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Production of recombinant dengue non-structural 1 (NS1) proteins from clinical virus isolates

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ABSTRACT

Dengue is a febrile disease caused by infection of dengue virus (DENV). Early diagnosis of dengue infection is important for better management of the disease. The DENV Non-Structural Protein 1 (NS1) antigen has been routinely used for the early dengue detection. In dengue epidemic countries such as Indonesia, clinicians are increasingly relying on the NS1 detection for confirmation of dengue infection. Various NS1 diagnostic tests are commercially available, however different sensitivities and specificities were observed in various settings. This study was aimed to generate dengue NS1 recombinant protein for the development of dengue diagnostic tests. Four Indonesian DENV isolates were used as the source of the NS1 gene cloning, expression, and purification in bacterial expression system. Recombinant NS1 proteins were successfully purified and their antigenicities were assessed. Immunization of mice with recombinant proteins observed the immunogenicity of the NS1 protein. The generated recombinant proteins can be potentially used in the development of NS1 diagnostic test. With minimal modifications, this method can be used for producing NS1 recombinant proteins from isolates obtained from other geographical regions.

1. Introduction

Dengue infection has become a major public health problem in many countries in the tropical and sub-tropical regions in the world, with an estimated 50 million infections occur per year. The disease affects approximately 2.5 billion people living in Southeast Asia, the Pacific, and the Americas [1,2]. The clinical manifestations of dengue infection are varies, ranging from a mild fever (Dengue Fever, DF) to the more severe forms of the disease, Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS) [3].

Dengue virus (DENV) is a member of Flaviviridae family. There is a substantial genetic diversity shown by the presence of four serotypes (DENV-1, -2, -3, and -4) and multiple genotypes (or subtypes) within each serotypes [4,5]. DENV is transmitted through human-mosquito cycle with the aid of *Aedes aegypti* and *Ae. albopictus* mosquito vectors. The genome consists of single-stranded positive-sense RNA which encodes three structural (C, prM/M, E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) [1].

Currently, there are no specific antiviral therapy for dengue while a new vaccine has just been licensed in several countries. Patient management relies on good supportive care. Prompt and early diagnosis of dengue viral infection remains crucial. Laboratory confirmation is important due to difficulties in making accurate diagnosis with the broad spectrum of clinical presentations. Among the available dengue diagnosis tools, the detection of virus NS1 protein antigen has become the basis for commercial diagnostic assays [2]. NS1 is a 44–49 kDa (353–354 amino acids) glycoprotein essential for viral replication and viability [6,7]. Many tests have been developed to diagnose DENV infections using NS1 due to the presence of the virus and their encoded NS1 protein in the blood during acute phase [8]. High level of viremia and NS1 antigenemia has also been associated with more severe clinical presentations [9].

In dengue hyper-endemic countries such as Indonesia, commercial NS1 protein antigen detection kits have been increasingly used and are becoming the tool of choice among clinicians to

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confirm DENV infection. The diagnostic accuracies of these commercial kits have been previously investigated [10,11]. A multi-country evaluation study suggested that even the best performing NS1 assay had moderate sensitivity (median 64%, range 34–76%), although 100% of specificity was achieved. We and other have recently evaluated the performance of NS1 diagnostic tests used in Indonesia, and also observed the relatively low sensitivities of the available NS1 diagnostic tests [12,13]. It has been suggested that the poor sensitivity of the assays was related to geographical regions of study sites, suggesting the need for further assessment [14]. The low sensitivities of the tests warrant further and continued development of dengue diagnostics tests that hopefully can achieve better sensitivity, especially when used in dengue endemic countries.

Researchers have attempted to employ different expression systems and strategies for recombinant NS1 protein production with limited success mainly due to problems in insolubility and the lack of post-translational modifications and altered secondary structure resulting the decreased immunogenicity [15,16]. The use of vector system that helps for high-level soluble recombinant protein production has been reported to overcome major obstacles associated with heterologous protein expression in *E. coli* [15]. We described our attempt to generate recombinant NS1 proteins from all four DENV serotypes using Indonesian local DENV isolates as the source of NS1 gene sequences and a universal cloning method based on the site-specific recombination properties of bacteriophage lambda that increase the solubility of the recombinant proteins generated and maintain their immunogenic properties. The method used in this study might be suitable for producing NS1 proteins from other geographical regions with no or minimal modification. The recombinant NS1 proteins can potentially be used to further study the NS1 antigen-based dengue diagnostics development.

2. Materials and methods

2.1. Dengue virus isolation and propagation

Dengue viruses (DENVs) were isolated from dengue patients and propagated in cell tissue culture as previously described [12]. Four isolates representatives of DENV serotypes were used as source of viral RNA i.e. DENV-1 SUB-025, DENV-2 SUB-011, DENV-3 SUB-030, and DENV-4 SUB-007. The NS1 gene sequences of each isolates have been described previously and deposited in GenBank database [12].

2.2. Virus RNA extraction and cDNA preparation

Virus RNA was extracted from cell tissue culture supernatant using MagNA Pure LC Total Nucleic Acid Isolation Kit and an automated MagNa Pure LC 2.0 Instrument (Roche, Mannheim, Germany) according to manufacturer's instructions. Extracted nucleic acid was reverse-transcribed into cDNA using Superscript III reverse transcriptase (RT) (Invitrogen-Life Technologies) and antisense primers as described elsewhere [17,18]. The resulting cDNA was used as templates for the generation of NS1 gene fragment using *Pfu* Turbo DNA Polymerase (Stratagene-Agilent Technologies, La Jolla, CA). PCR product was purified from 0.8% agarose gel using QIAquick gel extraction kit (Qiagen, Hilden, Germany) and then used as templates for subsequent *attB* PCR product generation.

2.3. Primers design and generation of attB PCR product

The Gateway BP primers were designed according to the recommended instructions from Gateway cloning kit's manufacturer (Invitrogen-Life Technologies). The forward and reverse BP primers were designed to incorporate four guanine (G) residues at the 5′ end followed by 25 bp attB1 site, additional nucleotides, and gene specific sequences according to the corresponding DENV serotypes. Four DENV reference sequences available on GenBank (accession numbers NC_001477, NC_001474, NC_001475, and NC_002640 for DENV-1, -2, -3, and -4, respectively) were used as the source for the gene specific sequences in primers. The primers used are listed in Table 1.

The *attB* PCR product for each DENV serotype was generated using BP primers and the corresponding NS1 gene fragment as template. PCR amplification was performed using *Pfu* Turbo DNA Polymerase using typical PCR conditions as follows: templates were denatured for 2 min at 95 °C, followed by 40 cycles of 30 s denaturation at 95 °C, 1 min annealing at 55 °C, and 3 min extension at 72 °C. An additional 10 min final extension step at 72 °C was applied and followed by storage at 4 °C. PCR products were then purified from 0.8% agarose gel using QIAquick gel extraction kit (Qiagen).

2.4. Site-specific recombinational cloning

The generated *attB* PCR products were used in subsequent site-specific recombinational cloning using Gateway technologies (Invitrogen-Life Technologies). The Gateway cloning kit consists of reagents for BP and LR reactions. The *attB* PCR product was first used in BP reaction incorporating 100 fmol of *attB* product and Gateway BP Clonase enzyme in each reaction, as recommended by the manufacturer. The plasmid pDONR221 (Invitrogen-Life Technologies) was used as donor vector to generate entry clones. DNA sequencing was used to verify the successful recombination, integrity of sequence, and in-frame position on the resulting entry clones. Cycle sequencing step was performed using BigDye Dideoxy Terminator sequencing kits v3.1 (Applied Biosystems, Foster City, CA) using pDONR-F (5′-TCGCGTTAACGCTAGCATGGATCTC) and pDONR-R (5′-GTAACATCAGAGATTTTGAGACAC) primers. Each purified DNA was run in an ABI 3130xl capillary sequencer (Applied Biosystems), using the method described by the manufacturer.

Confirmed entry clones were then subjected to LR reaction using LR Clonase enzyme. The destination plasmid pET-G20A (European

Table 1
Gateway BP primers used in the cloning of DENV NS1 gene. Sequences printed in bold are the gene specific sequences for the corresponding serotypes.

DENV serotype	Primer name	Direction	Sequence (5′-3′)
DENV-1	D1NS1BP-F	Sense	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCCTGGTGCCGCGTGGCAGC**GACTCGGGATGTGTAATCAAC**
	D1NS1BP-R	Antisense	GGGGACCACTTTGTACAAGAAAGCTGGGTTTA**TGCAGAGACCATTGACTTAA**
DENV-2	D2NS1BP-F	Sense	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCCTGGTGCCGCGTGGCAGC**GATAGTGGTTGCGTTGTGAGC**
	D2NS1BP-R	Antisense	GGGGACCACTTTGTACAAGAAAGCTGGGTTTA**GGCTGTGACCAAGGAGTTGA**
DENV-3	D3NS1BP-F	Sense	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCCTGGTGCCGCGTGGCAGC**GACATGGGGTGTGTCATAAAC**
	D3NS1BP-R	Antisense	GGGGACCACTTTGTACAAGAAAGCTGGGTTTA**CGCTGAGACTAAAGACTTTA**
DENV-4	D4NS1BP-F	Sense	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCCTGGTGCCGCGTGGCAGC**GACATGGGTTGTGTGGCGTCA**
	D4NS1BP-R	Antisense	GGGGACCACTTTGTACAAGAAAGCTGGGTTTA**GGCCGTCACCTGTGATTTGA**