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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).

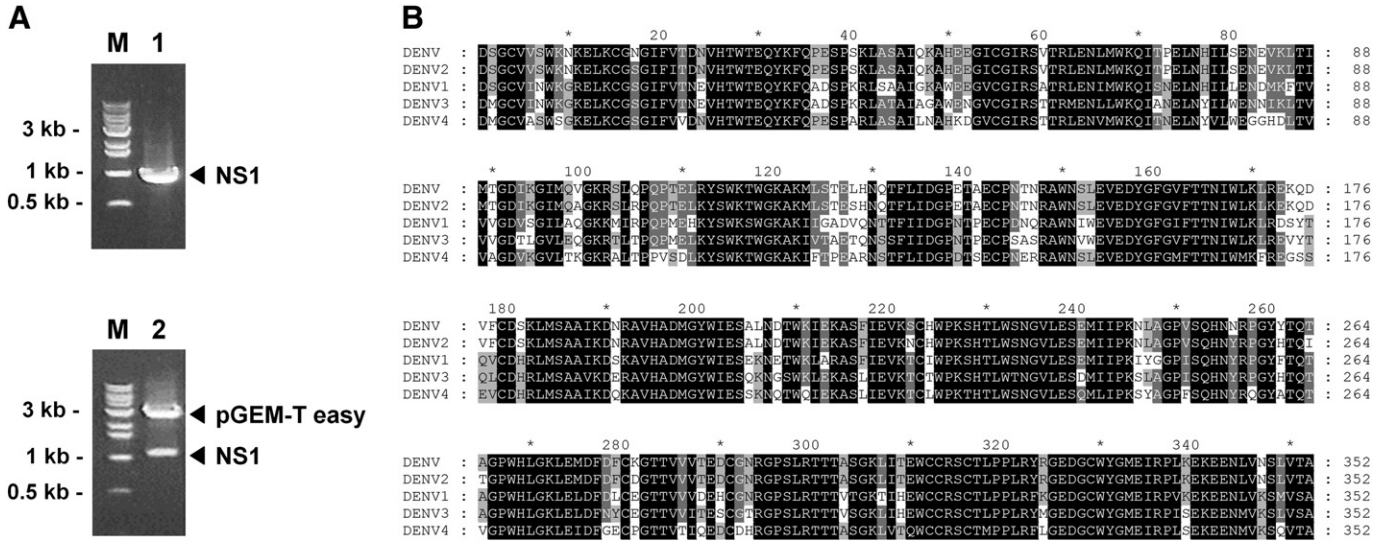

Fig. 1. Cloning of DENV2 NS1 gene. (A) Full length NS1 cDNA fragment amplified from a patient's plasma sample. M, DNA size markers; lane 1, the amplified gene of DENV2 NS1, lane 2, the DENV2 NS1 gene cloned into the pGEM-T easy vector was digested by EcoR I and Xho I. (B) Amino acid sequence alignment 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).

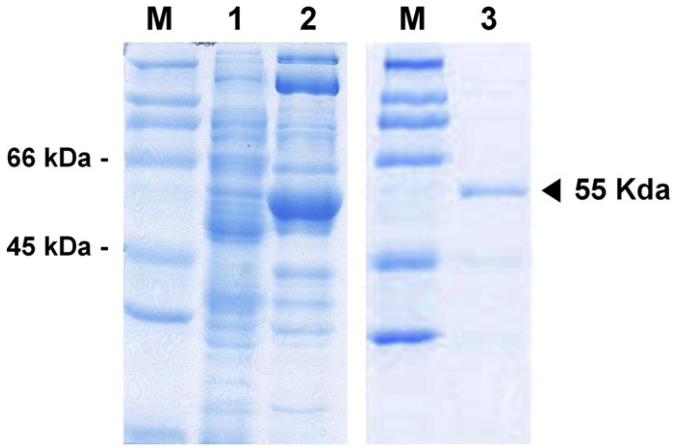

Fig. 2. Expression and purification of the soluble NS1 protein. M, size marker; lane 1, lysate of *E. coli* BL21(DE3) transformed with pET32a: :DENV2 NS1 after 0.5 mmol/L IPTG induction; lane 2, purified DENV2 NS1 protein with DEAE ion exchange chromatography (first step purification); lane 3, purified DENV2 NS1 protein with Ni-NTA Affinity chromatography (second step purification).

2.5. Determination of K_d

An indirect competitive ELISA was conducted to measure the affinity of mAbs, as previously described (Friguet et al., 1985). Various concentration of the recombinant NS1 antigen was incubated with mAbs for 1 h at room temperature (RT). This antigen–antibody mixture was then transferred to wells where recombinant NS1 antigens are coated. After 1-h incubation at RT, wells were rinsed with phosphate buffered saline containing 0.1% Tween-20. One-hour incubation was followed with antimouse IgG-horseradish peroxidase conjugate at RT and 3,3',5,5'-Tetramethylbenzidine solution was added after final washing. Finally, the absorbance was measured by a microplate reader (Benchmark plus; Bio-Rad, Hercules, CA) at 450 nm after the termination of coloring reaction by H_2SO_4. Dissociation constant (K_d) was calculated by generating Klotz plot as previously described (Friguet et al., 1985).

2.6. Colloidal gold preparation and antibody conjugation

Colloidal gold particles (40 nm mean diameter) were prepared as described (Frens, 1973). Briefly, 100 mL of 0.01% $HAuCl_4$ in a beaker was boiled thoroughly, and 1.8 mL of 1% trisodium citrate was then added under constant stirring. When the color of the solution is changed to wine-red, the solution was boiled for another 5 min. The heat source was removed, and the solution was stirred for another 10 min. The colloidal gold solution was stored in the dark at 4 °C and used as soon as possible.

The mAb was conjugated with 40 nm colloidal gold particles as described (Cramer et al., 1989; De Waele et al., 1989). The mAb was dialyzed with 1 mmol/L Borax (pH 9.0) for 5 h at 4 °C. The gold solution was mixed with the mAb after adjusting pH to 9.0 with 0.2 mol/L K_2CO_3. The mixture was then incubated for 30 min at RT. The mAb-gold conjugate was blocked with 1% bovine serum albumin for 30 min under the same conditions and then washed 3 times with phosphate buffered saline containing 1% bovine serum albumin. The OD of conjugate was measured at 540 nm.

2.7. Preparation of RDT strips

The 2 antibodies were dispensed and immobilized to the appropriate positions of a nitrocellulose membrane (control line, 1 mg/mL of goat anti-mouse IgG; test line, 1 mg/mL of anti-NS1 mAb), and the membrane was then dried for 1 day at RT. The mAb-conjugated gold colloid was dried on a glass fiber and used as the conjugator pad. The sample pad was prepared by treating a cellulose paper with 0.1 mol/L Tris-Cl (pH 8.0) containing 0.5% polyvinyl alcohol and Tween-20. The absorbance pad consisted of a cellulose paper without any treatment. The test strip was assembled, and all pads were partially overlapped to enable the migration of the sample solution along the strip.

2.8. RDT assay procedure and interpretation of the results

Total 142 human serum specimens were used to evaluate the RDT. Sensitivity and specificity of the RDT were calculated by the general diagnostic formulas (Cohen, 1960; Jacobson, 1998). One hundred microliters of specimen was loaded into the sample well of the device. Interpretation of the test result was done within 15 min and further checked until 30 min to observe a possible false positive. The control line should appear in all tests. If a red color band appears at the test line, it means that the specimen contains NS1 antigens. Positive test results were categorized into 4 groups according to the intensity of red band color appeared at the test line: 3+, 2+, 1+, and +w (weak). The result reading was cross-validated by at least 2 independent persons.

3. Results

3.1. Cloning of NS1 gene

The amplified NS1 gene was found to be 1056 bp in size (Fig. 1A). In order to specify the serotype of the gene, the multiple alignment of amino acids was performed using the reference NS1 sequences of each

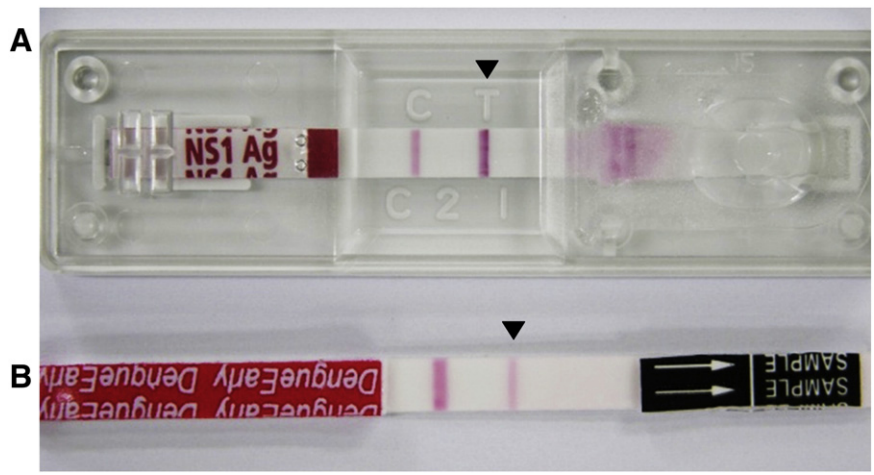

Fig. 3. Antigenicity analysis of the recombinant NS1 protein using dengue NS1 Ag kits. Test lines (arrow) were visible in both (A) BioLine Dengue NS1 Ag Kit and (B) PanBio Dengue NS1 Ag Kit.

Table 1
Properties of hybridomas developed in this study.

Hybridoma	Isotype	K_d[a]	Role in RDT	Performance
3E12E6	IgG_1	$7.032 \pm 0.322 \times 10^{-9}$	Conjugating Ab	
4A6A10	IgG_1	$7.512 \pm 0.419 \times 10^{-9}$	Capturing Ab	
8H4B10	IgG_{2b}	$9.006 \pm 0.324 \times 10^{-9}$	Conjugating Ab	Weak reactivity
10G3H5	IgG_1	$6.966 \pm 0.259 \times 10^{-9}$	Capturing Ab	False positivity

[a] Dissociation constant (K_d) was determined by a Klotz plot, which was fully followed by the method of Friguet et al. (1985).

serotype (Fig. 1B). The cloned NS1 gene was approximately 96% identical to the NS1 gene of DENV2 but 70% identical to those of other serotypes, suggesting that it is originated from DENV2.

3.2. Expression and purification of the recombinant DENV2 NS1 protein

The entire DENV2 NS1 protein was successfully expressed as a soluble recombinant protein and purified under native conditions. Strategic 2-step purification extremely elevated the purity of eluted NS1 protein. Approximately 40 mg of protein was obtained from 1 L of bacterial culture, and 1 mg/L of protein was obtained at high purity after final 2-step purification. The expressed recombinant NS1 protein was approximately 55 kDa in molecular mass (Fig. 2).

3.3. Antigenicity of the purified DENV2 NS1 protein

In order to test whether the purified recombinant NS1 protein retains its antigenicity similar to a native NS1 protein, the reactivity of the purified protein to commercial assays was examined by using SD BioLine and PanBio Dengue NS1 Ag Kit (Alere Inc., Waltham, MA). Both kits are RDT types. Ten nanogram of the purified recombinant NS1 protein was applied to strips, and the result was analyzed by observing the appearance of color band at the test line. Both kits were able to detect the purified recombinant NS1 protein as an antigen (Fig. 3), indicating that the recombinant NS1 protein purified under native conditions retains its antigenicity.

3.4. Generation of mAbs against the recombinant NS1 protein

Using the highly antigenic recombinant NS1 protein as an immunogen, mAbs were generated. Sixteen hybridoma cell lines stably producing independent mAb were established based on their high absorbances in ELISA screenings. Among them, 4 hybridoma cell lines were selected as they exhibited the highest reactivity with the recombinant NS1 protein: 3E12E6, 4A6A10, 8H4B10, and 10G3H5. The dissociation constant (K_d) was determined by measuring the affinity of mAbs to the recombinant NS1 antigen and generating the Klotz plot (Table 1 and Fig. 4). Hybridoma cell line 10G3H5 showed the lowest K_d value, meaning the highest affinity to the recombinant NS1 protein. Hybridoma cell lines 3E12E6 and 4A6A10 also displayed high affinity to the recombinant NS1 protein

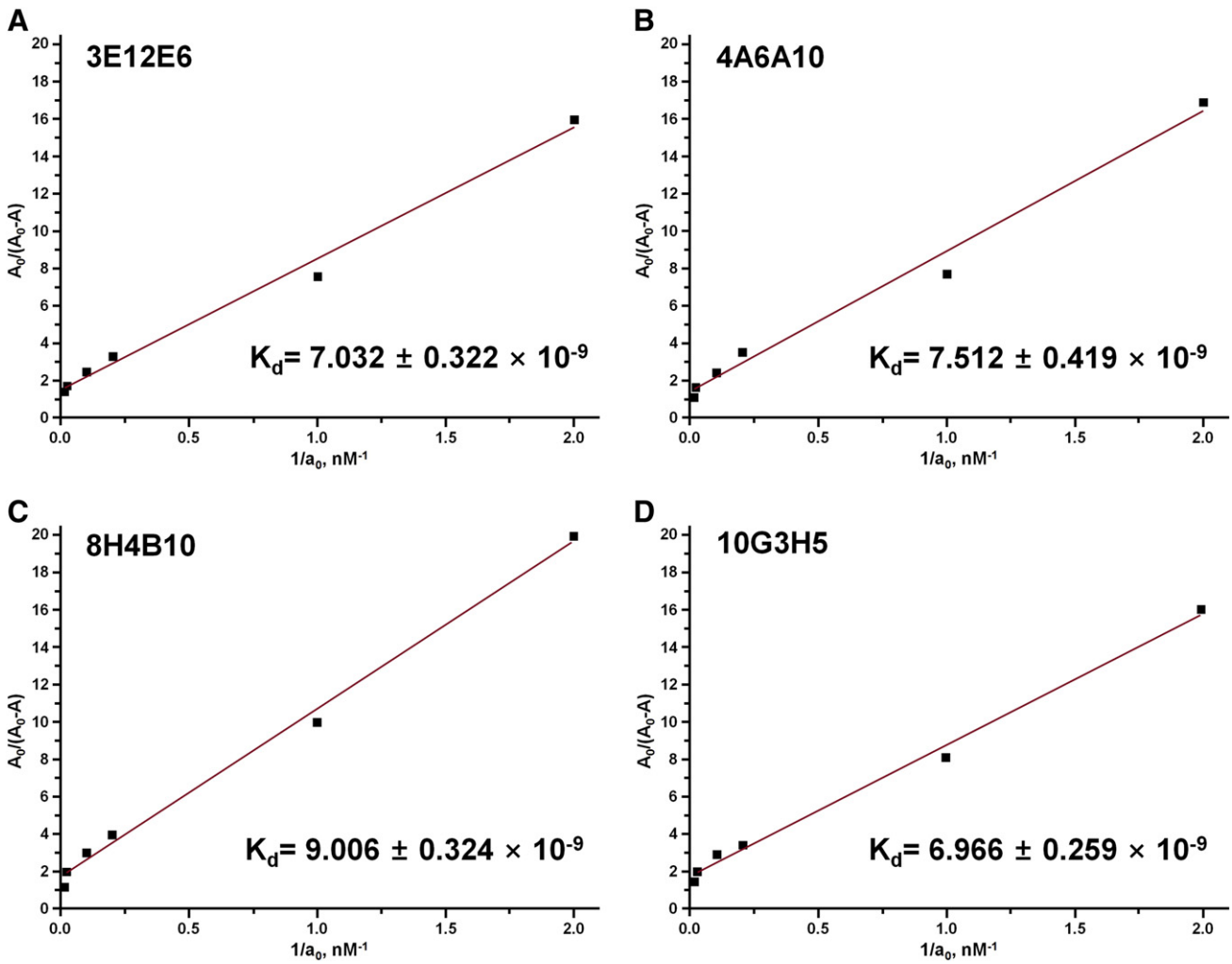

Fig. 4. Klotz plots of monoclonal antibodies binding to the recombinant NS1 protein by an indirect ELISA.

Table 2
Clinical evaluation of the dengue NS1-specific RDT kit developed in this study.

n = 142	RDT developed in this study		Total
	Negative	Positive	
Negative	100	0	100
Positive[a]	3	39	42
Total	103	39	142
Sensitivity = 92.9% (39/42), specificity = 100% (100/100)			

[a] Dengue NS1–positive sera were confirmed by RT-PCR.

similar to 10G3H5. Isotypes of these mAbs were all IgG_1 subclass. Hybridoma cell line 8H4B10 was IgG_{2b} subclass and exhibited the lowest affinity to the recombinant NS1 protein among 4 selected mAbs.

3.5. RDT construction

Each mAb was hired as a conjugating antibody for colloidal gold or a capturing antibody for an antigen. The effectiveness of the RDT was then tested using clinical sample of each DENV serotype (DENV1, 2, 3, and 4) and cultured viruses (DENV2, 3, and 4) in animal cells to finally select clinically practical monoclonal antibodies. The results showed that mAbs 4A6A10 and 10G3H5 were effective as capturing antibodies and 3E12E6 and 8H4B10 as conjugating antibodies (data now shown). However, 8H4B10 exhibited relatively weak reactivities and 10G3H5 displayed false positivity (data not shown). Thus, 3E12E6 and 4A6A10 were selected and applied as a conjugating and a capturing antibody, respectively, to construct an RDT for dengue NS1 detection in this study.

3.6. Clinical evaluation and performance comparison of the RDT

The constructed RDT was evaluated using clinical specimens kindly provided from several hospitals in Korea, Malaysia, Brazil, and India. One hundred dengue-negative and 42 dengue NS1–positive sera were examined. Especially, the diagnostic performance of the RDT for dengue NS1–positive sera was compared to that of a commercial RDT kit (SD BioLine Dengue NS1 Ag from Alere Inc.), which is widely used worldwide. As a result, all dengue-negative sera were diagnosed as negative by the constructed RDT, leading to 100% specificity (Table 2 and Fig. S1). The cross-reactivity was not observed with cultured viruses of the Flaviviridae family such as Japanese encephalitis virus (JEV), West Nile virus, and Chikungunya virus (data not shown). The noise signal was so low that the accurate interpretation was possible within 15 min. The result reading was further checked until 30 min, but no false positives were observed.

To compare the diagnostic performance against 42 dengue NS1–positive sera, the positive test results were categorized into 4 groups based on the intensity of band color that appeared at the test line. The strongest intensity was recorded as 3+, and the weakest intensity, as +w (Fig. 5). When the comparison is done in a target (object)–oriented way, both RDT kits showed equivalent diagnostic results for 18 samples out of 42: nos. 5, 8, 10–12, 20, 21, 23, 27, 29–32, 34, 38, and 40–42 (Table 3, Figs. 6A and S2). Three false negatives were included in the 18 samples: nos. 21, 23, and 30 (Table 3; Figs. 6A and S2). The RDT developed in this study showed a better performance for 15 samples: nos. 1, 2, 4, 6, 7, 9, 14–19, 24–25, 28, 33, 35, and 36. In particular, sample no. 14 was diagnosed as a false negative by the commercial kit. SD BioLine Dengue NS1 Ag kit scored only 5 samples as stronger lines: nos. 3, 13, 22, 37, and 39 (Table 3; Figs. 6A and S2). It also generated 4 false negatives in addition to 3 that were resulted from both RDTs: nos. 1, 6, 14, and 35 (Table 3; Figs. 6A and S2). When the overall performance is compared in an intensity focused way, it clearly shows that the RDT developed in this study can more effectively and stably diagnose dengue cases (Fig. 6B). To summarize, the constructed RDT in this study resulted in 92.9% (39/42) of sensitivity, whereas SD BioLine Dengue NS1 Ag kit resulted in 83.3% sensitivity (35/42) (Tables 2 and 3). The results indicate that the RDT kit developed in this study is more excellent to detect a dengue NS1 antigen.

4. Discussion

A DENV NS1 protein is known as a prospective antigen to diagnose early dengue infection. Although NS1-based indirect or capture ELISA tests were developed and commercialized (Bessoff et al., 2008; Blacksell et al., 2008; Dussart et al., 2008; Hsieh and Chen, 2009; Kumarasamy et al., 2007; Lima Mda et al., 2010), the sensitivity of test kits is still unsatisfactory. To improve the sensitivity of NS1-based assay, a DENV NS1 recombinant protein was purified with high antigenicity and purity in this study. Useful mAbs were generated using this functional antigen, and an RDT was developed using mAbs as an RDT format is valuable for a POC diagnosis of dengue infection (Hang et al., 2009). The design of an antigen is one of the most important steps in the development of immunoassay. A native protein is most likely to produce useful antibodies, but it is very difficult to obtain in sufficient amount of a native protein. Thus, an antigen is generally prepared as a recombinant protein due to its advantages such as higher protein productivity compared to a

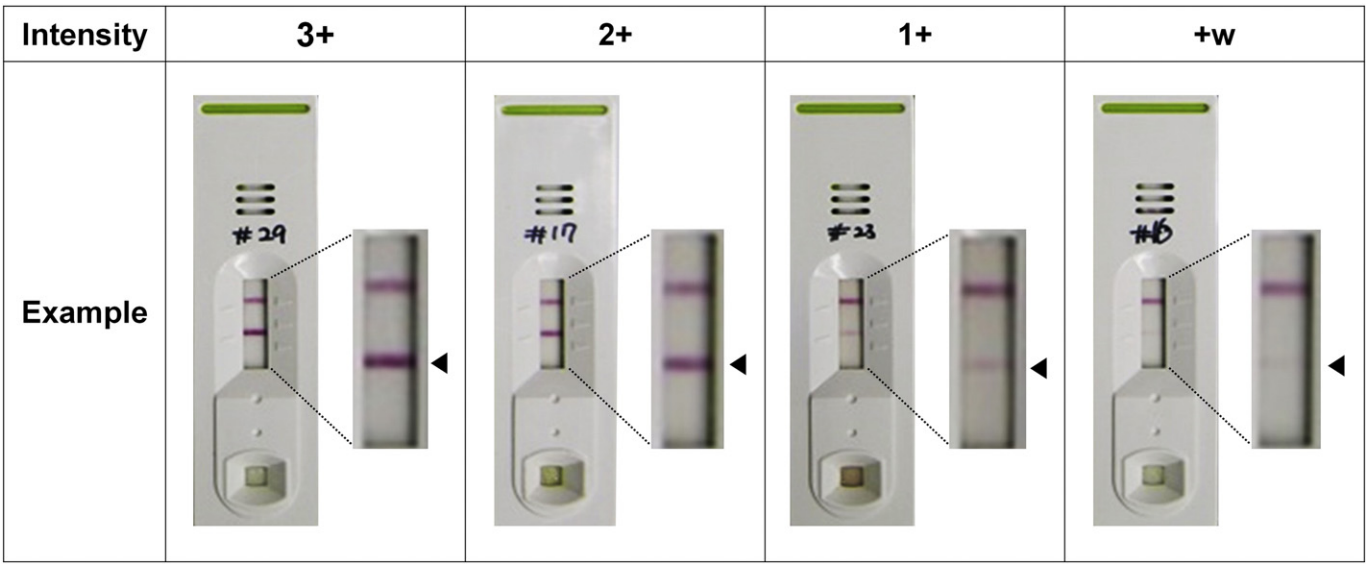

Fig. 5. Interpretation of diagnostic performance. The strongest band intensity at the test line was recorded as 3+, and the weakest, as +w.

Table 3
Comparison of diagnostic performance between the RDT developed in this study and a commercial RDT (BioLine Dengue NS1 Ag).

Sample no.	Collection site	RDT in this study	BioLine Dengue NS1 Ag
1	Malaysia	1+	−
2		3+	1+
3		+w	1+
4		3+	2+
5		2+	2+
6		2+	−
7		3+	2+
8		2+	2+
9		3+	2+
10		1+	1+
11		3+	3+
12		+w	+w
13		+w	1+
14		+w	−
15		3+	2+
16	India	2+	1+
17		3+	2+
18		2+	1+
19		2+	1+
20		2+	2+
21		−	−
22		2+	3+
23		−	−
24		3+	1+
25		1+	+w
26	Brazil	1+	+w
27		1+	1+
28		2+	1+
29		1+	1+
30		−	−
31		3+	3+
32		1+	1+
33		1+	+w
34		2+	2+
35		1+	−
36		3+	2+
37		+w	1+
38		1+	1+
39		1+	2+
40		1+	1+
41		1+	1+
42		2+	2+
Sensitivity (%)		92.9 (39/42)	83.3 (35/42)

−, Negative; +, positive (3+, the strongest band color intensity; +w, the weakest band color intensity).

native protein and better antigenicity compared to a peptide. Among various expression systems, the prokaryotic system using *E. coli* is usually adapted to express antigens in the aspect of cost, protein yield, and simplicity of process. Although there is still concern whether the recombinant antigens could elicit appropriate immune responses and, therefore, produce useful antibodies in place of native antigens, many recombinant proteins that contain immunodominant epitopes have been used successfully as antigens in the virus detection such as hepatitis C virus (Conry-Cantilena, 1997), human immunodeficiency virus 1 (Sohn et al., 1994), Hantan virus (Zoller et al., 1993), and JEV (Konishi et al., 1996). Until now, in most studies, the DENV NS1 protein expressed in *E. coli* was purified under the denaturing conditions due to the insolubility of a protein and further refolded to restore the antigenicity of a protein. However, refolding is a time- and effort-consuming process to be finely optimized. In addition, a perfect refolding is not guaranteed. Thus, in this study, the DENV NS1 protein was expressed and purified under the native conditions in *E. coli*.

Since the study aims to generate mAbs highly specific for the DENV NS1 protein, the priority considered in protein expression and purification was to obtain a natively antigenic protein at high purity rather than to produce protein with high yield, which many researches have focused on for years (Amorim et al., 2010; Das et al., 2009; Huang et al., 2001; Lazaro-Olan et al., 2008; Sankar et al., 2013). High purity of the DENV NS1 protein was successfully achieved by 2-step purification using ion exchange and affinity chromatographic techniques (Fig. 2). The antigenicity of the purified NS1 protein was confirmed by 2 commercial rapid test kits (Fig. 3). In fact, protein yield (40 mg/L) was also not poor. It was comparable (10–30 mg/L of Huang et al. (2001)) or better (8–12 mg/mL of Lazaro-Olan et al. (2008)) than previous reports, in which the same *E. coli* system is used. Recently, functionally active DENV2 NS1 recombinant protein was successfully expressed and purified without any *in vitro* posttranslational modification, that is, a soluble NS1 protein was purified under the native condition (Sankar et al., 2013). Authors selected a specialized vector (pBAD/Thio TOPO) to increase protein solubility in *E. coli* system and eventually achieved high-yield production of protein (155 mg/L). Although protein yield produced in this study is much lower than what Sankar et al. (2013) reported, their purification needs 1 more step to cleave 11.7 kDa of thioredoxin tag to get a sole DENV NS1 protein. In addition, the improvement of protein purity was not considered in their purification steps.

Using the highly pure NS1 recombinant protein, mAbs were generated to develop an RDT. Although commercial RDT kits are available, the specificity of those NS1-based dengue tests is reported to be variable

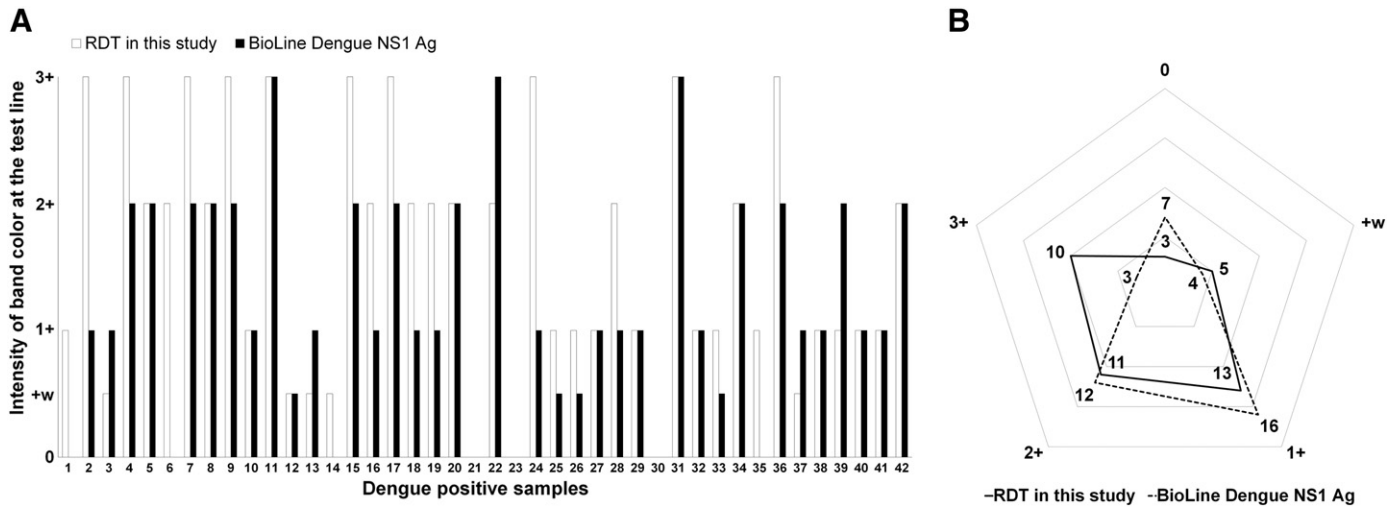

Fig. 6. Comparison of diagnostic performance. (A) A target (object)-oriented comparison. (B) Intensity-focused comparison. $P = 0.001$ (Student's t test).

ranging from 86.1% to 100%. The sensitivity is even more variable between 37% and 98.9% (Osorio et al., 2010; Tricou et al., 2010). The mAbs showing high affinity to the purified DENV NS1 antigen were successfully screened and applied to construct a novel RDT (Fig. 4 and Table 1). The RDT was further evaluated using patient samples collected from various regions worldwide (South America, South Asia, and Southeast Asia), in which DENV infection is frequent and major. Results showed that the constructed RDT displayed 100% of specificity with no cross-reactivity among viruses belonging to Flaviviridae family and 92.9% of sensitivity (Table 2). Comparison of the diagnostic performance with a widely used commercial kit, SD BioLine Dengue NS1 Ag confirmed that the constructed RDT is obviously superior to diagnose dengue infection in the aspect of sensitivity and accuracy (Table 3, Figs. 6 and S2). Probably, the preparation of pure antigen accounts for the development of an accurate diagnostic test, which is applicable for dengue diagnosis in various endemic areas. However, 3 samples (nos. 21, 23, and 30) were negative by both RDTs. They could be collected after day 7 post onset of illness, when NS1 antigens decreased. The constructed RDT was able to detect all 4 serotypes of DENV, suggesting that mAbs raised against recombinant DENV2 NS1 protein may recognize NS1 epitopes common for 4 serotypes of DENV.

In conclusion, a DENV NS1 antigen has been purely purified with retained antigenicity in a bacterial system. Two mAbs (3E12E6 and 4A6A10) showing high affinity to the antigen were developed and introduced to construct a novel RDT. Clinical evaluation demonstrated that the constructed RDT is relatively more sensitive and accurate than a commercial RDT. It could be used as a promising POC testing tool for diagnosing early DENV infections in remote areas and can contribute to the control of DENV-related diseases.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.diagmicrobio.2015.03.009.

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