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Development and clinical evaluation of a highly accurate dengue NS1 rapid test: from the preparation of a soluble NS1 antigen to the construction of an RDT

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ABSTRACT

Early diagnosis of dengue virus (DENV) is important. There are numerous products on the market claiming to detect DENV NS1, but these are not always reliable. In this study, a highly sensitive and accurate rapid diagnostic test (RDT) was developed using anti-dengue NS1 monoclonal antibodies. A recombinant NS1 protein was produced with high antigenicity and purity. Monoclonal antibodies were raised against this purified NS1 antigen. The RDT was constructed using a capturing (4A6A10, $K_d = 7.512 \pm 0.419 \times 10^{-9}$) and a conjugating antibody (3E12E6, $K_d = 7.032 \pm 0.322 \times 10^{-9}$). The diagnostic performance was evaluated with NS1-positive clinical samples collected from various dengue endemic countries and compared to SD BioLine Dengue NS1 Ag kit. The constructed RDT exhibited higher sensitivity (92.9%) with more obvious diagnostic performance than the commercial kit (83.3%). The specificity of constructed RDT was 100%. The constructed RDT could offer a reliable point-of-care testing tool for the early detection of dengue infections in remote areas and contribute to the control of dengue-related diseases.

1. Introduction

Dengue is the most important human disease caused by mosquito-borne dengue viruses (DENVs) existing as 4 known serotypes (DENV1-4), with over 1 billion people at risk in the subtropics and tropics (Simmons et al., 2012). The DENV genomic RNA is approximately 11 kb long and is composed of 3 structural proteins (core protein, C; membrane protein, M; and envelope protein, E) at the N terminus followed by 7 nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Clyde et al., 2006). As there is no available vaccine or specific antiviral treatment for prevention and control of dengue, the early diagnosis is very important in dengue-related diseases.

Dengue diagnosis includes the detection of virus by cell culture; the detection of virus antigen by enzyme-linked immunosorbent assay (ELISA); the detection of anti-DENV antibody by hemagglutination inhibition, complement fixation test, and neutralization test; and the detection of virus nucleic acids by reverse transcription (RT)-PCR or real-time RT-PCR (Guzman et al., 2010). A rapid diagnostic test (RDT), also known as a lateral flow rapid test, is a diagnostic assay designed for use at the point of care (POC) (Song et al., 2012). A RDT has several advantages, such as low cost, simple operation, easy readability, temperature stability, and fast result delivery. As a consequence, a RDT has been widely used in clinics and elsewhere (Noyola and Demmler, 2000). Anti-dengue immunoglobulin M (IgM)– or immunoglobulin G (IgG)–based RDTs are now available, but they have limitation to detect DENV at the early stage of infection.

The DENV NS1 protein, a highly conserved ~48-kDa glycoprotein, was initially described as essential for RNA replication (Mackenzie et al., 1996). High concentration of this antigen can be detected in patients with primary and secondary DENV infections up to 5 days after the onset of illness (WHO, 2009). Many studies have shown that the detection of NS1 antigen could be useful for the early confirmation of DENV infections (Alcon et al., 2002; Bessoff et al., 2008; Blacksell et al., 2011; Datta and Wattal, 2010; Dussart et al., 2006; Dussart et al., 2008; Hsieh and Chen, 2009; Kassim et al., 2011; Kumarasamy et al., 2007; Lima Mda et al., 2010; Tricou et al., 2011). Thus, many attempts were conducted to express and purify a highly antigenic recombinant NS1 protein in large quantities in order to develop useful antibodies against the DENV NS1 antigen. However, most attempts to express DENV NS1 proteins in the bacterial expression system (*Escherichia coli*) resulted in insoluble proteins (Amorim et al., 2010; Das et al., 2009; Huang et al., 2001; Lazaro-Olan et al., 2008; Sankar et al., 2013), requiring denaturation and refolding to harvest proteins from inclusion bodies. However, denaturants can alter the secondary structure of proteins and, therefore, result in decreased immunogenicity and antigenicity, which are eventually directed toward the reduced sensitivity of a kit. Refolding process is followed to restore the functionality of proteins, but it is still limited to obtain highly antigenic NS1 proteins.

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In this study, the DENV2 NS1 protein was expressed and successfully purified in a soluble form as well as at high purity using *E. coli* expression system. This antigenic NS1 protein was used to produce DENV NS1-specific monoclonal antibodies (mAbs). The use of the pure NS1 antigen enabled the production of useful mAbs displaying high affinities to the antigen. A novel RDT was constructed using isolated DENV NS1-specific mAbs. The RDT was clinically evaluated and compared to a commercial one. The result showed that the developed RDT can offer a reliable diagnostic tool for the early dengue detection in remote areas and can contribute to the control of dengue-related diseases.

2. Materials and methods

2.1. Clinical samples

One hundred sera of normal healthy donors were provided by Chungbuk National University Hospital (Cheongju, Korea), and 42 sera positive for dengue NS1 antigen were from General Hospital Kuala Lumpur in Malaysia (15 cases), Rio de Janeiro Hospital in Brazil (17 cases), and Bombay Hospital in India (10 cases). All the samples were tested by RT-PCR (Waggoner et al., 2013) to confirm DENV infection and to determine the serotype of DENV. Seventeen samples from Brazil were serotyped to DENV1, 8 Malaysian and 7 Indian samples to DENV2, 2 Malaysian and 3 Indian samples to DENV3, and 5 Malaysian samples to DENV4. Ethical approval was obtained from the Institutional Review Board of the General Hospital Kuala Lumpur in Malaysia, Rio de Janeiro Hospital in Brazil, Bombay Hospital in India, and Chungbuk National University Hospital in Korea. Written informed consent was also obtained from all subjects.

2.2. Cloning of DENV2 NS1 complementary DNA

DENV RNA was extracted from a Malaysian female patient plasma (2011), which was provided from General Hospital Kuala Lumpur using QiAamp Viral RNA Mini Kit (Qiagen, Valencia, CA). Complementary DNA (cDNA) was reverse transcribed using Super Script II First Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Full length of NS1 gene was amplified by PCR with a forward (5′-GCGAATTCATGA ATTCACGCAGCACCTC-3′) and a reverse primer (5′-GCCTCGAGCTGG CTGTGACCAAGGAGT-3′). The primers contain upstream EcoRI and downstream XhoI restriction sites (underlined). The amplified cDNA of NS1 was firstly inserted into the pGEM-T Easy vector (Promega, Madison, WI, USA) and then subcloned into the pET32a expression vector (Novagen, Merck Millipore, Darmstadt, Germany). The resultant construct was confirmed by sequencing.

2.3. Expression and purification of the recombinant NS1 protein

The recombinant construct was transformed subsequently into chemically competent *E. coli* BL21(DE3). Transformed cell was cultured in LB media (50 μg/mL ampicillin) at 37 °C until an OD_{600} reached 0.8–1.0. Induction was performed with 0.5 mmol/L Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 h at 37 °C. Cells were harvested by cold centrifugation at 10,000 rpm for 30 min and resuspended in 20 mmol/L Tris-Cl (pH 7.9). The bacterial cells were lysed by sonication (Sonics & Materials, Newtown, CT), and the soluble fraction was separated by cold centrifugation. The collected supernatant was introduced to pre-equilibrated DEAE-Cellulose column (Sigma-Aldrich, St. Louis, MO, USA). The bound proteins were eluted using the linear gradient of 0–100 mmol/L NaCl at a flow rate of 1 mL/min. The eluate was further purified by Ni^{2+}-nitrilotriacetic acid (Ni-NTA) resin (Qiagen). Elution was performed using 40 and 500 mmol/L imidazole in 20 mmol/L Tris-Cl (pH 7.9). The eluate was dialyzed using 100 mmol/L carbonate (pH 9.5) for 24 h by changing the buffer thrice.

2.4. Production and isotype determination of mAbs

Seven-week-old female BALB/c mice were immunized by injecting 50 μg purified recombinant NS1 protein mixed with the same volume of complete Freund's adjuvant (Sigma-Aldrich). The second and third injections were followed with the same amount of protein mixed with incomplete Freund's adjuvant (Sigma-Aldrich) in the same way at 3-week intervals. The titer of anti-DENV NS1 antibody was tested by the ELISA using 1 μg/mL of the purified recombinant NS1 protein as a coating antigen. Hybridoma cell fusion was performed 3 days after final immunization, as previously reported (Kohler and Milstein, 1975). Spleen cells obtained from immunized mice were fused with SP2/0 mouse myeloma cells (ATCC #CRL1581). Hybridomas producing specific antibodies were screened by an indirect ELISA. By limiting dilution, positive hybridomas were cloned. The mAbs were purified from ascitic fluid of mice using protein G-coupled Sepharose column (GE Healthcare Life Science, Pittsburgh, PA). Isotyping was carried out using goat anti-mouse immunoglobulins (Sigma-Aldrich).

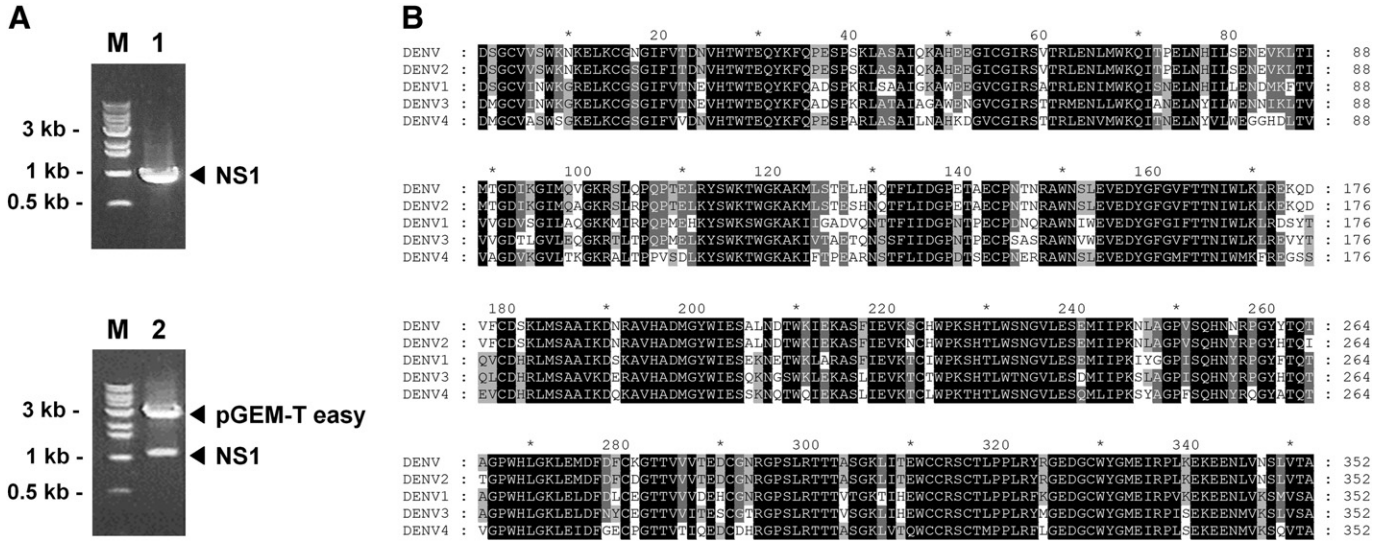

Fig. 1. Cloning of DENV2 NS1 gene. (A) Full length NS1 cDNA fragment amplified from a patient's plasma sample. M, DNA size markers; lane 1, the amplified gene of DENV2 NS1, lane 2, the DENV2 NS1 gene cloned into the pGEM-T easy vector was digested by EcoR I and Xho I. (B) Amino acid sequence alignment between the cloned DENV NS1 protein and NS1 proteins of 4 dengue virus serotypes (DENV1, DENV2, DENV3, and DENV4). Accession no. gi|25014063 (DENV1); gi|159024813 (DENV2); gi|164654855 (DENV3); gi|73671172 (DENV4).