PAGE .The recombinant E protein was purified and used as antigen in immunization of rabbit to evaluate its ability to stimulate immune responses. The results showed that the molecular mass of expression protein was 73 kDa and Western blotting analysis indicated that the antigenicity of the protein was specific.***

Keywords: West Nile virus, E protein, antibody, prevention

1. Introduction

West Nile virus (WNV) contains a single strand of positive sense RNA genome of 10-11 kb with an open reading frame encoding a single polyprotein precursor of 3430 amino acids [1]. It is a virus with single positive-strand RNA which encodes three structural proteins (the capsid C, the membrane M and the envelope E) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) in the ORF [2.3.4.5.6]

Glycoprotein (E) is shown to be the major antigen capable of inducing virus neutralizing antibody that could confer protective immunity against infection of WNV. Currently, no specific treatment or vaccine license is available for the prevention against this pathogenic virus. Therefore, it is urgent to develop an effective prophylactic vaccine to prevent WNV infection [7, 8, 9].

The objective of this present study was:

- To purify the proteins for preparing antigen to Immunize rabbits
- To stimulate immune responses capable of inducing and neutralizing antibodies that could confer protective immunity against WNV infection.
- To evaluate immunogenic properties of the glycoprotein E

2. Materials and Methods

2.1 Materials

a) Strains and plasmids

E. coli（DH5, BL21）and plasmids (pMD18-T, pET-32a (+)) (from South China Agricultural University College of Veterinary Medicine Department of Microbiology) were used in E. coli strains were cultured at 37°C in Luria-Bertani (LB) containing ampicillin (100μg/mL).

b) Enzyme reagent

Taq DNA polymerase, restriction enzymes *Bam*HI and *Sal*I, T4DNA ligase were purchased from Takara Biotechnology Co. Ltd; DAB display agent purchased from biotechnology companies, Tektronix Po; pre-stained protein molecular weight standards Fermentas biotechnology products.

c) Reagents

DNA gel extraction kit (EZNA ® Gel Extraction Kit I), a small amount of plasmid extraction kit (EZNA ® Plasmid Miniprep Kit I) are OMEGA products; ampicillin (Amp), IPTG was purchased from Tektronix Biotechnology Po Company; dNTPs, agarose, Tris base were purchased from Shanghai Biological Engineering Technology Public Health Services Limited; yeast extract, tryptone, agar powder, ammonium persulfate, N, N-methylene bisacrylamide, acrylamide, N, N, N', N'-tetramethylethylene- diamine (TEMED), Coomassie brilliant blue R-250, SDS, urea, 5×SDS loading-Buffer, glycine was purchased from Beijing Dingguo Biotechnology Co., Ltd.; Ethidium bromide (EB), Sigma; polyvinylidene fluoride (PVDF) membrane Millipore products; mouse anti-His monoclonal antibody, horseradish peroxidase (HRP) labeled goat anti-mouse IgG, soluble TMB color reagent for the day biochemical roots Technology (Beijing) Co., Ltd.; mouse anti-WNV antibody Boster products e reagent

2.2 Methods

a) **Preparation of the E gene:**

The extraction of E gene was performed in the pcDNA-E samples from our laboratory of Microbiology and Immunology (South China Agricultural University, Guangzhou), amplified by PCR. The E gene associated with a pcDNA was conserved in the filter paper; it was maintained in 100μl distillated water overnight and under cold conditions (20℃). The following day, 1ml alcohol at 99 ℃ was added to the solution and after centrifugation 1500rpm for 30 min, the supernatant was discarded and the pellet was dried. Then 10μl of distilled water was added, to recover the plasmid in the pellet of 1.5 ml tubes. This sample was used for PCR of the E gene.

b) PCR and cloning of the E gene

The first step of the manipulation was to cut the gene E of the pcDNA E plasmid by PCR. The complete E gene was amplified by PCR using the following sets of primers: NY99-Primer1:5'-GAGGATCCTTCAACTGCCTTGGAAT-3',

NY99-Primer2: 5'-AAGTCGACGAGCGTGCACGTTCA-3', of the glycoprotein gene, between the restriction sites BamH1 and SAL1. A total of 1500 bp were amplified under the following conditions: 28 cycles, predenaturation at 94°C for 5 min, denaturation at 94°C for 30s, annealing at 50°C for 30s, extension at 72°C for 2 min and final extension for 7 min at 72°C.

c) Construction of the pET32a-E expression plasmid

The cloned gene was then subcloned into the pET-32(a) and was introduced into Escherichia coli DH5. Then, the extracted plasmid was introduced into BL21 (DE3) for expression. The recombinant expression plasmid, designated as pET32a-E, was identified by restriction enzyme digestions BamH1, SAL1 and verified by sequencing.

d) DNA manipulation and transformation

DNA manipulation, plasmid isolation, and agarose gel electrophoresis were operated according to Sambrook et al. (2001). Transformation of E. coli was conducted according to Chung et al. (1989).

e)* Transforming *E. coli

The suspension is spread on the surface of agar containing ampicillin. The next day, a few cells- resistant to both antibiotics-wills have grown into visible colonies. Each colony represents a clone of transformed cells (figure 1).

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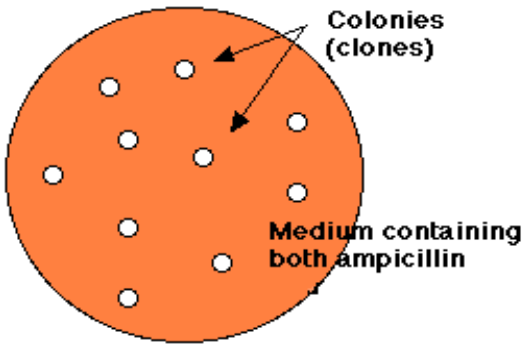

Figure 1: Solid media and colony in surface

3. Identification of gene E by PCR

The next day a single colony was selected, inoculated into 3 mL of LB liquid medium containing ampicillin (100 μg/mL) at 37℃, and stirred overnight at 200 rpm at about 12 h-16 h. The bacteria were directly as a template for PCR.

3.1 Expression and Purification of the protein

After induction by IPTG at the final concentration of 1.0 mmol.L-1, at 28 ℃ for 4 hours. The recombinant bacteria pET32-E was inoculated into 100 mL 2×YT in fresh liquid medium containing Amp (100 μg/mL) at the proportion of 1:100. After the culture was stirred to the OD_{600} value of 0.55 at 37℃, a final concentration of 1.0 mmol/L IPTG was added and stirred for another 4 h at 37 ℃. The culture was centrifuged at 4℃ for 20 min at 5 000 rpm and washed once with 100 mL of phosphate buffer. The resuspended bacteria was sonicated in the ice bath for 20 min with 4 s to 6 s ultrasound intervals; then centrifuged at 5 000 rpm for 20 min at 4℃. The protein was expressed and solublized according to the method of Kojima et al. (2003);

Guo et al. (2003).The expression product was identified by SDS-PAGE.

After precipitation, the supernatant was collected and processed after SDS-PAGE to determine proteins in soluble form or as inclusion bodies.

4. Western-blotting analysis of expression product

After SDS-PAGE, the gel was removed and prepared on the membrane "sandwich", and then the proteins were transferred on the membrane with 200 mA constant current of 1.5 hours. The PVDF membrane was remove after transfer, then put the PVDF membrane in the plate glass of the appropriate size, with 5% skim milk and TBS 4℃ overnight. After washing, discard the blocking solution; wash the membrane with TBS slowly shaking three times for 10 min. Then add mouse anti-His monoclonal antibody (1:1 000 dilution) and mouse anti-WNV antibody (1:100 dilution) with adding 1% skimmed milk and TBS, slow stirring for 5 h. After that, wash the membrane slowly shaking with TBS three times for 10 min. Then add HRP labeled goat anti-mouse IgG (dilution 1:1 000) with 1% skim milk and TBS, with gentle stirring for 3 h. Then wash the membrane with TBS for three times, slowly shaking for 10 min. Add a quantity of solution of horseradish peroxidase and DAB color for 3 to 5 minutes appears a specific reaction and then the membrane was visualized after a brief washing in distillated water.

4.1 Immunization of Rabbit

In the active immunization studies，two rabbits were immunized four times at intervals of 10 days for two consecutive months and each rabbit received 2 ml of the antigen-glycoprotein E. The antigen was prepared from a homogenate proteins fusions with 8 ml of sodium chloride (NaCl 0.9%), diluted with solution adjuvant; the homogeneous mixture was used to immunize rabbits

5. Results and Analysis

5.1 PCR amplification of the E gene

According to the sequence of the E gene, a pair of primers was designed. After PCR a DNA product with 1500 bp in size was amplified. It was verified by 1% agarose gel electrophoresis (Fig.2).

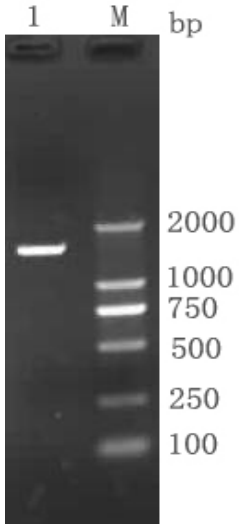

Figure 2: Identification of the E gene by PCR
M: DL2000; lane 1: gene E (1500bp)

5.2 PCR identification of bacteria (pMD18-T-E)

The purified PCR products were cloned to the pMD18-T Simple Vector multiple cloning sites and transformed into DH5α competent. The recombinant plasmid was extracted, and the E gene was identified by PCR. The result showed a 1500 bp DNA band can be obtained on1% Agarose gel electrophoresis (Figure 3).

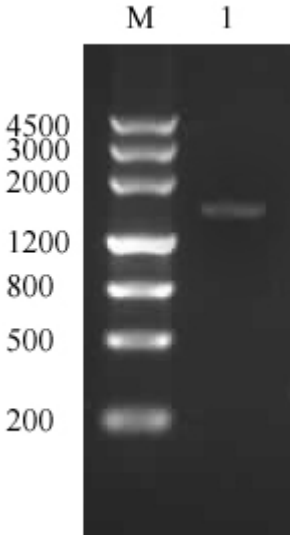

Figure 3: PCR identification of pMD18-T-Eproduct electrophoresis
M: DNA marker □; lane 1: PCR products

5.3 Identification of recombinant plasmid pMD18-T-E

After analysis of sequence DNA, the plasmid pMDl8-T-E was extracted and digested with restriction enzyme *Bam*HI and *Sal*I. The PCR product of approximate 1500 bp was inserted into the pMDl8-T vector, thus the correct recombinant plasmid was constructed (Figure. 4).

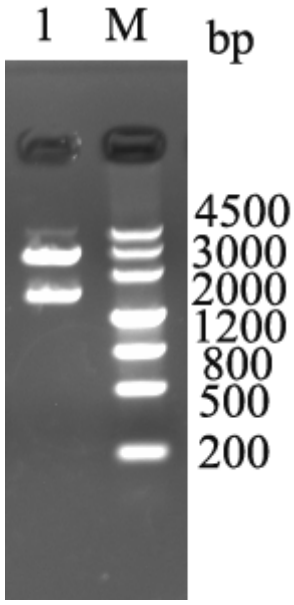

Figure 4: Identification of pMD18-T-E by double digestion (*Bam* HI and *Sal* I)
M: DNA marker □; lane 1: gene E (1500bp)

5.4 PCR identification of bacteria (pET32-E)

The DNA extracted as template for PCR amplification. PCR products with 1% Agarose gel electrophoresis, obtained a specific fragment size of about 1500bp, consistent with the expected size of products (Figure.5)

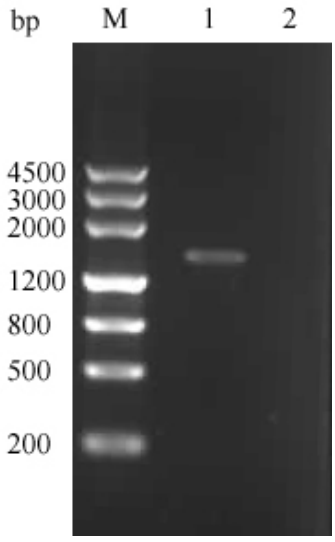

Figure 5: PCR identification of pET-E
M: DNA marker □; lane 1: PCR products;
lane 2: PCR negative control

5.5 Identification of pET32-E by double digestion

The expression vector pET- E was verified by restriction enzyme *Bam*HI and *Sal*I, the results suggested there were two DNA on the gel with the size of 5 900 bp and 1 500 bp, all together was a 7.4kb, the results were consistent with expectations, showing that the gene fragment cloned in pET-32a (+) vector is positive (Figure.6).

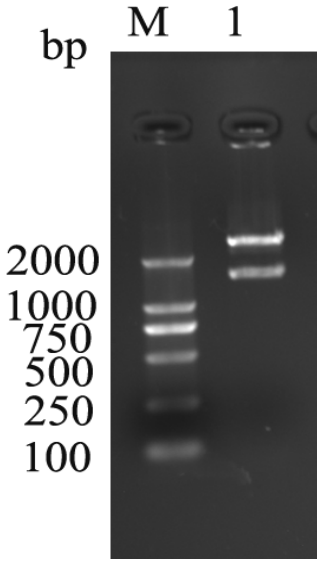

Figure6: Identification of the PET-32(a) plasmid by restriction enzymes (BamHI and SalI) digestion.
M: DNA marker-DL 2000; lane 1: PET-32(a) 5900 bp
Analysis and sequence of E gene from West Nile Virus

The DNA fragment was separated by electrophoresis on a 1% agarose gel, excised, and purified. The amplified fragment was digested with the BamH1 and SAL1 enzymes, cloned into pET-32a (+) vector and confirmed by sequencing. The results of sequencing the recombinant plasmid amino acid analysis and sequence homology, and using DNASTAR software with the sequencing results of original genes have been analyzed and compared.

5.6 SDS-PAGE analysis of the recombinant protein pET-E

The plasmid pET-E was transformed into E. coli BL21 (DE3), and the E protein was expressed inducing by IPTG at 28 ℃ for 4 hours. The samples were analyzed by SDS-PAGE and the proteins were visually by staining with Coomassie blue. The molecular weight of expressed protein was about 73 kDa. (Fig .7)

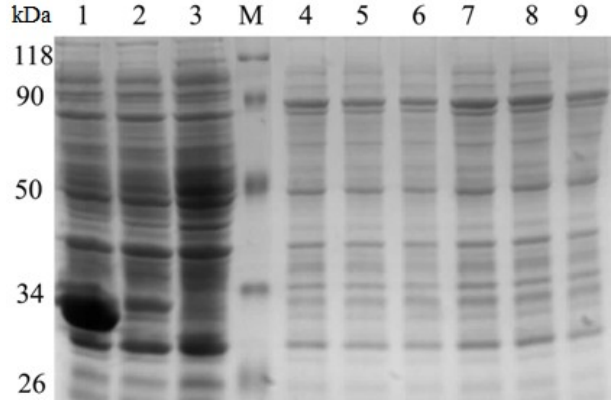

Figure 7: Analysis of the expression of E protein by SDS–PAGE

M: molecular mass protein markers, Lane 1: PET32 (a), Lane 2: pET32a-E without IPTG induction (control), Lane 3: cell lysates from *Escherichia coli* BL21, Lane 4-9: pET32a-E with IPTG-induced (Full-length (73 kDa).

5.7 Western-blotting analysis of the recombinant protein pET-E

Expression of the product obtained after transformation of strain supernatant, the preparation of the primary antibody and anti-mouse as secondary antibody **(a)** and the preparation of the primary antibody and specific WNV antibody as secondary antibody **(b)** for Western blot analysis. The results showed a protein band estimated at 73 kDa (Fig .8)

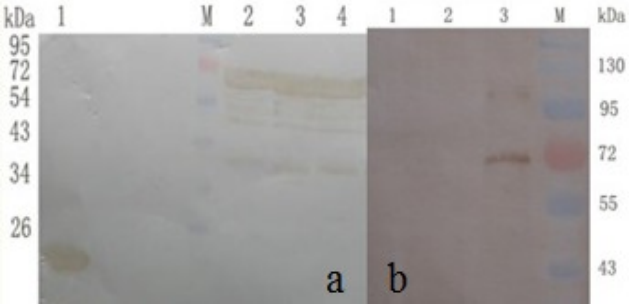

Figure 8: Analysis of the expression of pET-E by western blot
M: Marker
a: Lane 1: PET32 (a), Lane 2-3-4: pET32a-E with IPTG-induced
b: Lane 1: PET32 (a), Lane 2:BL21,Lane 3: pET32a-E with IPTG-induced

6. Discussion

The current epidemic of WNV is expected to continue over the coming years as the virus develops its geographical distribution. This impacts significantly both human and veterinary concerns. There is a lack of effective and specific antiviral treatment. The high production costs and limited scalability associated with mammalian-cell culture production may restrict the use of therapeutic mAbs against WNV and other flaviviruses in resource-poor settings [11, 12]. Moreover, Plasmid DNA is an emerging experimental vaccine, based on the plasmid ability to express a protein antigen which, in turn, promotes antibody production and

confers host immunity. A recent review presents the various strategies followed to develop vaccines against WN virus and other flaviviruses [13] .It was reported that highly expressed level of the fusion pET32a-E protein was transformed into E.coli BL21 (DE3), and optimized the condition for induction [14] .Several groups are developing vaccines against WN virus. Two veterinary vaccines are now approved for use in the United States. One, manufactured by Fort Dodge Animal Health, consists of a killed virus preparation [15]. The other, a recombinant canarypox virus, is marketed by Merial [16] .Other vaccine development strategies are based on naked plasmid DNA, attenuated WN strains, non-infectious RNA vaccines and chimeric viruses [17,18,19,20,21;23] and [13] .A recent review presents the various strategies followed to develop vaccines against WN virus and other flaviviruses [13] .It has been shown that truncated envelope protein from dengue virus, when expressed in Drosophila S2 cells, folds into a conformation resembling that of native envelope protein [22]. Here we show the results of experiments with a truncated form of WN virus envelope protein produced in Drosophila S2 cells. Mice vaccinated with this antigen were protected against a lethal challenge with WN virus. Vaccination of horses elicited neutralizing antibodies. The antigen was immunogenic when administered with aluminum hydroxide, the only adjuvant currently approved for human use. We propose that this form of truncated E protein is a candidate antigen for further development as equine and human vaccines to protect against WN virus infection. However, the risk of immune enhancement of heterologous flavivirus infection with inactivated virus and the legitimate safety concerns with the chimeric flaviviruses are highly debatable. In this study, the glycoprotein E was used as antigen in rabbits. The serum was able to neutralize West Nile virus. Our present indicated that Glycoprotein (E) plays an important role in diagnostic tests, inhibition of hemagglutination and induces protective immunity.

7. Conclusions

The results indicated that WNV neutralizing antibodies alone are considered sufficient to impart protective immunity to vaccines.

8. Acknowledgements

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