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DEVELOPMENT OF IMMUNOCHROMATOGRAPHIC STRIP TEST FOR DENGUE VIRUS DETECTION

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ABSTRACT

Dengue is a major public-health problem in Thailand, where one of the challenges in dengue management is the unavailability of highly sensitive and reliable bedside diagnostic test kits. This research aimed to develop a rapid immunochromatographic strip test for dengue virus (DENV) detection using human monoclonal antibodies (HuMabs) cross-reactive to all four dengue-virus serotypes and specific to the viral envelope protein. From several clones of previously produced HuMabs, 12 were screened, of which two clones, HuMab 19 and HuMab 54, were selected based on dot-blot analysis. The two HuMabs were prepared, purified, and determined their binding ability by ELISA. The lateral flow strip tests were constructed using HuMab 54 as a gold conjugated antibody, while the captured antibody on the test and control lines were HuMab 19 and goat anti-human antibody, respectively. Finally, these immunochromatographic strip tests were tested with four DENV serotypes obtained from culture fluid of infected C6/36 cell and later DENV-spike human serum sample (pseudo positive sample), pre-treated with low pH glycine buffer. The strip tests detected four DENV serotypes in infected culture fluid within 15 minutes with no false positives. However, they could only detect DENV in a pseudo-positive sample when the sample had been treated with glycine buffer, requiring 30 minutes' reading time. Further studies and improvements are needed to enhance the detection efficiency of this in-house immunochromatographic strip test with pseudo-positive samples, with subsequent evaluation of detection efficiency with dengue patient samples.

Keywords: Dengue virus, human monoclonal antibodies, immunochromatographic strip test

INTRODUCTION

Mosquito-borne dengue virus (DENV) infection is endemic in tropical and subtropical countries, including Southeast Asia, the Western Pacific, and South and Central America, where it affects both children and adults. DENV is responsible for various spectrums of infection, ranging from self-limited dengue fever (DF) to potentially life threatening dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) (Luo et al., 2013). This febrile illness has neither preventive vaccination nor specific medication for treatment. Recently, there has been an increase in dengue infection worldwide due to increased urbanization, population growth, migration and international travel, inadequate wastewater management, climate change, and ineffective vector control (Malavige et al., 2004). According to the World Health Organization (WHO), approximately 2.5 billion people, or 40% of current world's population reside in dengue high-risk areas. The WHO estimated 50 to 100 million cases of dengue infection occur each year, including 500,000 DHF cases resulting in

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22,000 deaths, especially among young children (WHO, 2009); of these, 70% have occurred in Asia (Limkittikul et al., 2014).

Since the majority of dengue infections have occurred in Asia, where several countries are considered under-developed and developing, there is a great demand for inexpensive, rapid, user-friendly, robust, and reliable tools, such as enzyme-linked immunosorbent assay (ELISA) and rapid immunochromatography assays. Rapid immunochromatographic strip tests are also known as lateral flow immunoassays (LFIA) or simply as strip tests. LFIA have recently gained very high popularity in disease diagnosis especially in point-of care diagnosis (POC). This is because LFIA are low cost, easy to operate, stable over a wide range of weather conditions, have long shelf lives, require a small sample volume, and have a short reading time (Sajid et al., 2015).

LFIA have the advantages of both immunoassays and chromatography in which the capillary flow facilitates the reaction between antibodies and antigens after chromatographic separation through the nitrocellulose membrane (Preechakasedkit et al., 2012). Another important component in LFIA is the labels for detection. One of the most common labels used in immunoassays are enzymes such as alkaline phosphatase and horseradish peroxidase, fluorescent probes, chemiluminescent substances, metals and metals chelates such as gold nanoparticles, and liposomes (Darwish, 2006).

Hence, various commercial LFIA are available to detect dengue antigens such as non-structural protein 1 (NS1) or the patients' antibodies IgM, IgG, and IgA specific to dengue virus (Blacksell, 2012). Several LFIA are using NS1 as a target protein for dengue diagnosis as NS1 are produced in detectable quantities in the first 5-6 days post-onset of illness in primary infection, and 6-12 days for secondary infection (Blacksell, 2011). Although NS1 LFIA are inexpensive, easy to perform, and can quickly identify a patient who presents at a hospital, the issue of low sensitivity still remains. Various studies have shown that in dengue-endemic regions where secondary infection is common, NS1 LFIA have low sensitivities towards DENV2 and 4, and to severe dengue or patients' samples that were taken after 3 days of fever onset (Osorio et al., 2010). It has been suggested that in secondary infection, detection of NS1 is more difficult due to the pre-existing IgG leading to lower sensitivity of the strip test (Shen et al., 2015).

On the other hand, DENV envelope (E) protein is a highly conserved glycoprotein of approximately 55 kDa and is a major structural protein disclosed on the viral surface, serving as a target for neutralizing antibodies (Lai et al., 2008; Chen et al., 1996). Several studies have shown that E protein may be used as a candidate for sub-unit vaccine or as a target for dengue diagnosis (Jaiswal et al., 2004). DENV E protein can be detected during the viremic period or in the acute phase, leading to rapid patient identification (Vaughn et al., 2000). In addition, the recent development of therapeutic HuMabs to reduce dengue severity by reducing the virus in the blood circulation may be possible with the aid of rapid identification of acute dengue patients (Sasaki et al., 2013; Sukupolvi-Petty et al., 2010; Srikiatkhachorn et al., 2012). This is potentially beneficial for the treatment of dengue patients during acute infection.

Hence, this study aims to develop rapid immunoassays using human monoclonal antibodies (HuMabs) specific to the envelope protein of all four DENV serotypes. These HuMabs were previously produced by hybridoma technology (Setthapramote et al., 2012), and were tested with four serotypes of dengue virus obtained from culture fluid of infected C6/36 cell and later in spike human serum sample (pseudo-positive sample).

MATERIALS AND METHODS

Reagents and materials

Twelve HuMab clones specific to the envelope protein of all four DENV serotypes (HuMabs # 8, 25, 165, 183, 358, 19, 54, 118, 131, 135, 411, and 533) were previously produced by hybridoma technology (Setthapramote et al., 2012). Goat-anti-human polyclonal IgG (2 mg/ml) was obtained from Merck (Merck, Germany). The sources of

DENV used in the present study were DENV-1 virus (Mochizuki strain), DENV-2 (New Guinea C strain and 16681 strain), DENV-3 virus (H87 strain), and DENV-4 (H241 strain), and stored as viral stock at -80°C. These viruses were used in an immunofluorescence assay (IFA) and in constructing pseudo-positive human serum samples. All components of the rapid lateral flow strip test were purchased from Merck Millipore's Hi-Flow Plus Membrane, including HF120 membrane, Hi-Flow Plus HF120 backing sheet, glass fiber conjugate pads, and cellulose Absorbent sample pads (Merck, Germany). Gold colloid solution (40 nm) (Cosmo Bio, Japan) was kindly provided by Dr. Isao Miyazaki from Biomedical Research Institute Company Limited at Chiba University, Japan. The Lightning-Link® Antibody Labeling Kit for HRP (horseradish peroxidase) enzyme was obtained from Innova Biosciences, USA.

Screening of 12 HuMabs by Dot-blot analysis

Initially, all 12 clones of previously produced HuMabs were roughly screened using dot blot analysis. All HuMabs were diluted to 1 µg/ml. The starting titer of all four serotypes of dengue virus was as follows: 4.9×10^5 FFU/ml for DENV1, 2×10^5 FFU/ml for DENV2, 1.63×10^5 FFU/ml for DENV3, and 1.89×10^5 FFU/ml for DENV4, followed by 2-fold serial dilution. The polyvinylidene difluoride (PVDF) membrane was soaked in methanol for 10 seconds before washing in distilled water for 5 minutes and left to dry on filter paper at room temperature. Then three µl of each dengue virus were applied onto nitrocellulose membranes, waiting 30 minutes to dry then immersed in blocking buffer (5% skimmed milk in Tris buffer saline with tween (TBST)) for 1-2 hours at room temperature. Next, the membranes were washed three times with TBST for 3 minutes each before incubating with diluted HuMabs in blocking buffer at room temperature for 1 hour. Then, the membranes were washed again followed by incubation with peroxidase labeled goat-anti-human IgG (H+L) (diluted at 1:10,000 in blocking solution) at room temperature for 1 hour before repeating the same washing step. The interaction signal was developed using DAB solution kit (KPL, USA) according to the manufacturer's instructions. Finally, the HuMAbs that showed highest sensitivity towards DENV1-4 were selected for further testing in rapid immunochromatography strips.

HuMabs production

HuMabs were produced by culturing the hybridoma clones secreting those HuMAbs in Dulbecco's Modified Eagle Medium (DMEM), with 4.5 g/L glucose, plus L-glutamine, and sodium pyruvate in a 10-cm cell culture dish. The cells were grown in a humidified chamber of 5% CO_2, at 37°C, and passaged when the cells reached 90% confluence. Next, the HuMabs containing supernatant were collected and their specificity towards all four serotypes of DENV was tested by Immunofluorescence Assay (IFA) as described by Setthapramote et al., (2012). Next the HuMAbs were purified using ÄKTA™ prime plus machine and HiTrap™ Protein A column. Later, western blot and SDS-PAGE were carried out to determine purification efficiency of the purified HuMabs. Then, the concentration of purified HuMabs was measured using Pierce™ BCA Protein Assay as instructed by the manufacturing protocol. Lastly, the purified HuMabs were stored in PBS at -80°C.

Enzyme Linked Immunosorbent Assay (ELISA)

Sandwich ELISA was set up to determine whether the selected purified HuMabs are suitable to be used on immunochromatographic strip test for DENV detection. Each ELISA well was modified to fit the testing purpose as shown in Figure 1. Prior to ELISA, HuMab 54 was labeled with HRP (horseradish peroxidase) enzyme using Lightning-Link® Antibody Labeling Kit (Innova Biosciences, USA) as instructed by the manufacturer.

On a 96-well high-binding Maxisorp Immunoplate (Nunc, USA.), 100 µl of HuMab19 (captured antibody)(5 ng/µl), DENV, BSA, and non-infected culture fluid were coated at 4°C overnight. Then wells were washed five times with

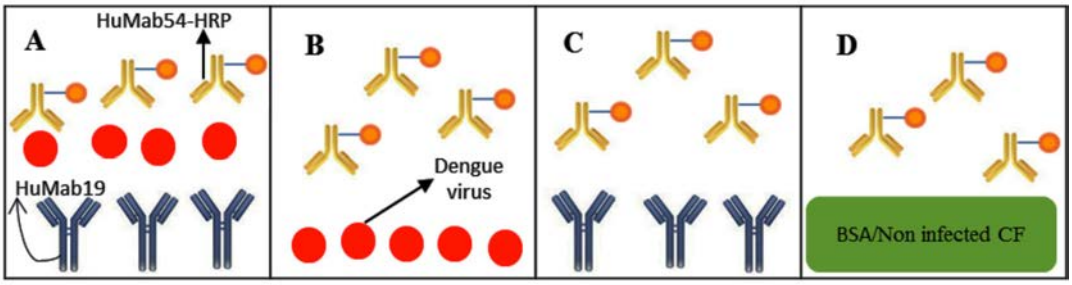

Fig. 1- Configuration of the selected HuMabs 19 and 54in each ELISA well specifically designed to test the pairing of both HuMabs with the 4 serotypes of DENV. (A) Determines the pairing of 2 HuMAbs in binding with DENV. (B) Determines the binding of HuMab54-HRPwith coated DENV (used as a positive control). (C) Determines the interaction of 2 HuMabs in the absent of DENV. (D) Determines the binding ofHuMab54-HRPwith BSA and non-infected culture fluid (used as a negative control).

0.05% TBST (washing buffer) before blocking with 1% BSA for 1 hour at room temperature. In the first well (A) only, 100µl of dengue virus was added and incubated for 1 hour at room temperature before repeating the same washing step. Next, HuMab54 labeled with HRP at a ratio of 1:2,000 was added and incubated for 1 hour at room temperature followed by the same washing step. Then, 100 µl/well of TMB reaction reagent was added and the reaction was allowed to develop for about 15 minutes before it was stopped with 25 µl of 2 M sulfuric acid. Using a plate reader (TECAN machine), the optical density was then measured at 450 nm.

Preparation of gold conjugated antibody

Based on the ELISA result using HuMAb54 as HRP-labeled antibody, HuMab 54 was chosen as the gold-conjugated antibody. Prior to the gold labeling process, salt particles in the storage buffer were removed by high-speed centrifugation, as salts interfere with the gold labeling process. Initially, the antibody concentration was adjusted to 1 mg/ml before it was transferred into a spin down-filter tube and centrifuged at 14,000 g at 4°C for 15 minutes. Next, 500 µl of 10 mM Tris-HCl, pH 9.0 were added and centrifuged again at the same speed. This process was repeated three times before the concentrations of the antibodies were measured using Nano Drop 2000 UV-Vis Spectrophotometer.

HuMab 54 was then labeled with gold colloid (Cosmo Bio, Japan) according to the manufacturer's instructions. Initially, 100 µl of gold colloidal stock solution was added to 100 µl diluted HuMab54 (in 10 mM Tris-HCl, pH 9.0) and left in the tube to stand at room temperature for 15 minutes. Next, 200 µl of 1% BSA-PEG solution was added and centrifuged at 15,000 g at 4°C for 15 minutes. Then, the supernatant was discarded and the gold pellet was re-suspended with 200 µl of 1% BSA-PEG solution followed by centrifugation at 15,000 g at 4°C for 15 minutes. Finally, the supernatant was discarded and the gold pellet was re-suspended with 200 µl of 5% sucrose.

Construction of immunochromatographic strip test

The immunochromatographic strip test was developed based on the method of Preechakasedkit et al., (2012) with slight modifications. The membrane strip was placed on the adhesive surface of the backing sheet followed by a glass fiber conjugate pad, and lastly by an absorbent pad on both ends of the sheet. With the help of a cutter, the assembled sheet was cut into strips of 4 mm and stored for future use in an aluminum foil packaging bag with silica gel to avoid humidity.

Evaluation of constructed immunochromatographic strip test

1. **Testing of HuMab54-gold conjugated antibody**

After a successful labeling with gold colloids, the gold conjugated HuMab 54 was tested on the constructed immunochromatographic strip test. On the membrane of the strip tests, 1 μl of 2

mg/ml goat-anti human polyclonal IgG was dotted and left to dry at room temperature for 1 hour. Next in ELISA well, 90 μl of 5% BSA solution and 10 μl of HuMab 54-gold conjugate was added before the strip test was placed in an upright position. The successful strip test construction was observed by the color detected at the control dot. Within 5 minutes, the red signal was observed indicating a complete and successful working of the gold conjugated antibody on the strip test.

2. Selecting the concentration of BSA in running buffer

To select the concentrations of BSA in running buffer, different BSA percentages, between 10% (w/v) BSA in PBS, 5% (w/v) BSA in PBS and, 0% BSA-PBS, were tested. Based on ELISA results, HuMab 54 was selected to label with gold colloid while HuMab 19 was used as a captured antibody on the test line of the strip test. On the membrane of the strip tests, 1 μl of 2 mg/ml goat-anti human polyclonal antibody was dotted as a control dot and one μl of HuMab 19 was dotted as the test dot and dried for about 1 hour at room temperature. For the dengue positive conditions, 100 μl of DENV2 with a titer of 7.8×10^6 FFU/ml was added into the ELISA well with 10 μl HuMab 54-gold conjugate, 90 μl of 5% and or 10% BSA, making a total volume of 200 μl. Similarly, for dengue negative conditions, 100 μl of C6/36 non-infected culture fluid was added with 10 μl of HuMab 54-gold conjugate, and 90 μl of 5% and or 10% BSA. Then the constructed strip tests were placed vertically on ELISA wells until the colored signals appeared on the strip tests.

3. Evaluation of the detection of strip test with four serotypes of DENV

Using the same pairing of HuMabs, the constructed strip tests were further tested with four serotypes of DENV obtained from the culture fluid of infected C6/36 cells. On the membrane of the immunochromatographic strip tests, 1 μl of 2 mg/ml goat-anti human polyclonal antibody was dotted to form a control dot and 1 μl of HuMab 19 was dotted as a captured antibody on the test dot. The strip tests were left to dry for about 1 hour at room temperature. Based on previous BSA concentration results, 5% BSA buffer was used for this evaluation.

For dengue positive conditions, 100 μl of each dengue serotypes was added into the ELISA well with 10 μl HuMab 54-gold conjugate, and 90 μl of 5% BSA, making a total volume of 200 μl. Similarly, for dengue negative conditions, 100 μl of C6/36 non-infected culture fluid was added with 10 μl of HuMab 54-gold conjugate, and 90 μl of 5% BSA. The titer of each dengue virus serotype used were; 5.16×10^6 FFU/ml for DENV1, 7.8×10^6 FFU/ml for DENV2, 1×10^7 FFU/ml for DENV3, and 1.87×10^7 FFU/ml for DENV4. Next, the constructed strip tests were placed vertically onto the ELISA wells until the colored signals appeared on the strip tests.

4. Evaluation of dengue detection by strip test in pseudo positive human serum samples

Human serum samples from healthy donors (certificate of ethical approval MUTM 2016-032-01) were ethically approved, collected, and stored at the Center of Excellence for Antibody Research. This human serum was used to create pseudo-positive serum samples with DENV. Initially, the human serum was treated with low pH glycine buffer following the protocol stated by Shen et al., (2015). To obtain a pseudo positive human serum sample, 100 μl of DENV2 (7.8×10^6 FFU/ml) was added with 10 μl HuMab 54-gold conjugate, 45 μl of 5% BSA, and 45 μl of treated human serum into the ELISA well. Similarly, the negative human serum sample was obtained by adding 100 μl of C6/36 non-infected culture fluid with 10 μl HuMab 54-gold conjugate, 45 μl of 5% BSA, and 45 μl of treated human serum. Next, the prepared immunochromatographic strip tests were placed vertically and observed for colored signals on the control and test regions.

RESULTS

Selected HuMabs

Dot blot analysis was used to screen 12 clones of previously produced HuMabs. Two clones (HuMab 19 and 54) were selected. Both clones of HuMabs effectively detected DENV1-4 with their

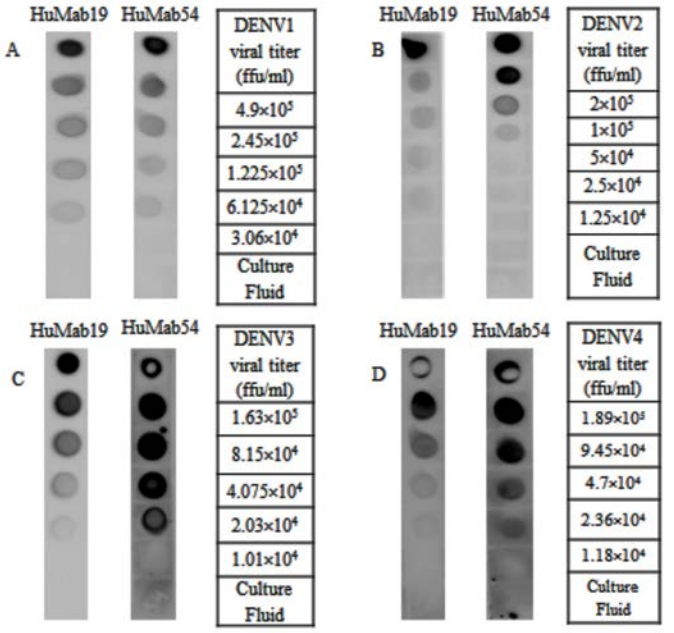

Fig. 2- Results of dot blot-analysis showing reactions of HuMab 19 and HuMab 54 tested with two-fold serially diluted dengue virus (A) DENV1, (B) DENV2, (C) DENV3, and (D) DENV4.

lowest detection at 3.06×10^4 FFU/ml for DENV1 (Figure 2A), 1.25×10^4 FFU/ml for DENV2 (Figure 2B), 1.01×10^4 FFU/ml for DENV3 (Figure 2C), and 1.18×10^4 FFU/ml for DENV4 (Figure 2D). Then, the selected HuMabs were propagated from hybridoma secreting cells. From the result of IFA, HuMabs 19 and 54 showed high specificity towards all the four serotypes of DENV (Figure 3) while SDS-PAGE showed that the purified HuMabs were about 230 kDa (Figure 4). The concentration of purified HuMab 19 and HuMab 54 was 1.75 mg/ml and 1.36 mg/ml, respectively.

ELISA results

Based on the result of the dot blot analysis, HuMab 54 was chosen to label with HRP conjugate and tested with different conditions in ELISA wells as described in the materials and methods section. ELISA results showed that HuMab 54-HRP when paired with HuMab 19, effectively detected and captured DENV1-4 (Figure 5). In addition, HuMabs 19 and 54 showed high specificity towards DENV because neither interacted with their surroundings (BSA and non-infected culture fluid used as negative control) nor with each other (the green column in the graph indicated poor binding activity between both HuMabs without dengue virus). Although a single HuMab54-HRP was adequately able to detect dengue virus 1-4 (as a positive control) the signals observed were significantly weaker when compared with the sandwich format of the the antibodies. In conclusion, both HuMabs 19 and 54 were suitable as a pair to distinctly detect DENV1-4 in ELISA format.

Evaluation of the constructed immunochromatographic strip test

1. **Testing of HuMab54-gold conjugated antibody**

Gold conjugated HuMab 54 was tested on the constructed immunochromatographic strip test by placing it vertically in the ELISA well containing mixed solutions as described previously. Slowly the solution mixture with HuMab54-gold conjugate migrated upwards until

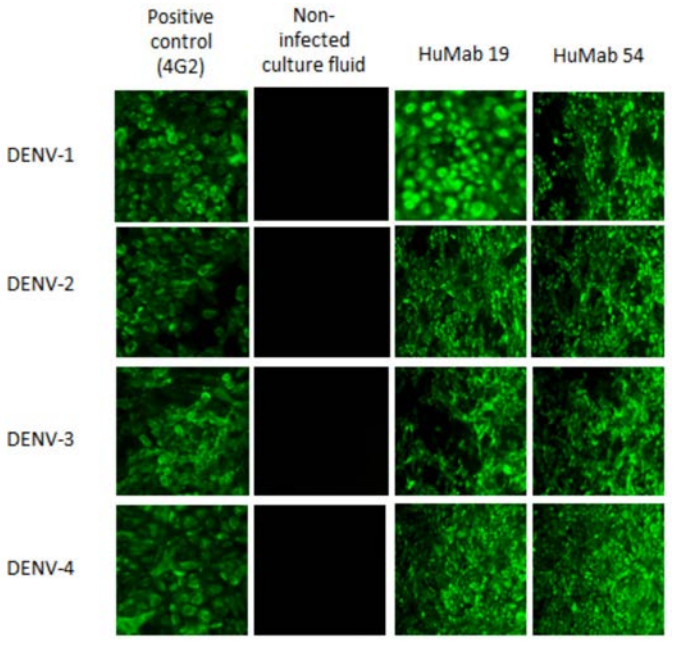

Fig. 3- Immunofluorescence staining of HuMabs 19 and 54, showing high specificity towards four serotypes of dengue virus.

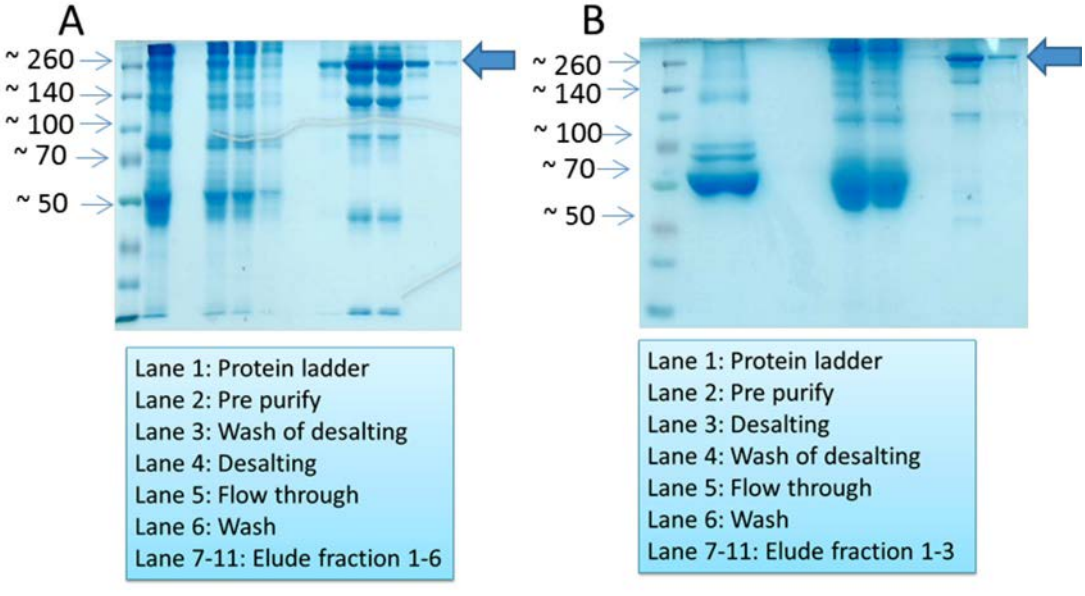

Fig. 4- SDS-PAGE of purified HuMabs (A) HuMab 54 and, (B) HuMab 19, in which both the HuMabs were about 230 kDa

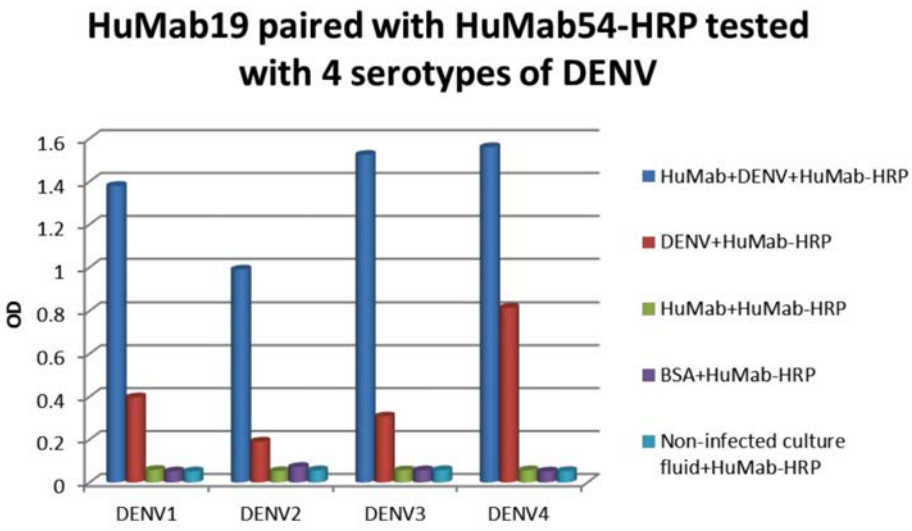

Fig. 5- ELISA results showing HuMabs 19 and 54 distinctly detecting DENV1-4 without binding to each other or with their surroundings. The Y-axis shows optical density at 405 nM.

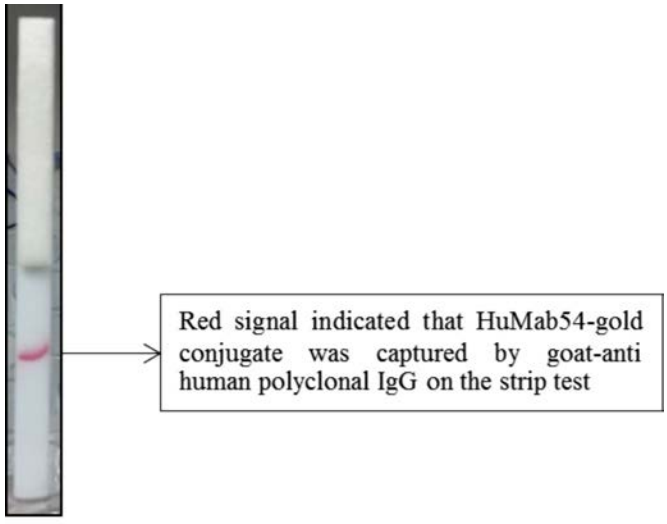

Fig. 6- Testing of gold conjugated HuMab 54 on constructed immunochromatographic strip test in which the red signal was observed within 5 minutes.

reaching the absorbent pad. Within 5 minutes, a red dot could be observed on the membrane of the strip test indicating that goat-anti human polyclonal IgG had captured a sucessfully labeled HuMab54-gold conjugated antibody was now ready to be used for further testing of DENV samples (Figure 6).

2. Selected percentage of BSA buffer

BSA was used as a blocking buffer to block non-specific protein and control the flow rate of the solution (Preechakasedkit et al., 2012). Hence, different BSA percentages were tested, 0% (PBS alone), 5% BSA, and 10% BSA. Within 5 minutes, red signals were formed on the test and control lines of both 5% and 10% BSA on DENV2 positive

strips, and only control signals appeared on DENV2 negative strips for both types of BSA (Figure 7). On the other hand, no signals were formed on the strip tests using PBS alone as a running buffer (data not shown). On leaving the strip tests upto 30 minutes, the colored signals got darker but the intensity of colored signals were clearly distinct in which 5% BSA showed stronger color signals compared to 10% BSA (Figure 7B and 7D), in addition, the flow rate of 10% BSA was longer due to higher viscosity. Hence, 5% BSA was selected as the optimum condition of the running buffer.

3. Detection of DENV1-4 in infected culture fluid and DENV2 pseudo human serum sample

The results showed that within 5 minutes, red signals were formed on the test and control lines on DENV1-4 positive strips and only control signals appeared on all DENV negative strips (Figure 8). On leaving the strip tests upto 30 minutes, the colored signals got darker for all strip tests. The intensity of colored signals were almost all the same for DENV2-4 (Figure 8B, 8C, and 8D) however, DENV1 showed weaker colored signals (Figure 8A). Hence, HuMab 19 and HuMab 54-gold conjugated antibody, when used on immunochromatographic strip tests, effectively detected DENV1-4 in infected culture fluid, showing high sensitivity and specificity with no false positive readings.

On the other hand, the strip tests evaluated with DENV2 pseudo human serum sample revealed weaker signal readings on both the positive and negative strips. Within 30 minutes, test and control dots appeared faintly on the positive strip and only the control dot appeared on the negative strip in pseudo positive human serum samples (Figure 9). On leaving the strip test upto 1 hour, the color signals got slightly darker.

DISCUSSION

Dengue is a major public health problem in Thailand. One of the challenges in dengue management is the unavailability of highly sensitive and reliable bedside diagnostic test kits. LFIA have recently gained very high popularity in disease diagnosis especially in point-of care

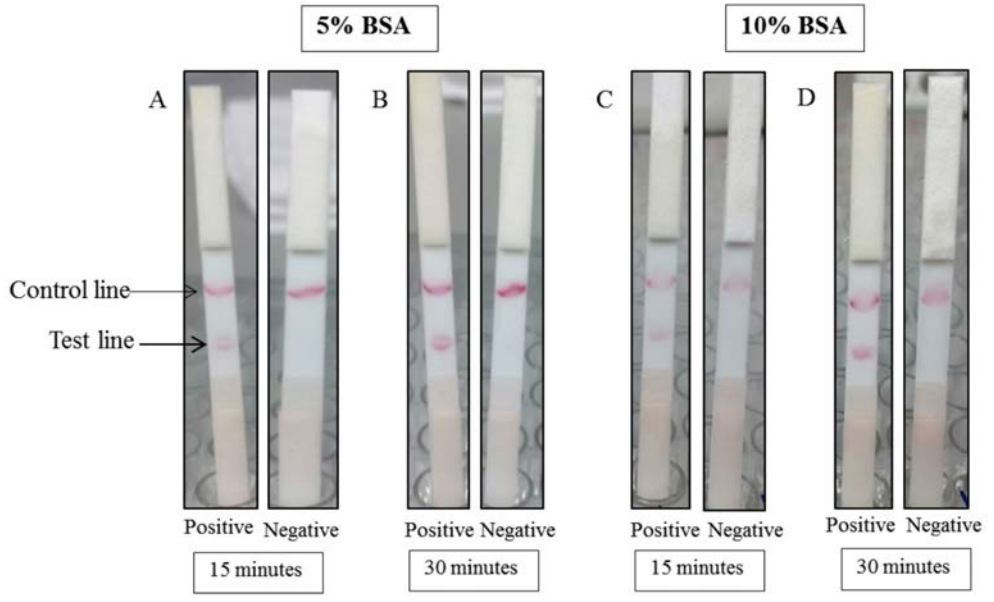

Fig. 7- Testing different concentrations of BSA in running buffers with dengue positive and dengue negative strips. (A) Positive and negative strip test using 5% BSA as running buffer observed at 15 minutes, (B) Positive and negative strip test using 5% BSA as running buffer observed at 30 minutes, (C) Positive and negative strip test using 10% BSA as running buffer observed at 15 minutes. (D) Positive and negative strip test using 10% BSA as running buffer observed at 30 minutes.

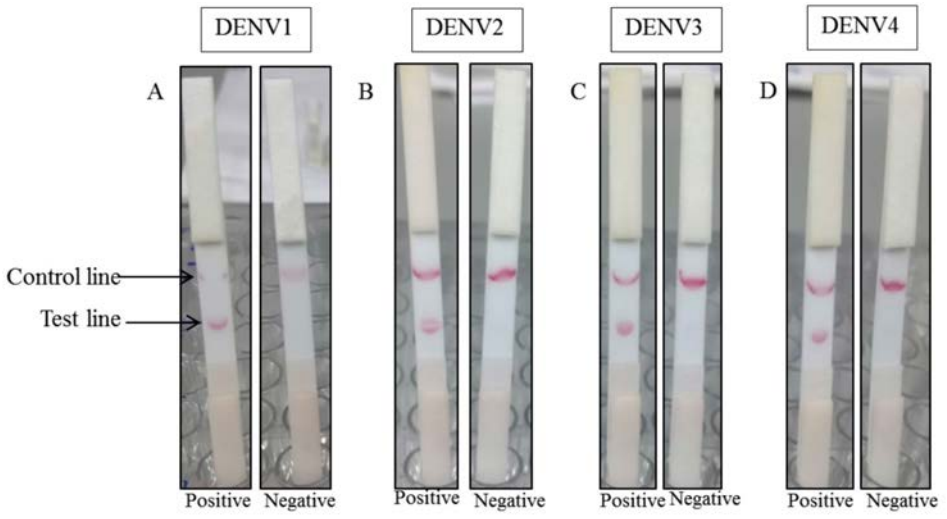

Fig. 8- Evaluating the in-house immunochromatographic strip test with (A) DENV1, (B) DENV2, (C) DENV3, (D) DENV4, in infected C6/36 culture fluid. The control and test signals could be observed on DENV1-4 positive strips and only control signals were observed on DENV1-4 negative strips.

diagnosis (POC). We aimed to develop in-house immunochromatographic strip tests using HuMabs specific to the envelope protein of all four serotypes of DENV.

In the present study, we used colloidal gold to label with HuMab 54 forming stable gold conjugated antibody. For the captured antibody on the strip tests, we used HuMab19 and goat anti human antibody as the test and control dot, respectively. Both HuMab 54 and 19 effectively and distinctly detected DENV1-4 as observed from our ELISA and IFA results. We also studied different amount of gold conjugated HuMab 54 used on the strip tests between 10 μl and 20 μl (data not shown). We observed that both volume of gold conjugated antibody could efficiently detected DENV1-4 in infected culture fluid with no differences in signal intensity, hence, 10 μl gold conjugated HuMab 54 was selected as adequate amount to be used on the strip tests.

Another important factor for the proper performance of a strip test is the attachment reaction between antigen and antibody, which can be enhanced by the use of running buffers. Generally, running buffers contain BSA or skimmed milk to block non-specific binding and regulate the flow rate of the solution (Paek et al., 2000). Other factors that also influence the flow rate are surfactant ratio and concentration of the blocking buffer used in running buffer (Preechakasedkit et al., 2012). Therefore, we

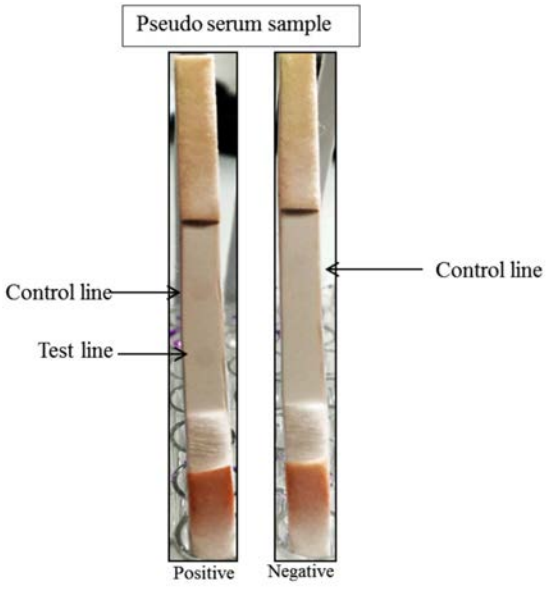

Fig. 9- Evaluating the in-house immunochromatographic strip test with pseudo human serum sample (spiked with DENV2) in which the control and test signals were visible on the positive strip and weak control signal on the negative strip.

studied the effect of flow rate containing different concentrations of BSA in running buffers, consisting of 0% BSA in PBS, 5% (w/v) BSA in PBS, and 10% (w/v) BSA in PBS using DENV2 with a viral titer of 7.8×10^6 FFU/ml.

From our results, red signals were formed on the test and control lines of both 5% and 10% BSA but no signals were formed on the strip tests using PBS alone as a running buffer (data not shown). This is probably due to the faster flow rate of PBS not allowing optimum interactions between antigens and the antibodies. We also observed that the flow rate of 10% BSA was longer due to higher viscosity. Fortunately, the intensity of colored signals was stronger in 5% BSA compared to 10% BSA. Hence, 5% BSA was selected as the optimum condition of the running buffer. However, our BSA optimum condition is different from previous study by Preechakasedkit et al., (2011) in which 10% BSA was selected. These differences are probably due to the distinct target of detection (bacteria) in which polyclonal rabbit antibodies were used instead of monoclonal antibodies.

Prior to producing pseudo positive human samples, we treated normal human serum with nonionic detergent (low pH glycine buffer). This was done to dissociate immune complexes that may interfere with the binding process between target antibody and dengue virus (Shen et al., 2015). Then DENV2 with a titer of 7.8×10^6 FFU/ml was added to the treated normal human serum and tested with our in-house immunochromatographic strip tests. Within 30 minutes, we could weakly detect DENV2 in pretreated pseudo positive human serum samples in which the test and control signals appeared for the positive strip and only control signal was observed for the negative strip. We also investigated our in-house strip tests with non-treated pseudo positive human samples and in non-treated 2-fold serial diluted pseudo samples. We found that without treating the human serum, the ability of the strip test to detect dengue virus was greatly reduced (data not shown). In diluted pseudo samples, our in-house strip tests could detect DENV2 at the dilutions of 1:16 and 1:32 for positive strips and only control signal was observed at 1:32 for negative strips (data not shown). The intensity of color signals were much weaker and the time taken for the color to be distinguished was also longer. Hence, the pretreatment of human serum with low pH glycine buffer was required in our study. As previously described, for the effect of antibody-virus immune complex, the low pH buffer is required to dissociate immune complexes and enhance the detection efficiency (Shen et al., 2015). In contrast, this requirement may not be necessary for the detection of NS1 in blood samples (Lapphra et al., 2008). However, as this is the first study to demonstrate the use of HuMabs in immunochromatographic strip tests to detect 4 serotypes of the dengue viral envelope protein, comparison of detection efficiency to detect E and NS1 maybe further required.

Although our strip tests succesfully detected DENV1-4 in the infected culture fluid, the problems of weak detection in pseudo positive human serum sample is one of the main limitations. In addition, most of the construction of our in-house strip tests, such as the application of antibodies on the membrane, was manually operated, which creates opportunities for human errors that may affect the performance of the strip tests. Possible improvements including the characterization of gold conjugated antibodies (Lin et al., 2011), blocking of the strip test components with blocking buffer, employing an accurate and precise method for injecting the antibodies onto the membrane, and testing the stability of the strip test after prolonged storage can be improve the performance of our strip test. (Wang et al., 2014). In further studies, we plan to enhance the signal readings of the strip tests in pseudo positive samples and use DENV1-4 patient samples.

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REFERENCES

Blacksell SD. Commercial Dengue Rapid Diagnostic Tests for Point-of-Care Application: Recent Evaluations and Future Needs? *J Biomed Biotechnol* 2012;2012:151967.

Blacksell SD, Jarman RG, Bailey MS, *et al*. Evaluation of Six Commercial Point-of-Care Tests for Diagnosis of Acute Dengue Infections: the Need for Combining NS1 Antigen and IgM/IgG Antibody Detection To Achieve Acceptable Levels of Accuracy. *Clin Vaccine Immunol* 2011;18(12):2095-101.

Chen Y, Maguire T, Marks RM. Demonstration of binding of dengue virus envelope protein to target cells. *J Virol* 1996;70(12):8765-72.

Darwish IA. Immunoassay Methods and their Applications in Pharmaceutical Analysis: Basic Methodology and Recent Advances. *Int J Biomed Sci* 2006; 2(3):217-35.

Jaiswal S, Khanna N, Swaminathan S. High-level expression and one-step purification of recombinant dengue virus type 2 envelope domain III protein in *Escherichia coli*. *Protein Expr Purif* 2004;33(1):80-91.

Lai CY, Tsai WY, Lin SR, *et al*. Antibodies to Envelope Glycoprotein of Dengue Virus during the Natural Course of Infection are Predominantly Cross-Reactive and Recognize Epitopes Containing Highly Conserved Residues at the Fusion Loop of Domain II. *J Virol* 2008;82(13):6631-6643.

Lapphra K, Sangcharaswichai A, Chokephaibulkit K, *et al*. Evaluation of an NS1 antigen detection for diagnosis of acute dengue infection in patients with acute febrile illness. *Diagn Microbiol Infect Dis* 2008;60(4):387-91.

Limkittikul K, Brett J, L'Azou M. Epidemiological Trends of Dengue Disease in Thailand (2000-2011): A systemetic Literature Review. *PLoS Negl Trop Dis* 2014;8(11):e3241.

Lin T, Shao JJ, Du JZ, *et al*. Development of a serotype colloidal gold strip using monoclonal antibody for rapid detection type Asia1 foot-and-mouth disease. *Virol J* 2011;8:418.

Luo YY, Feng JJ, Zhou JM, *et al*. Identification of a novel infection-enhancing epitope on dengue prM using a dengue cross-reacting monoclonal antibody. *BMC Microbiol* 2013;13:194.

Malavige GN, Fernando S, Fernando DJ, *et al*. Dengue Viral Infections. *Postgrad Med J* 2004;80(948):588-601.

Osorio L, Ramirez M, Bonelo A, *et al*. Comparison of the diagnostic accuracy of commercial NS1-based diagnostic tests for early dengue infection. *Virol J* 2010;7:361.

Paek SH, Lee SH, Cho JH, *et al*. Development of rapid one step immunochromatographic assay. *Methods* 2000;22(1):53-60.

Preechakasedkit P, Pinwattana K, Dungchai W, *et al*. Development of a one-step immunochromatographic strip test using gold nanoparticles for the rapid detection of Salmonella typhi in human serum. *Biosens Bioelectron* 2012;31(1):562-6.

Sajid M, Kawde AN, Daud M. Designs, formats and applications of lateral flow assay: A literature review. *J Saudi Chem Soc* 2015;19(6):689-705.

Sasaki T, Setthapramote C, Kurosu T, *et al*. Dengue virus neutralization and antibody-dependent enhancement activities of human monoclonal antibodies derived from dengue patients at acute phase of secondary infection. *Antiviral Res* 2013;98(3):423-31.

Setthapramote C, Sasaki T, Puiprom O, *et al*. Human monoclonal antibodies to neutralize all dengue virus serotypes using lymphocytes from patients at acute phase of the secondary infection. *Biochem Biophys Res Commun* 2012;423(4):867-72.

Shen WF, Galula JU, Chang GJ, Wu HC, King CC, Chao DY. Improving dengue viral antigens detection in dengue patient serum specimens using a low pH glycine buffer treatment. *J Microbiol Immunol Infect* 2015;pii:s1684-1182(15)00762-8.

Srikiatkhachorn A, Wichit S, Gibbons RV, *et al*. Dengue Viral RNA Levels in Peripheral Blood Mononuclear Cells Are Associated with Disease Severity and Preexisting Dengue Immune Status. *PLoS One* 2012;7(12):e51335.

Sukupolvi-Petty S, Austin SK, Engle M, *et al*. Structure and function analysis of therapeutic monoclonal antibodies against dengue virus type 2. *J Viral* 2010;84(18):9227-39

Vaughn DW, Green S, Kalayanarooj S, *et al*. Dengue Viremia Titer, Antibody Response Pattern, and Virus Serotype Correlate with Disease Severity. *J Infect Dis* 2000;181(1):2-9.

Wang Y, Wang L, Zhang J, *et al*. Preparation of Colloidal Gold Immunochromatographic Strip for Detection of Paragonimiasis skrjabini. *PLoS ONE* 2014;9(3):e92034.

WHO. Dengue guidelines for diagnosis, treatment, prevention, and control. *Geneva*. 2009.