Generation and Characterization of a Chimeric Viral Like Particle (VLP)-based Vaccine Candidate against Dengue Virus Serotype 2

การสร้างและตรวจสอบคุณลักษณะของวัคซีนตัวเลือกแบบอนุภาคเสมือนไวรัสลูกผสมต่อเชื้อไวรัสเด็งกี่ซีโรไทป์ 2

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ABSTRACT

This study was aimed to create a new chimeric viral-like particle (chimeric VLP)-based vaccine against DENV-2. The woodchuck hepatitis virus core antigen (WHcAg) was used as vaccine platform to expose the fusion loop with the entire domain III of DENV-2 (as a prototype) E protein on the particle surface and was designated as WHc-FL+ EDIII DENV-2. As control, the WHcAg (Wild-type VLP) was also generated. Our results showed that the vaccine candidate as well as control could be expressed in *Escherichia coli* as soluble proteins. Electron microscope analysis is clearly demonstrated the VLP formation in both chimeric VLP and wild-type VLP. Studies are underway to purify and evaluate the immunogenicity of this chimeric VLP *in vivo*.

บทคัดย่อ

งานวิจัยนี้มีวัตถุประสงค์เพื่อสร้างวัคซีนชนิดใหม่แบบอนุภาคเสมือนไวรัสลูกผสมต่อไวรัสเด็งกี่ซีโรไทป์ 2 โดยใช้แอนติเจนส่วนแกนกลางของไวรัสตับอักเสบชนิดบีที่ติดเชื้อใน Woodchuck (WHcAg) เป็นฐานวัคซีนในการนำเสนอส่วนของ fusion loop ร่วมกับ domain III ในโปรตีน E ของไวรัสเด็งกี่ซีโรไทป์ 2 (ทดลองใช้เป็นต้นแบบ) บนผิวของอนุภาค โดยให้ชื่อว่า WHc-FL+EDIII DENV-2 ทั้งนี้ได้สร้างตัวควบคุม คือ WHcAg (อนุภาคเสมือนไวรัสแบบไม่ดัดแปลง) ผลการศึกษาแสดงให้เห็นว่าทั้งวัคซีนตัวเลือกและตัวควบคุมสามารถแสดงออกเป็นโปรตีนใน *Escherichia coli* ในรูปแบบที่ละลายน้ำได้ โดยจากการวิเคราะห์ด้วยกล้องจุลทรรศน์อิเล็กตรอนแสดงให้เห็นอนุภาคเสมือนไวรัสอย่างชัดเจนทั้งอนุภาคเสมือนไวรัสแบบลูกผสมและแบบไม่ดัดแปลง ขณะนี้กำลังดำเนินการทำให้อนุภาคมีความบริสุทธิ์และทดสอบประสิทธิภาพของวัคซีนอนุภาคเสมือนไวรัสลูกผสมในการกระตุ้นภูมิคุ้มกันในสัตว์ทดลองต่อไป

Key Words: Viral-like particle, Dengue virus serotype 2, Woodchuck hepatitis B virus core antigen (WHcAg)
คำสำคัญ: อนุภาคเสมือนไวรัส ไวรัสเด็งกี่ซีโรไทป์ 2 แอนติเจนส่วนแกนกลางของไวรัสตับอักเสบชนิดบีที่ติดเชื้อใน Woodchuck

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Introduction

Dengue virus (DENV) is a member of genus Flavivirus which uses infected *Aedes Aegypti* mosquito as the main propagation vector. The DENV contains four different serotypes (DENV1, 2, 3 and 4), which causes dengue fever (DF), the severe clinical symptoms (dengue hemorrhagic fever (DHF)) and dengue shock syndrome (DSS) (Halstead, 2007). The disease severity is dependent on the subsequent virus infection as well as the antibody-dependent enhancement (ADE) through a suboptimal heterotypic antibody (enhancing antibody) triggered by previous DENV infection. These issues represent the major obstacles in the development of vaccines against DENV. Moreover, an ideal vaccine candidate must be protective against all serotypes. The major DENV antigen is the Envelope protein (E). Our vaccine candidate is composed of the envelope domain III (EDIII), which is the primary target of the most potent neutralizing antibody responses against DENV (Megret et al., 1992) and the fusion loop (aa 98-110) from domain II (EDII) that represents a cross-reactive epitope, which could trigger neutralizing antibodies against all four DENV serotypes (Whitehead et al., 2007).

One strategy to improve the immunogenicity of any vaccine candidate could consist in the display multiple copies of the target antigen on the surface of a genome-free viral particle. Viral-like particle (VLP) as a carrier can be generated through the heterologous expression of specific viral proteins which can self-assemble once produced (Yildiz et al., 2011). Hepatitis B virus core antigen (HBcAg)-based VLP vaccine candidates for malaria and influenza A have been evaluated in clinical trials (Gregson et al., 2008; Blokhina et al., 2013). The usage of the core particles from related hepadnaviruses that infect species besides human, for example woodchuck and duck, could avoid the pre-existing immunity against previously exposed to human hepatitis B virus infection, which may adversely affect HBcAg-based vaccine immunogenicity. Moreover, woodchuck-derived WHcAg has proven to be at least as immunogenic as the human-derived HBcAg and such VLP can still be produced in *Escherichia coli* (*E.coli*), a cost-effective expression system (Billaud et al., 2005). The WHcAg-based VLP vaccines demonstrated protection against malaria and influenza (Billaud et al., 2007; Ameiss et al., 2010). According to the advantage of WHcAg-based vaccine, we developed a chimeric WHcAg VLP, which exposed the fusion loop and EDIII on the VLP surface. This vaccine candidate represents a new promising DENV vaccine platform.

Objective of the study

To produce and characterize a new recombinant chimeric VLP vaccine against DENV serotype 2.

Methodology

Construction of expression vector encoding chimeric VLP

To express a chimeric WHc-FL+EDIII DENV-2 protein, a consensus amino acid sequence of DENV-2 fusion loop (E aa 98-110) + DIII amino acid sequence (E aa 295-395) was generated through alignment of E sequence from DENV-2 strains isolated between 1974 and 2004 (source: GENBANK database). A $(Gly_4Ser)_3$ linker jointed the two E

fragments. This sequence was finally flanked at the N- and the C-terminus by the same linker. The resulting Linker-FL-Linker-DomainIII-Linker was inserted at the level of the amino acid position 78, in the external loop of WHcAg. The C-terminal of containing RNA/DNA binding motifs (RRR/SPXX motifs) were deleted (aa 150-188) . The chimeric WHc-FL+EDIII DENV-2 was cloned into the *E.coli* expression vector, pET15b (containing a T7-LacZ promoter). As a positive control of VLP formation, we cloned into the same expression vector as the wild-type WHcAg.

Expression in *E.coli* host strains

The *E.coli* strains carry the DE3 gene encoding T7 RNA polymerase in order to activate the T7 promoter. In this study, two commercially available *E.coli* strains, *SHuffle* (New England Biolab, UK) and *Origami* (Millipore, USA) were used for plasmid transformation and protein expression. These strains were selected for their faculty to fold efficiently the expressed proteins through deletion of the glutaredoxin reductase (*gor*) and thioredoxin reductase (*trxB*) genes, to promote disulfide bonds formation and consequently to improve protein solubility. The *E.coli* host strains were transformed with the two VLP-expressing plasmids. Transformed *E.coli* were cultured in LB medium with 100 µg/ml Ampicillin as a starter and further inoculated into fresh LB medium at 30 ^{o}C until the OD_{600} reached 0.4 to 0.6. Protein expression was induced by addition of IPTG. Different concentrations of the inducer (0.04 - 0.5 mM), temperatures (16 - 37 ^{o}C) and durations of induction (1 - 17 h) were tested to optimize the protein solubility. Whole *SHuffle* and *Origami* cells were lyzed with a high pressure homogenizer (TS series benchtop 0.75 kW model, UK) and the lysates were subsequently ultracentrifuged (40,000 rpm, 1 hour) to separate the soluble (cytoplasmic) and insoluble (inclusion bodies/membrane associated) proteins. The protein fractions were analyzed by SDS-PAGE and Western Blotting (WB). In WB, the monoclonal antibody (mAb) 14E11 (mouse mAb, Millipore, USA) and mAb 3H5 (kindly provided by Dr.Chunya Puttikhunt, National Center for Genetic Engineering and Biotechnology) were used to detect the WBc and DENV-2 EDIII, respectively.

Purification of VLP by sucrose density gradient

The cytoplasmic fraction was applied onto 10 – 60% (w/v) sucrose gradient and ultracentrifuged at 41,000 rpm, 2 hours at 4 ^{o}C. The gradients were then harvested and the 14 collected fractions, from the top to the bottom, were analyzed by SDS-PAGE and WB.

Characterization of VLP by Electron Microscopy (EM)

To assess VLP formation, the WB-positive fractions were dropped on to carbon-Formvar coated copper grid and negatively stained with 2% uranyl acetate. The transmission electron microscope (Hitachi HT7700 model, Japan) at 100 kV was used to analyze the morphology of VLPs.

Results

Expression of recombinant WHcAg in *E.coli* host strains

We evaluated the conditions of induction temperatures (37 ^{o}C, 22 ^{o}C and 16 ^{o}C), periods of induction (1, 3, 7 and 17 h) and concentration of IPTG (0.04 - 0.5 mM) to optimize the solubility of

chimeric and WT WHcAg proteins (data not shown). The expression assay was clearly demonstrated that highest solubility level of chimeric WHcAg protein was obtained when the protein was in *SHuffle* using the following conditions: 0.04 mM IPTG, induction at 16 °C for 17 hours. Wild-type WHcAg was highly soluble when expressed in *Origami* for 17 hours with 0.5 mM IPTG induction at 22 °C (Figure 1).

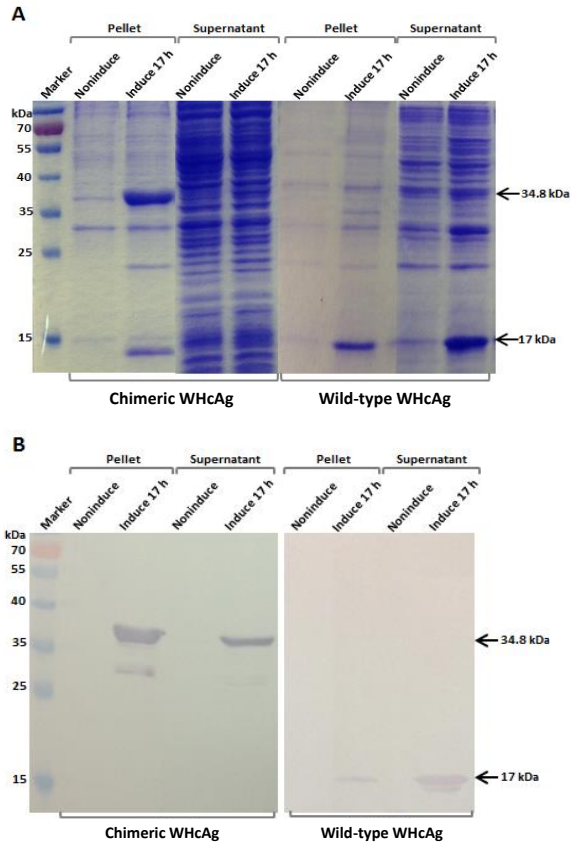

Figure 1 SDS-PAGE (A) and WB (B) analysis of ultracentrifuged soluble (supernatant) and insoluble (pellet) expressed WHcAg. Chimeric WHcAg 34.8 kDa (3H5 mAb) and Wild-type WHcAg 17 kDa (14E11 mAb) expressed in *SHuffle* and *Origami* strains, respectively. Arrows indicate the expected size of expressed WHcAg proteins.

Evidence of VLP formation by ultracentrifugation on sucrose density gradient

To demonstrate the self-assembly of chimeric and WT WHcAg into VLP particles, soluble fractions of the recombinant bacteria were submitted to a zonal ultracentrifugation using a sucrose density gradient. Typically, such density gradient contains three zones: fractions 1 - 6 corresponding to monomeric proteins, fractions 7 - 10 corresponding to VLP-like structures and fractions 11 - 14 to large protein aggregates. Whereas the WT WHcAg was clearly detected in fractions 7 - 9, the chimeric WHcAg was distributed in fractions 2 - 10. These results showed that expressed WT WHcAg produces clearly VLP whereas the chimeric WHcAg can produce monomreic as well as VLP-like structures (Figure 2).

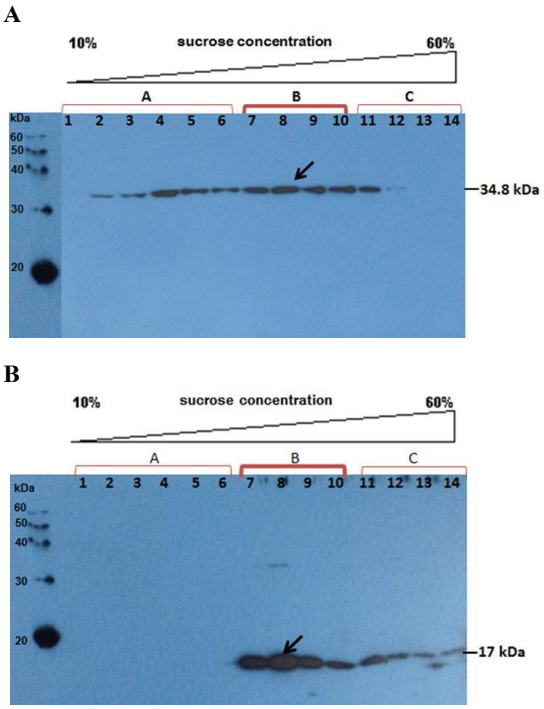

Figure 2 Distribution of the chimeric WHcAg (A) and WT WHcAg (B) in a 10% – 60% (w/v) sucrose gradient ultracentrifugation, The proteins were detected in WB by

chemiluminescence using mAb 3H5, anti-DENV-2 EDIII (B) and mAb 14E11, anti-WHc (A) and horseradish peroxidase as a detection system. The arrows indicate the purified proteins in VLP zone (zone B). The sizes of chimeric and WT WHcAg are indicated as 34.8 and 17 kDa, respectively.

Characterization of the purified chimeric and WT WHcAg by Electron Microscopy (EM)

To confirm that the insertion of DENV E protein fragments maintains the self-assembly capacity of WHcAg to create VLP the fraction 8 from the two density gradients shown in Figure 2 was analyzed by transmission electron microscopy. The electron micrograph demonstrated both chimeric WHcAg and wild-type isolated from fraction 8 formed VLP particles with a diameter of approximately 35 nm as which is the expected size of VLP based on hepatitis core antigen. Images were recorded at x100000 microscope magnification (Figure 3).

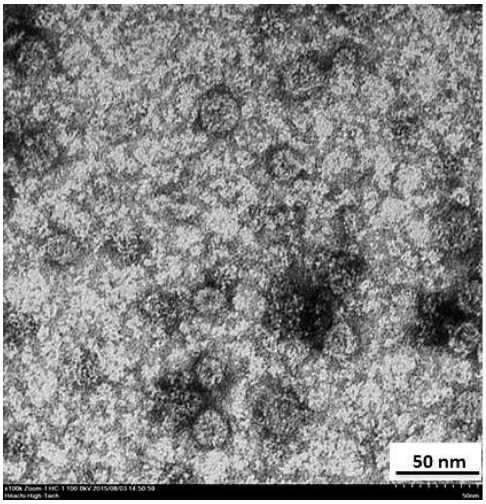

Figure 3A Electron micrographs of the purified chimeric WHcAg-based VLP from the fraction 8 (Figure 2A). Scale bar, 50 nm.

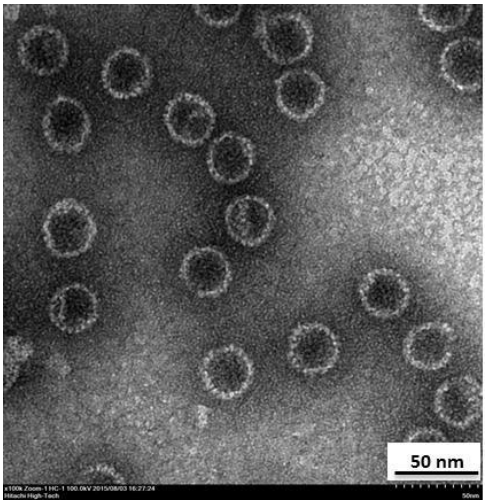

Figure 3B Electron micrographs of the purified wild-type VLP from the fraction 8 (Figure 2B). Scale bar, 50 nm.

Discussion and Conclusion

In the context of DENV vaccine development, numerous of non-replicating (naked DNA, subunit protein or viral-like particles) vaccine candidates had been explored (Prompetchara et al., 2014; Hermida L et al., 2006; Arora et al., 2012; Arora et al., 2013). In this study, we, for the first time, created chimeric dengue vaccine VLP based on woodchuck hepatitis core antigen (WHcAg) as this protein can self-assemble into VLP in bacteria and as the use of this protein instead of HBcAg would avoid pre-existing immunity issue against human hepatitis B virus. Recently, the EDIII has been reported as a promising vaccine immunogen that is the major target of neutralizing antibody response (Roehrig et al., 2004; Guzman et al., 2010; Swaminathan et al., 2010). Consequently, the EDIII as well as the fusion loop (cross-reactive neutralizing epitope) of DENV-2 (as a prototype) was inserted into the WHcAg with the aim to presents multiple copies of the E protein fragments on the surface of the WHcAg-based VLP.

Under our experimental conditions, WHcAg-fusion loop + DIII DENV-2 E were mostly expressed in the *E.coli Origami* strain as an insoluble protein. Such insolubility to be detrimental to the VLP formation due to the necessity to solubilize the protein under denaturing conditions and to refold appropriately. To optimize the solubility level of the chimeric WHcAg, a new *E.coli* strain (*SHuffle*) was used because this strain was modified to generate disulfide bonds and consequently to trigger proper folding of the expressed protein. Through the use of the *SHuffle* strain, we could detect some soluble chimeric WHcAg. Such yield was however quite low and could be explained by the large hydrophobicity of the fusion loop as well as of some area of the domain III. The WHcAg (Wild-type VLP) was also generated as control, our results showed that this construct could be predominantly expressed as soluble proteins in *E.coli*. Analysis of zonal centrifugation and electron micrographs are clearly showed that the entire soluble WT and partial chimeric WHcAg proteins were self-assembled for the VLP formation. Both VLPs display the expected diameter (30-40 nm). Further purification steps such as gel filtration and anion-exchange chromatography will be required to achieve the purity of our VLP vaccine candidate (Skrastina et al., 2013).

In conclusion, a new vaccine candidate against DENV-2, based on the WHcAg VLP was successfully produced in bacteria. The immunogenicity of this chimeric VLP will be further evaluated in mice through its capacity to induce the stimulation of neutralizing antibodies.

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